REPORT

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Dihydrofolate Reductase and Thymidylate Synthase Transgenes Resistant to Methotrexate Interact to Permit Novel Transgene Regulation*

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Background: Methotrexate targets dihydrofolate reductase (DHFR) and thymidylate synthase (TYMS) to prevent cancer growth, and increases DHFR expression when applied.
Results: TYMS resistant to methotrexate inhibits the methotrexate induced increase in DHFR expression.
Conclusion: Thymidine synthesis regulates post-transcriptional expression of DHFR and TYMS.
Significance: Methotrexate-resistant DHFR and TYMS can be used to regulate *cis* and *trans* transgene in primary T cells.

Methotrexate (MTX) is an anti-folate that inhibits *de novo* purine and thymidine nucleotide synthesis. MTX induces death in rapidly replicating cells and is used in the treatment of multiple cancers. MTX inhibits thymidine synthesis by targeting dihydrofolate reductase (DHFR) and thymidylate synthase (TYMS). The use of MTX to treat cancer also causes bone marrow suppression and inhibits the immune system. This has led to the development of an MTX-resistant DHFR, DHFR L22F, F31S (DHFR^{FS}), to rescue healthy cells. 5-Fluorouracil-resistant TYMS T51S, G52S (TYMS^{SS}) is resistant to MTX and improves MTX resistance of DHFR^{FS} in primary T cells. Here we find that a known mechanism of MTX-induced increase in DHFR expression persists with DHFR^{FS} and *cis*-expressed transgenes. We also find that TYMS^{SS} expression of *cis*-expressed transgenes is similarly decreased in an MTX-inducible manner. MTX-inducible changes in DHFR^{FS} and TYMS^{SS} expression changes are lost when both genes are expressed together. In fact, expression of the DHFR^{FS} and TYMS^{SS} *cis*-expressed transgenes becomes correlated. These findings provide the basis for an unrecognized post-transcriptional mechanism that functionally links expression of DHFR and TYMS. These findings were made in genetically modified primary human T cells and have a clear potential for use in clinical applications where gene expression needs to be regulated by drug or maintained at a specific expression level. We demonstrate a potential application of this system in the controlled expression of systemically toxic cytokine IL-12.

Antifolate drugs have been in use for seven decades in the treatment of cancer (1). MTX[3] is a commonly used agent from this class and inhibits several folate-dependent enzymes including DHFR and TYMS. Inhibition of these proteins adversely affects the *de novo* synthesis of purine and thymidine nucleotides, which is vital for survival of rapidly replicating cells. MTX has proven valuable for treating rapidly replicating cancers such as leukemia, but the impact of MTX on healthy, replicating tissue leads to dose-limiting toxicities such as bone marrow suppression (2, 3). This disadvantage of MTX has led to intense study into mechanisms of resistance (4) and alternative antifolates for the treatment of cancer (3).

Stemming from the discovery of DHFR mutants that conferred MTX resistance, a mutant that weakly binds MTX, DHFR^{FS}, was developed (5). A strategy was formulated where bone marrow cells would be genetically modified *ex vivo* with DHFR mutants resistant to MTX (6). The use of DHFR^{FS} was later implemented in genetically modified T cells. T cells modified with DHFR^{FS} are desired following bone marrow transplant for relapsed leukemia to overcome immune suppression of MTX, allowing genetically modified T cells to survive and target cancer (7, 8). An advantage of DHFR^{FS} is that it can be used to select for transgenes useful for therapeutic efficacy such as tumor targeting proteins such as chimeric antigen receptors (8), suicide genes (9), or imaging genes (10). In an effort to broaden the application of DHFR^{FS}, our group added a human TYMS mutant, discovered in a bacterial screen, resistant to 5-fluorouracil (5-FU) (11). Upon the addition of human mutant TYMS^{SS} with DHFR^{FS} in human T cells, we demonstrated

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[3] The abbreviations used are: MTX, methotrexate; DHFR, dihydrofolate reductase; TYMS, thymidylate synthase; 5-FU, 5-fluorouracill; PBMC, peripheral blood mononuclear cell; CoOp, codon-optimized; iC9, inducible caspase 9; ANOVA, analysis of variance; THF, tetrahydrofolate; 5,10-CH_2THF, 5,10-methylenetetrahydrofolate; MFI, mean fluorescence intensity.

improved resistance of T cells to 5-FU and increased resistance to MTX beyond that of $DHFR^{FS}$ alone, in agreement with a series of experiments performed in murine bone marrow cells where linking of $DHFR^{FS}$ to a mutant of TYMS led to improved resistance to 5-FU and the antifolate pemetrexed (12, 13). We suggested that the addition of $TYMS^{SS}$ restores thymidine synthesis in MTX-treated T cells (9).

In working with DHFR and TYMS, it was noted that human DHFR is known to bind and inhibit translation of DHFR mRNA as a post-translational mechanism of regulation. Furthermore, MTX was shown to prevent this binding (14) and lead to an increase in DHFR protein expression. The nucleotide sequence to which DHFR protein binds DHFR mRNA within the coding domain is known (15, 16), and the specific amino acids that DHFR protein utilizes to bind DHFR mRNA have been elucidated (17). However, subsequent studies found that a mutation to mRNA binding amino acids of DHFR still resulted in increased DHFR protein expression in the presence of MTX (18). Conversely, mutations to amino acids unrelated to DHFR binding mRNA (17) prevented MTX-inducible expression of DHFR (18). This suggests that another mechanism of post-transcriptional regulation for DHFR is in effect, and findings detailed in this study lead us to propose that a mechanism independent of mRNA binding is affecting MTX-induced DHFR up-regulation. Notably, we demonstrate that this mechanism can be utilized for regulation of transgene expression when transgenes are located in a 3′ *cis* arrangement with $DHFR^{FS}$.

By codon-optimizing $DHFR^{FS}$ to remove the mRNA binding sequence previously identified (15, 16), we find that MTX continues to induce an increase in $DHFR^{FS}$ expression independent of the mRNA sequence and that the addition of $TYMS^{SS}$ unexpectedly blunts this increase. This finding suggests a novel method of post-transcriptional DHFR expression that is regulated by the enzymatic action of $TYMS^{SS}$. In performing these experiments in primary human T cells, we also identified that the application of MTX decreases $TYMS^{SS}$ expression in an MTX-dependent manner, likely due to TYMS binding its own mRNA to repress translation (19). Translational repression of TYMS is lost in the presence of tetrahydrofolate (19), and MTX prevents the synthesis of tetrahydrofolate (3). Consistent with this hypothesis, restoration of tetrahydrofolate synthesis in T cells by expressing $DHFR^{FS}$ along with $TYMS^{SS}$ prevented the MTX-induced expression decreases for $TYMS^{SS}$. Based on these findings, we were led to discover that expression of $DHFR^{FS}$ and $TYMS^{SS}$ is linked post-transcriptionally and results in correlated expression of DHFR and TYMS within human T cells. Here we present methods to increase, decrease, or link the expression of transgenes based on the use of mutant $DHFR^{FS}$ and $TYMS^{SS}$ in a clinically relevant context. This system will permit new forms of post-translational regulation to be utilized for improved control of gene therapy vectors.

Experimental Procedures

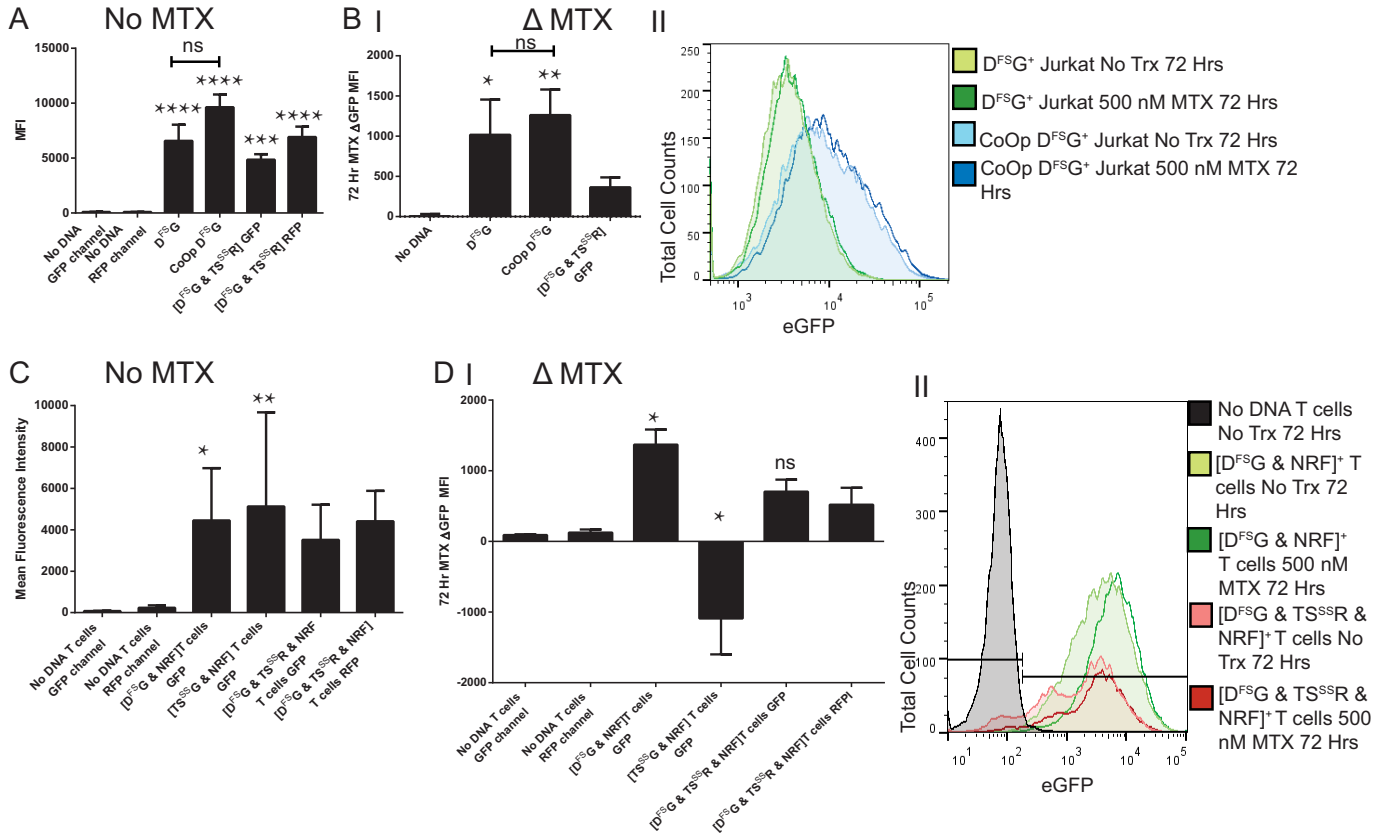
Cells—The Jurkat human T cell line was used for controlled expression experiments. Cell line origin and attributes have been previously discussed (9). T cells were derived from healthy donor-derived peripheral blood mononuclear cells (PBMC) from the MDACC Blood Bank, Houston, TX. PBMC were isolated and cryopreserved until use (9).

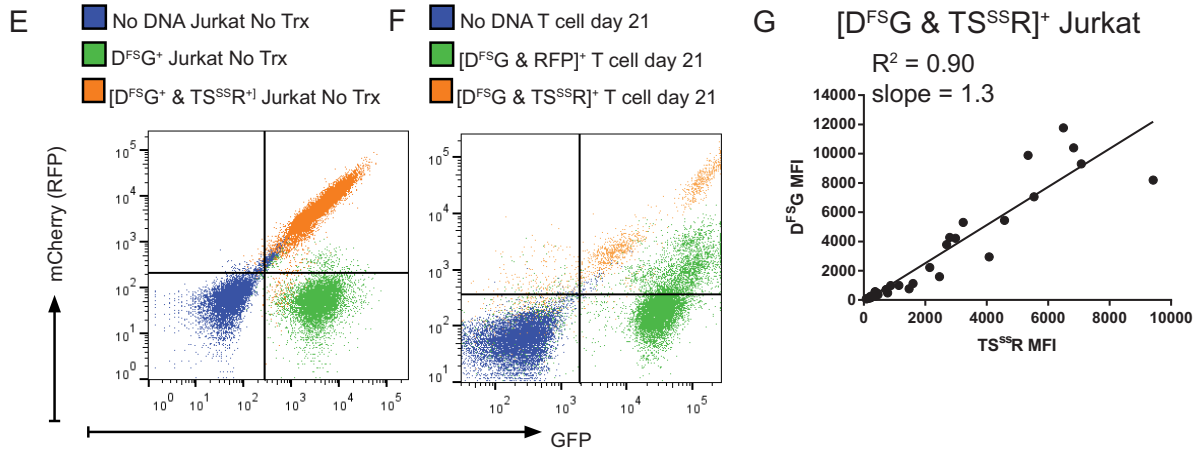
Genetic Constructs—A mutant $DHFR^{FS}$ containing the original DHFR mRNA codon sequence with point mutations to L22F,F31S was designed to co-express eGFP C terminus to a T2A amino acid sequence. This construct is referred to as FLAG-$DHFR^{FS}$-2A-eGFP pSBSO ($D^{FS}G$). The $DHFR^{FS}$ was also codon-optimized to remove the original codon sequence, with specific attention paid to the coding region where DHFR is known to bind its own mRNA (15, 16). This codon-optimized (CoOp) $DHFR^{FS}$ otherwise had the same design as $D^{FS}G$ and is known as FLAG-CoOp $DHFR^{FS}$-2A-eGFP pSBSO (CoOp $D^{FS}G$). $TYMS^{SS}$ without codon optimization was co-expressed with a C terminus eGFP or RFP. The constructs are referred to as FLAG-$TYMS^{SS}$-2A-GFP pSBSO ($TS^{SS}G$) and FLAG-$TYMS^{SS}$-2A-RFP pSBSO ($TS^{SS}R$). A selection-free RFP plasmid NLS-mCherry pSBSO (RFP) was utilized as a control. The construct Myc-ffLuc-NeoR pSBSO (NRF) was electroporated, and G418 (InvivoGen, Ant-gn-1) was used for selection as described below when alternative selection was desired. A construct to select for inducible caspase 9 (iC9) FLAG-$DHFR^{FS}$-2A-iC9 pSBSO (D^{FS}iC9) was also used. The design and synthesis of these constructs have been described elsewhere (9). The construct FLAG-$TYMS^{SS}$-2A-IL-12p35-2A-IL-12p40 pSBSO (TS^{SS}IL-12) was synthesized from codon-optimized (GeneArt, Life Technologies) IL-12 p35 and IL-12 p40 transgenes and digested within the 2A regions to ligate IL-12 p35 and IL-12 p40 with a $TYMS^{SS}$ fragment also digested within the 2A region. $TS^{SS}G$ backbone digestion points 5′ to the start site of $TYMS^{SS}$ and 3′ to the IL-12p40 stop site ligated the three components into the $TS^{SS}G$ backbone in a four-part ligation. All constructs described integrate into the cellular genome with the aid of Sleeping Beauty transposase (20).

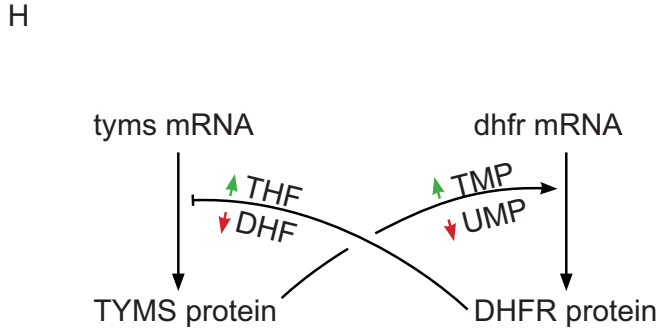
Culture Conditions—Both Jurkat cells and human PBMC were electroporated, maintained, and selected as described previously (9). Briefly, Jurkat cells were electroporated with transgene, and 48 h later, drug was applied for 2 weeks. Surviving cells were rested in complete media for another 3–5 weeks before testing. Human PBMC were electroporated, and the following day, T cells were propagated with activating and propagating cells at a 1:1 ratio including recombinant human IL-2. 48 h after co-culture was initiated, drug was applied to culture for a 2-week period. Propagation of PBMC with activating and propagating cells and IL-2 selectively expanded T cells and was repeated weekly. The T cells surviving drug treatment from days 2 to 14 were co-cultured without drug for another 3 weeks (9, 20, 21). On day 35 of propagation, T cells were stimulated with anti-CD3, anti-CD28, and IL-2 (9). MTX (Hospira, Lake Forest, IL), pemetrexed (Lilly, Indianapolis, IN), and 5-FU (APP Pharmaceuticals, Schaumburg, IL) were attained from the MDACC pharmacy, whereas raltitrexed (Abcam Biochemicals, Cambridge, MA, catalog number Ab142974) was not.

Flow Cytometry—T cells were prepared for flow cytometry by washing once in FACS buffer, and surface-stained for anti-human CD3-APC (BD Pharmingen, 340661, 1:33 dilution), as described previously (21). Intracellular staining for Myc tag Alexa Fluor 488 (MBL International, M047-A48, 1:33 dilution) and anti-human IL-12 p40/p70-PE (BD Pharmingen, 554575,

1:20 dilution) also proceeded as described previously (21). Transgene expression was analyzed on either a BD LSRFortessa or a BD FACSCalibur (BD Biosciences), with the same flow cytometer used consistently within a given experiment. FlowJo v 10.0.5 (TreeStar Inc., Ashland, OR) was used to analyze flow cytometry data.







Legend
D^FSG: DHFR^FS-2A-eGFP
TS^SSG : TYMS^SS-2A-eGFP
TS^SSR: TYMS^SS-2A-RFP
NRF: ffLuc-2A-NeoR
RFP: NLS-mCherry

Statistical Analysis—Statistical analysis and graphical representation were performed as before (9). Briefly, non-Gaussian distributions of a multivariate nature were analyzed by one-way ANOVA Kruskal-Wallis test followed by Dunn's multiple comparison test. Univariate tests (experimental *versus* control) utilized *t* tests. Two-way ANOVA was performed when appropriate and followed by Šidák's multiple comparison test. The analysis is denoted in context. Pearson's correlation and linear regression analysis were performed where denoted. Statistical significance was designated as $\alpha < 0.05$. Means with standard deviations are depicted within each figure

Results and Discussion

Mayer-Kuckuk *et al.* (22) demonstrated that DHFR linked to a transgene of interest can be used to induce an increased expression of that transgene *in vitro* and *in vivo* when MTX is administered. We attempted to recapitulate an increase in the expression of MTX-resistant $DHFR^{FS}$ after the administration of MTX within human T cells, which Skacel *et al.* (18) has shown in a hamster cell line. As a control for our studies, $DHFR^{FS}$ transgene was codon-optimized to remove any of the mRNA sequence or structure that contributed to mRNA binding (16). It was hypothesized that this modification would increase $DHFR^{FS}$ expression by removing DHFR protein binding to $DHFR^{FS}$ mRNA and prevent the increase of $DHFR^{FS}$ expression previously noted in the presence of MTX. The expression of $DHFR^{FS}$ and CoOp $DHFR^{FS}$ selected for uniform expression in Jurkat T cell line is shown in Fig. 1*A*. CoOp $DHFR^{FS}$ did not contribute to a significantly higher expression of $DHFR^{FS}$ as indicated by a *cis*-expressed eGFP, nor did it prevent MTX-induced increases in transgene expression as noted in Fig. 1*B*. This was unexpected. However, the loss of MTX-induced increase in $DHFR^{FS}$ expression was noted when $TYMS^{SS}$ was co-expressed with $DHFR^{FS}$, as seen in Fig. 1, *A* and B (9). The addition of $TYMS^{SS}$ led to an insignificant reduction in the expression of un-optimized $DHFR^{FS}$ in the absence of MTX. The addition of MTX was unable to induce the same increase in $DHFR^{FS}$ expression seen during the sole expression of either $DHFR^{FS}$ version. Thus, $TYMS^{SS}$ is playing a role in the MTX-inducible increase of $DHFR^{FS}$, and likely through the restoration of thymidine synthesis.

Expression of these transgenes in primary T cells was evaluated to recapitulate the findings of MTX-inducible increases in $DHFR^{FS}$ expression that were prevented by $TYMS^{SS}$. Expression of $DHFR^{FS}$, $TYMS^{SS}$, or [$DHFR^{FS}$ & $TYMS^{SS}$] was achieved with stability and purity by selecting from days 2–14 of propagation with the respective drugs MTX, 5-FU, and G418 when the selection vector containing neomycin resistance was included (9). The expression of $DHFR^{FS}$-linked eGFP and $TYMS^{SS}$-linked RFP can be noted in Fig. 1*C* for $DHFR^{FS}$, $TYMS^{SS}$, or [$DHFR^{FS}$ & $TYMS^{SS}$]. Again it is noted that $DHFR^{FS}$ expression is increased in the presence of MTX (Fig. 1*D*), but this increase is blunted and no longer significant when $TYMS^{SS}$ is co-expressed with $DHFR^{FS}$, as in Jurkat cells. Of note, expression of $TYMS^{SS}$ without $DHFR^{FS}$ was successfully achieved in primary T cells by selection with 5-FU and a *trans* neomycin resistance gene selected by G418 (9). When $TYMS^{SS}$ was tested for inducible changes in the presence of high doses of MTX (Fig. 1*D*), it was found that $TYMS^{SS}$-linked RFP decreased significantly. MTX induced a reduction in the expression of $TYMS^{SS}$ that MTX-resistant $DHFR^{FS}$ restored. Although not definitive, these findings could indicate that $TYMS^{SS}$ is being repressed by a lack of 5,10-methylenetetrahydrofolate (5,10-CH_2THF). This is consistent with the findings of Chu *et al.* (19) that 5,10-CH_2THF prevents TYMS-mediated repression of TYMS mRNA translation. Those findings indicate that in a healthy cell where DHFR activity is uninhibited, TYMS is not significantly bound to its mRNA. However, we propose that MTX, which leads to a drop in 5,10-CH_2THF, is causing TYMS protein to bind TYMS and $TYMS^{SS}$ mRNA, preventing expression (23). It should be noted that $TYMS^{SS}$ is equivalent to the native sequence with the exception of the point mutations (9).

FIGURE 1. **$DHFR^{FS}$ 3′ *cis*-transgene expression increases in the presence of MTX independent of mRNA sequence, and the increase is suppressed by restoration of thymidine synthesis.** *A*, Jurkat cells were genetically modified to express FLAG-$DHFR^{FS}$-2A-eGFP pSBSO ($D^{FS}G$) with resistance to MTX ($n = 4$), CoOp $D^{FS}G$, with known mRNA binding elements $D^{FS}G$ removed ($n = 5$), and [$D^{FS}G$ & FLAG-$TYMS^{SS}$-2A-RFP pSBSO ($TS^{SS}R$)], with enhanced resistance to MTX beyond $D^{FS}G$ alone through the addition of MTX-resistant $TYMS^{SS}$ ($n = 7$). Genetically modified Jurkat cells were selected for 2 weeks in 1 μM MTX before culturing without MTX for 3–5 weeks. In *A*, the stable fluorescent protein expression, in the absence of MTX, is depicted by MFI. *B*, *panel I*, Jurkat cells were treated for 72 h with 0.5 μM MTX or no treatment. The MFI difference (Δ = eGFP MFI MTX-treated − eGFP MFI-untreated) is depicted. *B*, *panel II*, a histogram representative of the data used in *panel I* demonstrates the MTX-induced change in eGFP MFI for $DHFR^{FS}$ and CoOp $DHFR^{FS}$ in Jurkat cells. *No Trx*, no treatment. *C*, in primary T cells, transgenes $DHFR^{FS}$, $TYMS^{SS}$, or the combination were selected with ffLuc-NeoR pSBSO (NRF) for 2 weeks in the presence of cytotoxic drug and then propagated without selection for 3 weeks (see "Experimental Procedures"). On day 35, T cells were stimulated with anti-CD3, anti-CD28 antibodies, and 50 IU/ml IL-2 in the presence or absence of MTX for 72 h. The fluorescent protein MFI of untreated cells is shown. *D*, *panel I*, depicts the Δ MFI after 72 h of treatment with 0.5 μM MTX in comparison to no treatment. In *D*, *panel II*, a representative histogram demonstrates the observed shift in eGFP fluorescence for $DHFR^{FS+}$ T cells in the presence or absence of MTX ($n = 5$). *E*, a *trans* regulatory pattern of DHFR- and TYMS-linked proteins was observed. A representative flow plot from the 1 μM MTX-selected Jurkat cells left untreated, as in *A*, demonstrates that in untreated Jurkat cells of either unselected mock-electroporated (*No DNA*) or $D^{FS}G^{+}$, there is a globular appearance in the RFP channel. However, co-expression of $DHFR^{FS}$ with $TYMS^{SS}$ in [$D^{FS}G$ & $TS^{SS}R$]$^{+}$ Jurkat cells leads to a linear clustering. *F*, primary T cells were electroporated with $DHFR^{FS}$ and co-transformed with either RFP control or FLAG-$TYMS^{SS}$-2A-RFP pSBSO ($TS^{SS}R$) before propagation, as in *C*. A representative flow plot of primary human T cells from the same donor where [$D^{FS}G$ & RFP], [$D^{FS}G$ & $TS^{SS}R$], and untransformed T cells is shown on day 21, after 7 days without selection in MTX. A linear clustering of $DHFR^{FS}$ is again noted when co-expressed with $TYMS^{SS}$ that is not noted with RFP alone. *G*, further studies to identify a *trans* pattern of linked expression between $DHFR^{FS}$ and $TYMS^{SS}$ were identified in the selection of [$D^{FS}G$ & $TS^{SS}R$] electroporated Jurkat cells in antifolates MTX (0, 0.01, 0.1, 0.5, 1, 5 μM), pemetrexed (0, 10, 50, 100 μM), and raltitrexed (0, 1, 5, 10 μM). The MFI of $D^{FS}G$ and $TS^{SS}R$ for each expression pattern was plotted after days 2–14 in selection. The values were plotted, and a linear fitting was performed with the R^2 from the Pearson's correlation and the slope of the linear regression provided on the graph. These data are assembled from four technical replicates. *H*, a model of post-transcriptional regulation of DHFR and TYMS mRNA into the respective proteins is depicted with *colored arrows* indicating increase (*green*) or decrease (*red*) in specific small molecules. *Black pointed arrows* indicate promotion, while *black bars* indicate inhibition of protein translation. All experiments other than *G* were independently repeated twice. Kruskal-Wallis test was used to determine significant differences with multivariate analyses. Unmarked statistically significant results refer to comparison between experimental cells and the "No DNA" untreated control cells of equivalent measure. *ns* = not significant; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$. *TMP*, thymidine monophosphate; *UMP*, uridine monophosphate; *DHF*, dihydrofolate.

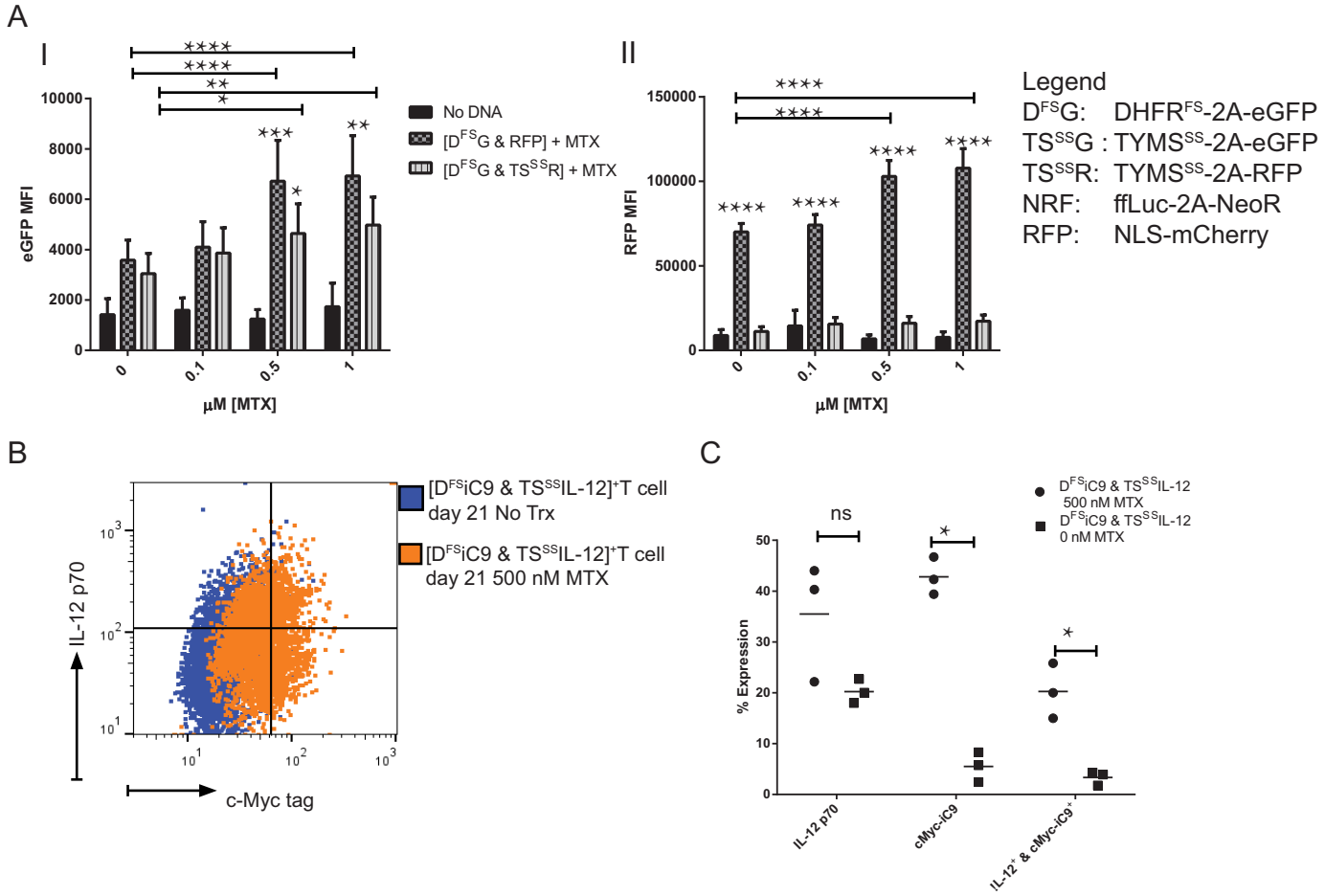


FIGURE 2. **Co-expression of DHFR^{FS} with TYMS^{SS} leads to controlled expression of TYMS^{SS} and *cis* transgenes in the presence of MTX.** *A*, primary T cells from the experiment described in Fig. 1*F* were propagated to day 35. T cells were stimulated for 72 h with anti-CD3, anti-CD28 antibodies, 50 IU/ml IL-2, and varying concentrations of MTX. The MTX-induced change in eGFP MFI for DHFR^{FS} is shown in *panel I*, while the influence of MTX on RFP and RFP co-expressed with TYMS^{SS} (TS^{SS}R) is shown in *panel II* ($n = 6$, repeated independently twice, analyzed by two-way ANOVA with Šidák's multiple comparison test). *B*, this regulatory pattern was applied to a clinically relevant problem: the cytokine IL-12 is a strong promoter of anti-tumor activity in T cells, but is highly toxic. A construct expressing IL-12 following TYMS^{SS}, called TS^{SS}IL-12, was used to modulate IL-12 expression in conjunction with the construct D^{FS}iC9. D^{FS}iC9 is capable of selecting T cells with DHFR^{FS} and depleting those T cells with iC9. A representative flow diagram of the same donor depicts intracellular expression of IL-12 and c-Myc-iC9 in [D^{FS}iC9 & TS^{SS}IL-12]-expressing T cells. These cells are shown on day 21 after selection from day 2–14 in 0.1 μM MTX and subsequent treatment with 0.5 μM MTX or no treatment from days 14–21. Cellular secretion of IL-12 was blocked for 6 h before intracellular staining. Gating is based on staining of untransformed, unselected primary T cells stained in the same way. *C*, three donors were treated as in *B*, and the change in transgene expression noted after 7 days of treatment with 0.5 μM MTX is shown. Each measure was analyzed by *t* tests. *ns* = not significant; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$.

Based on findings in Fig. 1, *A* and B, we propose that DHFR^{FS} expression is also regulated by the synthesis of thymidine. Likewise, based on findings in Fig. 1, *C* and D, we propose that TYMS^{SS} expression is regulated by the synthesis of tetrahydrofolate (THF). As a derivative of THF is used to make thymidine, a logical conclusion was made that DHFR^{FS} regulates the expression of TYMS^{SS} and TYMS^{SS} regulates the expression of DHFR^{FS}. If this is the case, a correlated expression of DHFR^{FS} and TYMS^{SS} should be noted within individual cells. A correlated expression of DHFR^{FS} and TYMS^{SS} was indeed observed in flow cytometry plots of Jurkat cells in Fig. 1*E* and primary T cells in Fig. 1*F*. A control RFP vector co-expressed with DHFR^{FS}, but not modulated by *cis* expression with TYMS^{SS}, did not appear to have the same co-expression pattern (Fig. 1*F*). To quantify this observation, Jurkat cells expressing [DHFR^{FS} & TYMS^{SS}] were treated with antifolates MTX, pemetrexed, and raltitrexed at varying concentrations for 2 weeks. DHFR^{FS}-linked eGFP mean fluorescence intensity (MFI) and TYMS^{SS}-linked RFP MFI for each separate well were then plotted and correlated. The linked expression between DHFR^{FS} and TYMS^{SS} was significant and fit a linear regression (Fig. 1*G*). These findings support a general mechanism for regulation of DHFR and TYMS, which leads to a linear co-expression of DHFR^{FS} and TYMS^{SS} independent of MTX. This model is shown in Fig. 1*H*.

Based on the above model in Fig. 1*H*, it appears that TYMS^{SS} expression will be stabilized by DHFR^{FS} from strong expression changes in the presence of MTX. This was tested in Fig. 2*A* with primary T cells expressing DHFR^{FS} along with either RFP or TYMS^{SS} linked to RFP by applying increasing doses of MTX. As expected, DHFR^{FS}-linked eGFP was increased by rising concentrations of MTX, and this increase was blunted by the presence of TYMS^{SS} (Fig. 2*A*, *panel I*). This conserves the model in Fig. 1*H* where restoration of thymidine synthesis prevents the MTX-induced increase in DHFR^{FS}. Further conserving the model, RFP linked to TYMS^{SS} did not significantly increase

over any concentration of MTX used (Fig. 2*A*, *panel II*). When $DHFR^{FS}$-linked eGFP increased, so too did the control RFP, and a rise in the expression of RFP alone was not expected. A possible explanation is that this increase was noted above 0.5 μM MTX, and $DHFR^{FS}$ alone is only resistant to 0.5 μM MTX (9). This suggests that higher doses of MTX begin to select for cells with higher gene content of $DHFR^{FS}$ and associated transgenes. This is supported by Kacherovsky *et al.* (10) where greater $DHFR^{FS}$ expression was selected by the addition of MTX. Notably, $DHFR^{FS}$ co-expressed with $TYMS^{SS}$ is resistant to concentrations of up to 1 μM MTX. This further supports the use of $TYMS^{SS}$ to modulate transgene expression and prevent unwanted selection toward higher gene expression levels of genes expressed in *cis* or *trans* with $DHFR^{FS}$.

Our group previously described a construct of $DHFR^{FS}$ *cis* expressing an inducible suicide gene, iC9 (9). This construct, called D^{FS}iC9, selects for T cells expressing D^{FS}iC9 in the presence of MTX and ablates $D^{FS}iC9^{+}$ T cells in the presence of drug that activates iC9 to induce apoptosis (24). Based on the above findings, the $DHFR^{FS}$ in D^{FS}iC9 could be used to modulate and potentially ablate the expression of a transgene of interest that is otherwise too toxic to express without regulation. IL-12 is such a transgene. IL-12 is a cytokine capable of inducing a strong immune response against tumor from tumor-specific T cells (25). However, systemic IL-12 is highly toxic and hence of low efficacy (26). Groups seeking to apply IL-12 to a clinical setting have sought inducible systems to overcome the toxicity related to this cytokine where T cells express IL-12 after activation (27). Presented here is an alternative approach where IL-12 is expressed *cis* to $TYMS^{SS}$ to decrease and stabilize the expression level of IL-12. In Fig. 2*B*, a flow plot demonstrates the expression of IL-12 *cis*-expressed with $TYMS^{SS}$ and iC9 *cis*-expressed with $DHFR^{FS}$ expression. The primary T cells were either left untreated or treated with high doses of MTX for 7 days. This expression pattern appears to indicate that IL-12 can be stably expressed even in toxic doses of MTX. A further analysis of similarly manipulated donors (Fig. 2*C*) demonstrates the potential of $TYMS^{SS}$ when co-expressed with $DHFR^{FS}$ to stabilize the expression of the potentially toxic transgenes of interest (here IL-12).

As for the endogenous regulatory mechanism proposed in Fig. 1*H*, there is evidence to support a natural regulatory pathway leading to linked expression of TYMS and DHFR. Regulation of TYMS expression by TYMS binding its own mRNA (19) and regulation of DHFR expression by DHFR binding its own mRNA (14) are both translationally regulated by the presence of folates. Our findings indicate that a DHFR mRNA-independent translational or post-translational mechanism is also involved in directing the co-expression of DHFR and TYMS. In this study, $DHFR^{FS}$ and $TYMS^{SS}$ expression was tracked by fluorescent proteins linked by a 2A ribosomal "slip" site. These linkages express two independent proteins due to slippage of the completed N terminus peptide at the 2A sequence. In this study, the N terminus peptide was $DHFR^{FS}$ and $TYMS^{SS}$ (28). The above findings of MTX-inducible changes suggest that if the fluorescent protein is regulated by either $DHFR^{FS}$ or $TYMS^{SS}$, then this regulation is occurring before or during translation rather than after the proteins separate post-translationally. Thus, our findings add complexity to the translational regulation of DHFR and TYMS to meet an unknown biological need for combined expression of DHFR and TYMS. Findings by Anderson *et al.* (29) demonstrated that DHFR and TYMS co-localize as a multi-enzyme complex within the nucleus during replication of DNA. Stabilization and construction of this multi-enzyme complex within the cell may explain the need for tight translational control of DHFR and TYMS.

In conclusion, we have demonstrated an endogenous mechanism of post-transcriptional regulation for DHFR and TYMS that can be co-opted with either $DHFR^{FS}$ or $TYMS^{SS}$ to regulate expression of *cis*- or *trans*-expressed transgenes. The purpose of this study is to describe a novel system for transgene regulation in gene therapy. This system is of significant clinical interest as the genes described are human transgenes of low immunogenicity that utilize MTX to regulate transgene expression. MTX is readily available for use in both hospital and clinical settings. As these studies demonstrate that this phenomenon occurs in primary human T cells, this system can readily be used in the field of transgenic T cell immunotherapy to improve the safety and efficacy of T cell therapies used to treat cancer (30). What remains to be determined is how an improved understanding of the regulatory mechanism surrounding DHFR and TYMS expression may lead to a better understanding of developmental disorders and improved chemotherapeutics.

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