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主論文

# Impaired NFKBIE Gene Function decreases Cellular Uptake of Methotrexate by Down-regulating SLC19A1 Expression in a Human Rheumatoid Arthritis Cell Line.

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***Abstract.***

**Objective.**

A non-synonymous single nucleotide polymorphism (nsSNP, rs2233434, Val194Ala) in the *NFKBIE* gene is known to be a rheumatoid arthritis (RA) susceptibility polymorphism in the Japanese RA population and could be closely associated with NF-κB activity. Inflammation caused by RA is sometimes associated with changes in expression levels of MTX (methotrexate) pathway-related genes. It is of interest to examine whether the *NFKBIE* gene had any influences the mode of MTX action.

**Methods.**

Both knockdown of *NFKBIE* gene expression and overexpression of wild-type *NFKBIE* and Val194Ala mutation were performed. A transfected human rheumatoid arthritis synovial cell line was cultured and then gene expressions in the MTX pathway were measured. In addition, we measured the uptake and the efflux of MTX derivatives under the *NFKBIE* knockdown condition.

**Results.**

Knockdown of *NFKBIE* reduced the mRNA for *SLC19A1*, a main MTX membrane transporter, and the intracellular accumulations of MTX derivatives. Moreover, our experiments also confirmed that overexpression of Val194Ala mutant *NFKBIE* decreased the *SLC19A1* mRNA when compared to that of wild-type *NFKBIE*.

**Conclusions.**

We suggest that the impairment of *NFKBIE* gene function can reduce the uptake of MTX into cells, suggesting that the gene is an important factor for the RA outcome.

*Key Indexing Terms:*

METHOTREXATE

NFKBIE

SLC19A1

RHEUMATOID ARTHRITIS

MH7A

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease that affects many tissues and organs, which is especially characterized by persistent synovial tissue inflammation, leading to the progressive joint disorder. In most RA patients, the activation of nuclear factor kappaB (NF-κB), a hallmark of inflammatory responses, plays an important role in the expression of cytokines in the synovial tissue [1-3].

Methotrexate (MTX) is the most commonly used anchor drug for treating RA. MTX is a folate analog, and its anti-rheumatic mechanism has been discussed; the effect of MTX *in vivo* may be mediated by reducing cell proliferation, increasing the rate of apoptosis of T cells and endogenous adenosine release; altering the expression of cellular adhesion molecules; and influencing the production of cytokines, humoral responses and bone formation [4].

A non-synonymous single nucleotide polymorphism (nsSNP, rs2233434, Val194Ala) in the *NFKBIE* (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon) gene has been reported to be an RA susceptibility polymorphisms using a large-scaled GWAS meta-analysis in Japanese RA patients [5]. *NFKBIE* encodes I kappaB-epsilon (IκBε), a member of the Inhibitor of kappa B (IκB) family and its mRNA is widely expressed in different human tissues. Additionally the gene is known to inhibit the nuclear translocation of NF-κB by binding to NF-κB proteins [6, 7]. For this reason, the risk haplotype of this nsSNP in *NFKBIE* has been reported to show an enhancement of NF-κB activity in transfected cells compared with the wild-type construct [8], following changes in IκBε structure. In a study on the use of human peripheral blood cells, Blits et al. observed that the expressions of MTX pathway genes were increased in MTX-naive RA patients compared to those of healthy controls [9]. Additionally, from the study using synovial tissues obtained from RA patients, Stamp et al. noted a negative correlation between the ESR (erythrocyte sedimentation rate) and expressions levels of some MTX pathway genes [10]. These findings led us to wonder whether MTX pathway genes are influenced by the *NFKBIE* gene.

In the present study, we hypothesized that *NFKBIE* may play a role in regulating the expression levels of MTX pathway genes (i.e., drug membrane transporters and MTX-metabolizing enzymes). To test our hypothesis, we examined the effect of knockdown of *NFKBIE* or overexpression of wild-type *NFKBIE* and the mutant (Val194Ala) *NFKBIE* on the gene expression levels of "drug membrane transporters" that are closely related to the intracellular accumulations of MTX in a human rheumatoid arthritis synovial cell line. We also performed an investigation of "MTX-metabolizing enzyme" expression in the similar culture conditions. Knockdown of *NFKBIE* expression resulted in down-regulation of *SLC19A1* (solute carrier family 19 (folate transporter), member 1) expression and intracellular accumulations of MTX

derivatives. These results suggest that impairing the effect of the *NFKBIE* gene may reduce cellular uptake of MTX by down-regulating *SLC19A1* expression.

## MATERIALS AND METHODS

*Cell Culture.*

MH7A, a human rheumatoid arthritis synovial cell line, was purchased from Riken Cell Bank. MH7A is established by transfection with the SV40 T antigen, and the cells are fibroblast-like synoviocytes [11]. Cells were cultured in RPMI 1640 medium (Sigma-Aldrich) containing 10% heat-inactivated fetal bovine serum (FBS) (Gibco) in a humidified incubator at 37°C under 5% $CO_2$. For experiments, MH7A was trypsinized and passaged to 24-well dishes.

*Chemicals.*

MTX, TNF-α and IL-1β were purchased from Sigma-Aldrich. Stock solutions of MTX (dissolved in 0.1N NaOH and then diluted in PBS), TNF-α and IL-1β (diluted in PBS) were stored at -20°C. For these drugs, working concentrations were prepared by diluting stock solutions in culture medium immediately before use.

*Small interfering RNA (siRNA) transfection.*

To knockdown endogenous *NFKBIE*, cells were transfected with 20 pmol ON-TARGETplus *NFKBIE* siRNA (Dharmacon) using Lipofectamine 2000 (Invitrogen). Non-targeting control siRNA (Dharmacon) was used as a transfection control and knockdown efficiency was examined by reverse transcription quantitative real-time PCR (qRT-PCR). Cells were plated at $0.75 \times 10^5$ cells/well and cultured for 24 hours; then the cells were transfected for 24 hours. Subsequently, they were incubated with or without 0.01, 0.1, 1, 5 and 10 μM MTX for 24 hours in the presence or absence of 20 U/mL IL-1β and 20 U/mL TNF-α.

*Measurement of the uptake and efflux of MTX derivatives.*

After washing the cells with PBS, the cells were incubated in the presence of 5 μM fluorescein methotrexate (Life Technologies), an MTX derivative, for 5 hours. In another experiment, pulse chase analysis of the efflux of the MTX derivative was performed. Two groups of transiently transfected cells were incubated with 5 μM MTX derivatives for 5 hours and then washed with PBS, followed by chase for 0 and 6 hours exclusively in RPMI supplemented with 10% FBS. On the basis of these results, we studied differences in the efflux of the MTX derivative between control and *NFKBIE* knockdown cells. Before analysis, cells were treated with trypsin (Wako) to obtain a single cell suspension. Intracellular accumulation of MTX derivatives was measured with a Cell Lab Quanta SC (Beckman Coulter) flow cytometer and se calculated mean fluorescence intensities on the basis of our arbitrary drawing regions on the histogram. As a result, at least 6,000 cells were evaluated.

*Plasmid constructs and preparations.*

The full coding IκBε clone was constructed by fusing two overlapping cDNAs for residue 26-807 from HEK293, for residue 793-1528 from MH7A with appropriate primer pairs (HEK293 forward: 5'-ATG AAT CAA CGA AGG AGT GAG TCA AGG CC-3', HEK293 reverse: 5'-GTG TCT CCG TCC TCG GAG ATG TAA GTG AGT GC-3'; MH7A forward: 5'-CGA GGA CGG AGA CAC GCT GGT CCA CCT GGC AGT GA-3', MH7A reverse: 5'-TCA GTC GGT ACA CAG CAG CAG TTT CCC TG-3') and KOD-Plus-Neo (TOYOBO). PCR products were inserted into the pFLAG-CMV5 vector, which was generated by replacing the Myc-tag of pMyc_CMV5 (a gift from Dr. David W. Russell in UT Southwestern Medical Center, USA) with a Flag-tag, between Hind Ⅲ and BamHI using the 5× In-Fusion HD Enzyme Premix (Clontech). The 581T>C (Val194Ala) mutation was generated using the 5× In-Fusion HD Enzyme Premix with the wild-type *NFKBIE* construct as a template and the following primers (forward: 5'-CGG GAC CCG CCA AGG AAC CAC AGG AGA A-3'; reverse: 5'-CCT TGG CGG GTC CCG GAG GAT GGG TGC A-3'). Both wild-type and mutated constructs were sequenced to verify that only the desired constructs were present.

To overexpress wild-type or Val194Ala mutant *NFKBIE*, cells were plated at $1.50 \times 10^5$ cells/well and cultured for 24 hours. Cells were transfected with 0.8 μg of plasmid DNA using Lipofectamine 2000 and empty pFLAG-CMV5 plasmid was used as the transfection control (Mock) and then incubated for 18 hours. After changing the medium, the cells were incubated with or without 0.01, 0.1, 1, 5 and 10 μM MTX for 24 hours in the presence or absence of both 20 U/mL IL-1β and 20 U/mL TNF-α.

*Quantitative reverse transcription PCR (qRT-PCR).*

Total RNA extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions was used as a template for one-step qRT-PCR with One Step SYBR® PrimeScript™ RT-PCR Kit II (Perfect Real Time) (TaKaRa). Amplification of the cDNA was performed using specific oligonucleotide primers (Table 1) with Applied Biosystems 7500 Real-Time PCR system according to the supplier's recommendations. The program used for qRT-PCR amplification included a 10-s inactivation of reverse transcriptase at 95°C, a 5-s denaturation step at 95°C, a 10-s annealing step at 58°C, a 34-s extension step at 72°C (for 35 cycles), and a dissociation step (15 s at 95°C, 60 s at 60°C, and 15 s at 95°C). To determine the specificity of each primer set, we performed melting curve analysis after the completion of PCR amplification. The accumulated levels of fluorescence were analyzed by the fit-point method after the melting curve analysis. The expression levels of mRNA were calculated using the $2^{-\Delta\Delta CT}$ method as previously described [12]. *NFKBIE*, *SLC19A1*, *ABCC1* (ATP-binding cassette, sub-family C (CFTR/MRP), member 1), *ABCC5* (ATP-binding cassette, sub-family C (CFTR/MRP), member 5), *ABCG2* (ATP-binding cassette, sub-family G (WHITE), member 2), *FPGS* (folylpolyglutamate synthase), *GGH* (gamma-glutamyl hydrolase (conjugase, folylpolygammaglutamyl hydrolase)), *DHFR*

(dihydrofolate reductase), *TYMS* (thymidylate synthetase), *MTHFR* (methylenetetrahydrofolate reductase (NAD(P)H)) and *ATIC* (5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase) gene mRNA levels were normalized with housekeeping gene *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) in each sample.

*Total cell and viable cell counting.*

All cells in one well were collected after trypsinization. The cell proliferation and viability were evaluated by counting the cells and 0.4% trypan blue (Wako) staining using the TC20 automated cell counter (Bio-Rad Laboratories) according to the manufacturer's instructions. To evaluate the number of total cells and live cells, we mixed the cell suspension 1:1 with 0.4% trypan blue solution and pipetted 10 μl of this mixture onto a counting slide specific for the TC20 automated cell counter.

*Statistical analysis.*

All of the data were compared using Student's *t*-test.

## RESULTS

*Effects of NFKBIE knockdown by siRNA in drug membrane transporters.*

To investigate the effects of *NFKBIE* knockdown on the expressions of drug membrane transporters we confirmed expression by qRT-PCR. The *NFKBIE* expression level in the *NFKBIE* knockdown group was almost 12-20 % of the control expression level under many conditions (Figure 1A). *NFKBIE* knockdown could reduce the *SLC19A1* expression (Figure 1B), whereas there was no obvious alteration in the expressions of the ABC transporters (Figure 1C-E).

*Uptake and efflux of MTX derivatives under the knockdown condition of NFKBIE by siRNA.*

Because several research studies have estimated that both the uptake and accumulation of MTX derivatives in mammalian cells are strongly affected by differences in the *SLC19A1* expression [13-18], we examined both the uptake and efflux of MTX derivatives and measured functional *SLC19A1* expressed in transfectant MH7A in an inflammatory milieu that mimics RA joints using flow cytometry. To determine whether knockdown of *NFKBIE* by siRNA affects the accumulation of MTX, MH7A was transfected and pre-treated with cytokines; then MTX derivative uptake into cells was examined. As shown in Figure 2A, B, knockdown of *NFKBIE* reduced the accumulation of MTX derivatives. But, we found no significant differences in the efflux of MTX derivatives between control and *NFKBIE* knockdown (Figure 2C).

*Effects of NFKBIE Val194Ala overexpression on drug membrane transporters.*

We transiently overexpressed either wild-type, Val194Ala or Mock into MH7A and incubated as described above. Small but statistically significant differences in the *SLC19A1* expression levels at a significant MTX level (> 5μM) were noted; the wild-type overexpression group exhibited more *SLC19A1* expression than the Val194Ala overexpression group (Figure 3A). In addition, this graph shows that overexpression of the wild-type gene had significantly more upregulated expression of *SLC19A1* than *NFKBIE* Val194Ala under no cytokines condition (Figure 3A). There were no statistical changes between wild-type and Val194Ala in the expressions of ABC transporters (Figure 3B-D).

*MTX-metabolizing enzymes expressions under knockdown and overexpression conditions.*

The expression levels of MTX-metabolizing enzymes including *FPGS*, *GGH*, *TYMS*, *DHFR*, *MTHFR* and *ATIC* were observed under different conditions (Figures 4 and 5). Our data indicate that the expressions of MTX-metabolizing enzymes except for *FPGS* had no significant associations either between control and *NFKBIE* knockdown, or between wild-type and Val194Ala *NFKBIE* overexpression (Figures 4 and 5). In *FPGS* expression, we observed that there was a general tendency toward a difference between the overexpression of the wild-type and Val194Ala gene in the overexpression study alone (Figure 5A).

## DISCUSSION

We have shown that, in a human rheumatoid arthritis synovial cell line, knockdown of *NFKBIE* expression results in the down-regulation of *SLC19A1* but does not influence ABC transporters or MTX-metabolizing enzymes. In addition, we have demonstrated that *NFKBIE* knockdown of MH7A results in lower intracellular accumulation of MTX derivatives than control MH7A. Furthermore, overexpression of the Val194Ala mutant *NFKBIE* resulted in decreased *SLC19A1* levels when compared to that of wild-type *NFKBIE* both above a specified MTX concentration (more than 5 μM) with cytokines and at a cytokine-free environment. ABC transporters and MTX-metabolizing enzymes expressions except for *FPGS*, lacked a significant difference between the overexpressed wild-type and Val194Ala *NFKBIE* constructs. In *FPGS* gene expression, there was a tendency of slight upregulation in the Val194Ala overexpression group compared to the wild-type overexpression group.

IκBε is known to be expressed in human synovial cells [19]. In IκBε-deficient mice, the expression of inflammatory cytokines such as IL-6, IL-1β, IL-1α and IL-1Ra, was constitutively increased compared to that in wild-type mice; also, there was increased B cell proliferation and survival [20, 21]. On the other hand, overexpression of IκBε with the nsSNP (rs2233433; Val194Ala) vector had an enhancement of NF-κB activity compared with the wild-type construct [8].

*SLC19A1*, a reduced folate carrier, is the most dominant folate transporter with high affinity for reduced folates and the antifolate drug MTX, but it has very low affinity for folic acid [22-24]. MTX enters the mammalian cells largely via *SLC19A1* and it effluxes from cells via ATP-binding cassette (ABC) transporters, which is to say that cellular uptake of MTX via *SLC19A1* is considered as the initial step for exerting its effect [25, 26]. Once entering the cells mainly via *SLC19A1*, MTX is polyglutamated by *FPGS*, retained as a polyglutamated form within the cell [27]. Polyglutamated MTX can be reversed to the nonpolyglutamated form by *GGH* and then extruded from cells via ABC transporters [28]. Monoglutamated MTX and polyglutamated MTX have equal binding affinity for *DHFR*, but polyglutamated MTX rather exerts a stronger inhibition of *DHFR*, *TYMS*, and *ATIC* [29-31]. *DHFR* plays a key role in converting dihydrofolate (DHF) to their active form tetrahydrofolate (THF), which is needed for several one-carbon transfer reactions in de novo synthesis of varieties of essential metabolites (e.g., purines, pyrimidines, etc.) [32]. *TYMS* is an important gene in *de novo* pyrimidine synthesis and for DNA synthesis and repair [33]. *ATIC* is an enzyme involved in the de novo purine synthesis pathway that is responsible for the conversion of AICAR into formyl-AICAR (FAICAR) [34]. Intracellular accumulation of AICAR owing mainly to direct inhibition of *ATIC* by polyglutamated MTX leads to increased

intracellular adenosine, a potent anti-inflammatory agent, and its consequent release to the extracellular space [35]. Additionally, *MTHFR* is thought to be a key enzyme that regulates DNA synthesis and DNA methylation [36]. Although *MTHFR* is not directly inhibited by MTX or by polyglutamated MTX, its expression level is recognized to be involved in MTX chemosensitivity [37].

Within the cell, folates, known as essential vitamins that serve as one-carbon donors in many biosynthetic pathways, also undergo polyglutamylation catalyzed by *FPGS*. As a result, polyglutamylated folates are enhanced the cellular retention and thus exert stronger effects [38]. The mRNA level of *FPGS* is upregulated by cell proliferation and reduced by cell maturation [39, 40]. The results obtained in synovial cells demonstrated that activated NF-κB pathways play an important role in cell proliferation [2]. We measured cell proliferation of overexpressing wild-type *NFKBIE* and Val194Ala *NFKBIE* by a cell counter. As a result, it was found that cells overexpressing Val194Ala constructs have a tendency for more cell-proliferative activity than those overexpressing wild-type constructs (Appendix 1). This observation is in agreement with our result of *FPGS* expression in an overexpression study.

The ABC transporter genes including *ABCC1*, *ABCC5* and *ABCG2* utilize the energy of ATP hydrolysis to translocate their substrates across biological membranes [24]. We have chosen these ABC transporter genes was because, in previous studies, transfectant cells overexpressing *ABCC1* and *ABCC5* displayed more MTX resistance levels than those of other ABC transporters as well as because *ABCG2* is a transporter of both MTX monoglutamates as well as di- and triglutamate conjugates of MTX, which is an important feature differentiating *ABCG2* from other transporters [24]. We found little differences in the mRNA expressions of those efflux transporters.

Although the molecular mechanisms for the reduction of *SLC19A1* expression are still unknown, our results strongly suggest that impairment of *NFKBIE* gene function suppressed *SLC19A1* expression. Studies in mouse synovial cells show that *SLC19A1* expression was down-regulated by inflammatory components such as IL-6 [18]. In RA patients, there was a significant negative correlation between *SLC19A1* expression and ESR [10]. These studies lead us to believe that *SLC19A1* downregulation is associated with inflammation. MTX itself is closely related to *SLC19A1* expression [18, 41]. More recently, it has become more obvious that MTX induces proinflammatory cytokines, such as IL-6, which may be one of the reasons MTX has some effects on the expression of *SLC19A1* [42, 43]. As previously mentioned, IκBε-deficient mice have increased expression of some inflammatory cytokines [20, 21]. *SLC19A1* expression is reportedly upregulated under the folate-restricted condition, but increased folate availability suppresses its expression [44, 45]. Jansen et al. identified that, upon growth in a low folate

medium, a mutation in the *SLC19A1* gene results in increasing affinity of transporters for (anti)folates and that in folate-replete medium, the preferential folate transport results in a markedly expanded intracellular folate pool impairing antifolate metabolism [46]. We demonstrated that in an overexpression study, there were no significant differences in the *SLC19A1* expression level between wild-type and Val194Ala at a low MTX concentration (0.01 – 1 μM) of cytokines. Because the medium used in our study contained far (approximately 100 times) higher levels of folate than the *in vivo* folate level [46], the dose-effect relationship between MTX and *SCL19A1* suppression may also be shifted to the higher concentration side.

In conclusion, the results obtained in this paper support the hypothesis that *NFKBIE* may play a role in regulating the expression of some MTX pathway genes in a cell culture model which reproduced the synovial cell context in RA joints as accurately as possible by the addition of inflammatory cytokines, and the results may be helpful for further understanding of the MTX-resistant mechanism. This study provides an important contribution because these findings were detected in *NFKBIE* (rs2233434, Val194Ala), an RA susceptibility gene locus in a large-scaled GWAS meta-analysis for RA in the Japanese population. The present study suggests that impairment of *NFKBIE* gene function may result in the downregulation of *SLC19A1*, reduction of MTX uptake and MTX-resistance, although further research with additional *in vivo* studies is also needed.

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*Figure 1.*

**Effects of *NFKBIE* knockdown on the expression levels of SLC19A1 and ABC transporters.**

Quantitative gene expressions of *NFKBIE* (A), *SLC19A1* (B), *ABCC1* (C), *ABCC5* (D) and *ABCG2* (E) genes in control (gray) and *NFKBIE* knockdown (black) MH7A treated with or without 0.01, 0.1, 1, 5 and 10 μM methotrexate (MTX) application for 24 hours in the presence or absence of 20 U/mL IL-1β and 20 U/mL TNF-α were measured by qRT-PCR. Statistical significance was analyzed using the unpaired *t*-test (*$P < 0.05$). The results are presented as the mean ± standard error of the mean (SEM) of at least three independent experiments.

*Figure 2.*

**Uptake and efflux of a methotrexate (MTX) derivative under knockdown of *NFKBIE* by siRNA in MH7A.**

MH7A cells were cultured for 24 hours, and then transfected for 24 hours. They were further incubated with 20 U/mL IL-1β and 20 U/mL TNF-α for 24 hours. After washing the cells with PBS, the cells were incubated in the presence of 5 μM of MTX derivatives for 5 hours. The uptake of MTX derivatives was quantified by measuring the fluorescence emission for each sample. (A) provides the representative histogram of control (blue) and knockdown of *NFKBIE* (dark red). The mean fluorescent intensity (MFI) per cell, expressed in arbitrary units, was then determined using a flow cytometer. (B) shows the summary results. In addition to these experiments, after incubating with MTX derivatives (5 μM) for 5 hours and washing cells with PBS, cells were chased for 6 hours exclusively in RPMI supplemented with 10 % FBS. Subsequently, the MFI per cell was measured at 0 and 6 hours. On the basis of these results, differences in the efflux of MTX derivatives between control and *NFKBIE* knockdown were studied (C). Each column and its corresponding vertical lines represent the mean and the standard error of the mean (SEM) of triplicate cultures. Statistical significance was analyzed with the unpaired *t*-test (* $P < 0.05$).

*Figure 3.*

**Effects of overexpression of Mock, wild-type *NFKBIE* and Val194Ala mutant *NFKBIE* on the expressions of *SLC19A1* and the ABC transporters.**

Quantitative gene expressions of *SLC19A1* (A), *ABCC1* (B), *ABCC5* (C) and *ABCG2* (D) genes in Mock (white), overexpression of wild-type *NFKBIE* (gray) and Val194Ala mutant *NFKBIE* (black). Those cells were treated with or without 0.01, 0.1, 1, 5 and 10 μM methotrexate (MTX) application for 24 hours in the presence or absence of 20 U/mL IL-1β and 20 U/mL TNF-α. The expression levels of each gene were measured by qRT-PCR. Statistical significance was analyzed using the unpaired *t*-test (*$P < 0.05$). The

results are presented as the mean ± standard error of the mean (SEM) of at least three independent experiments. *$P < 0.05$ *vs.* wild-type.

*Figure 4.*

**Effects of *NFKBIE* knockdown on the expressions of MTX-metabolizing enzymes.**

Quantitative gene expressions of *FPGS* (A), *GGH* (B), *DHFR* (C), *TYMS* (D), *MTHFR* (E) and *ATIC* (F) genes in control (gray) and *NFKBIE* knockdown (black) MH7A. Those cells were treated with or without 0.01, 0.1, 1, 5 and 10 μM methotrexate (MTX) application for 24 hours in the presence or absence of 20 U/mL IL-1β and 20 U/mL TNF-α. The expression levels of each gene were measured by qRT-PCR. Statistical significance was analyzed by the unpaired *t*-test (*$P < 0.05$). The results are presented as the mean ± standard error of the mean (SEM) of at least three independent experiments.

*Figure 5.*

**Effects of overexpression of Mock, wild-type *NFKBIE* and Val194Ala mutant *NFKBIE* on MTX-metabolizing enzyme expressions.**

Quantitative gene expressions of *FPGS* (A), *GGH* (B), *DHFR* (C), *TYMS* (D), *MTHFR* (E) and *ATIC* (F) genes in Mock (white), overexpression of wild-type *NFKBIE* (gray) and Val194Ala mutant *NFKBIE* (black). Those cells were treated with or without 0.01, 0.1, 1, 5 and 10 μM methotrexate (MTX) application for 24 hours in the presence or absence of 20 U/mL IL-1β and 20 U/mL TNF-α. The expression levels of each gene were measured by qRT-PCR. Statistical significance was analyzed using the unpaired *t*-test (*$P < 0.05$). The results are presented as the mean ± standard error of the mean (SEM) of at least three independent experiments. *$P < 0.05$ *vs.* wild-type.

*Appendix 1.*

**Effects of overexpression of Mock, wild-type *NFKBIE* and Val194Ala mutant *NFKBIE* on cell proliferation.**

The effects of in Mock (white), overexpression of wild-type *NFKBIE* (gray) and Val194Ala mutant *NFKBIE* (black) on cell proliferation were measured. MH7A cells were plated at $1.50 \times 10^5$ cells/well and cultured for 24 hours. In turn, cells were transfected for 18 hours as explained above. After changing medium, they were incubated with or without 0.01, 0.1, 1, 5 and 10 μM methotrexate (MTX) for 24 hours in the presence or absence of both 20 U/mL IL-1β and 20 U/mL TNF-α. All cells in a single well were collected after trypsinization and measured by a TC20 automated cell counter. The number of cells was used to determine the proliferation of transfected MH7A. The results are presented as the mean ±

standard error of the mean (SEM) of at least four independent experiments. Statistical significance was analyzed by the unpaired *t*-test (*$P < 0.05$). *$P < 0.05$ *vs.* wild-type.

Figure 1

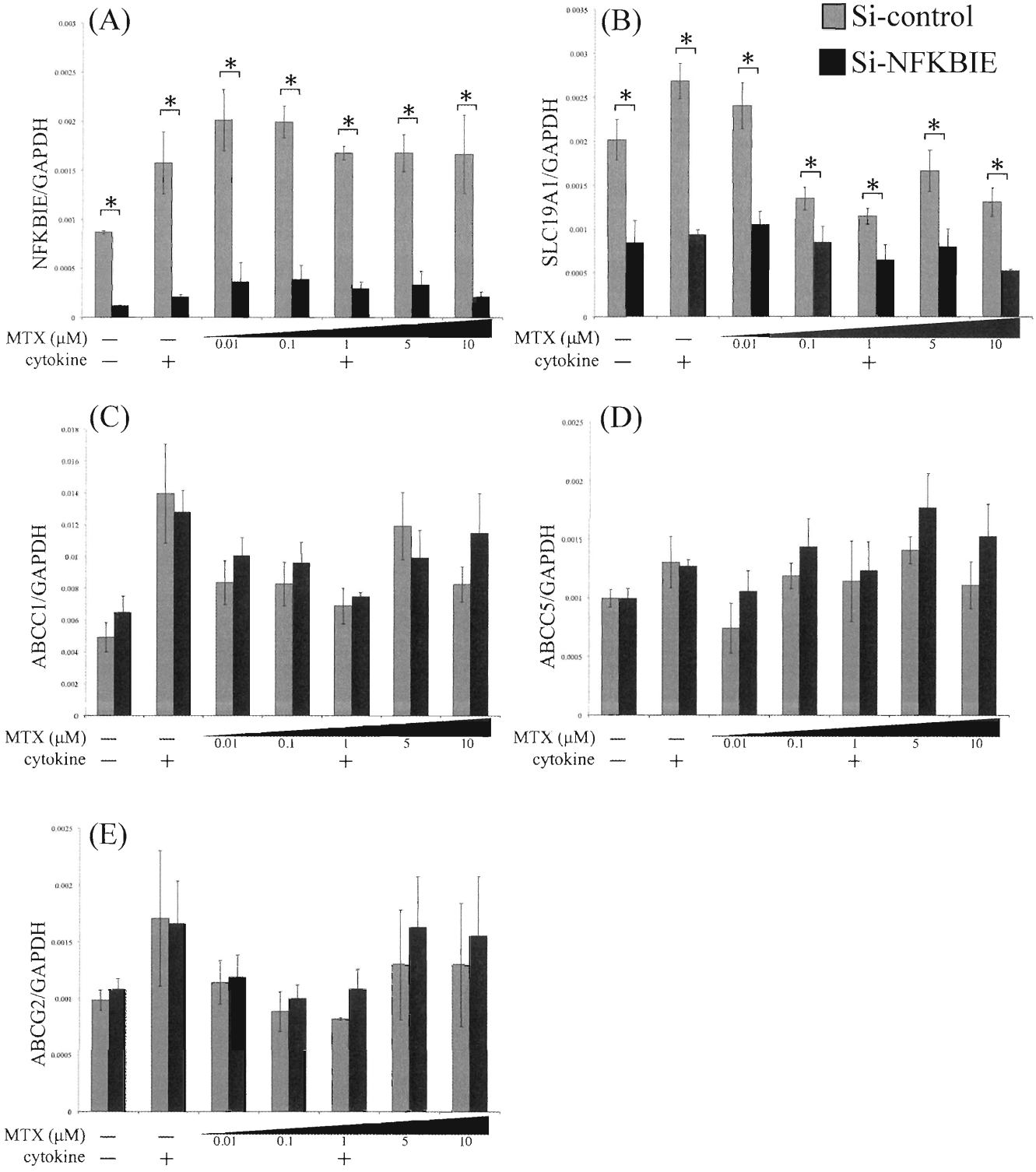

(A)
NFKBIE/GAPDH
MTX (μM) — — 0.01 0.1 1 5 10
cytokine — + +
(B)
Si-control
Si-NFKBIE
SLC19A1/GAPDH
MTX (μM) — — 0.01 0.1 1 5 10
cytokine — + +
(C)
ABCC1/GAPDH
MTX (μM) — — 0.01 0.1 1 5 10
cytokine — + +
(D)
ABCC5/GAPDH
MTX (μM) — — 0.01 0.1 1 5 10
cytokine — + +
(E)
ABCG2/GAPDH
MTX (μM) — — 0.01 0.1 1 5 10
cytokine — + +

Figure 2

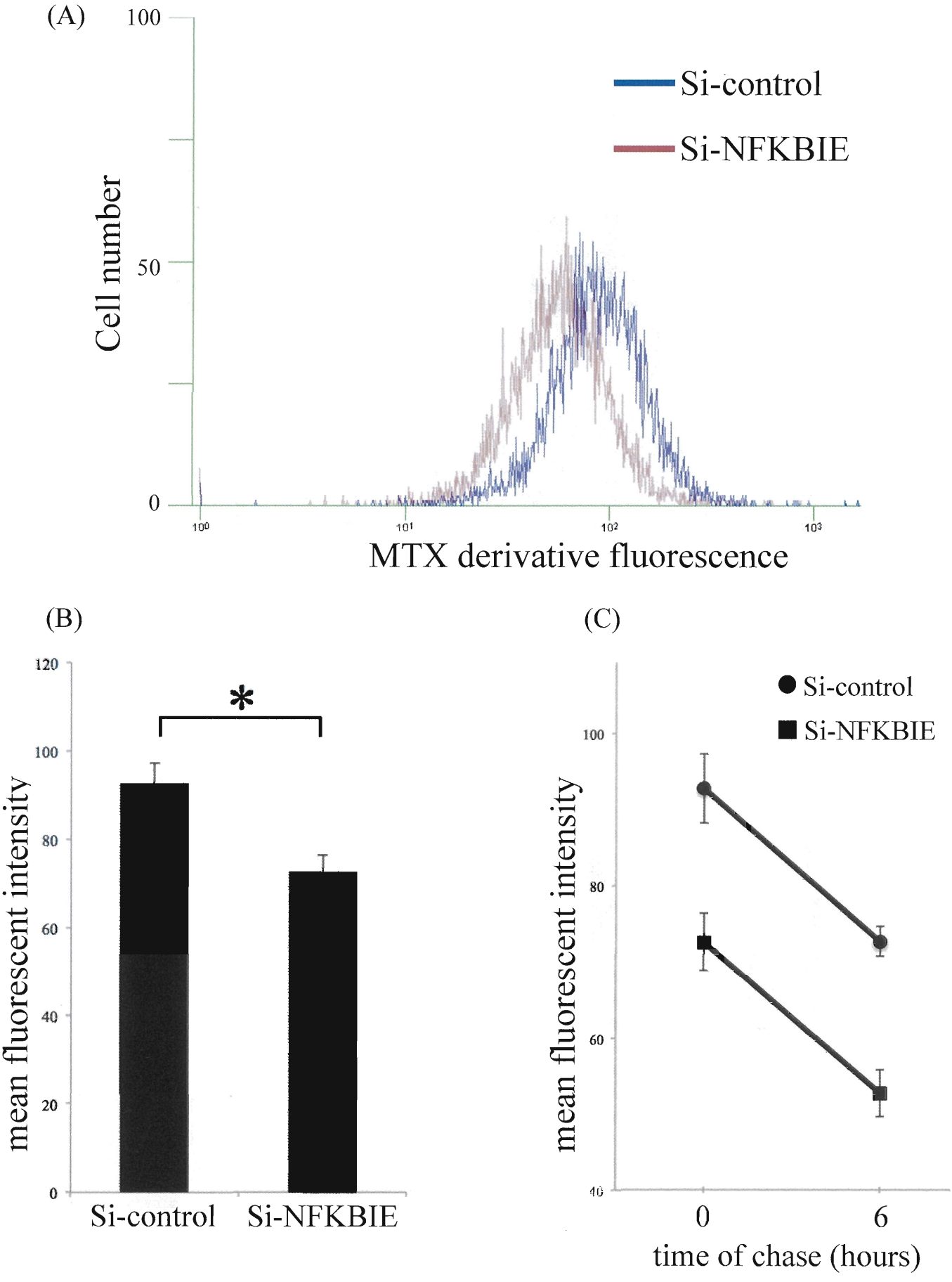

(A)
100
50
0
Cell number
Si-control
Si-NFKBIE
10⁰
10¹
10²
10³
MTX derivative fluorescence
(B)
120
100
80
60
40
20
0
mean fluorescent intensity
*
Si-control
Si-NFKBIE
(C)
Si-control
Si-NFKBIE
100
80
60
40
mean fluorescent intensity
0
6
time of chase (hours)

Figure 3

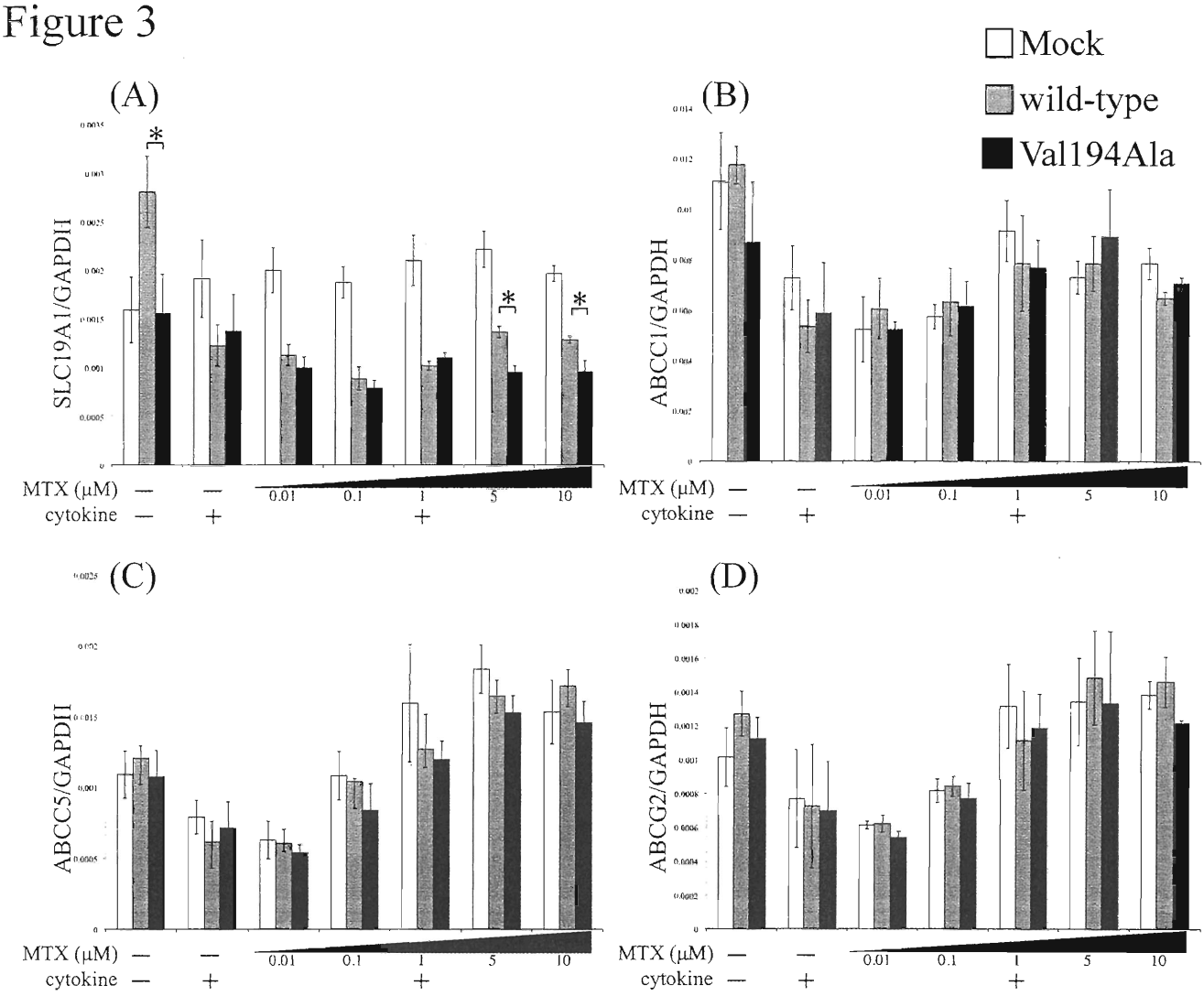

Mock
wild-type
Val194Ala
(A)
SLC19A1/GAPDII
*
*
*
MTX (μM) — — 0.01 0.1 1 5 10
cytokine — + +
(B)
ABCC1/GAPDH
MTX (μM) — — 0.01 0.1 1 5 10
cytokine — + +
(C)
ABCC5/GAPDII
MTX (μM) — — 0.01 0.1 1 5 10
cytokine — + +
(D)
ABCG2/GAPDH
MTX (μM) — — 0.01 0.1 1 5 10
cytokine — + +

Figure 4

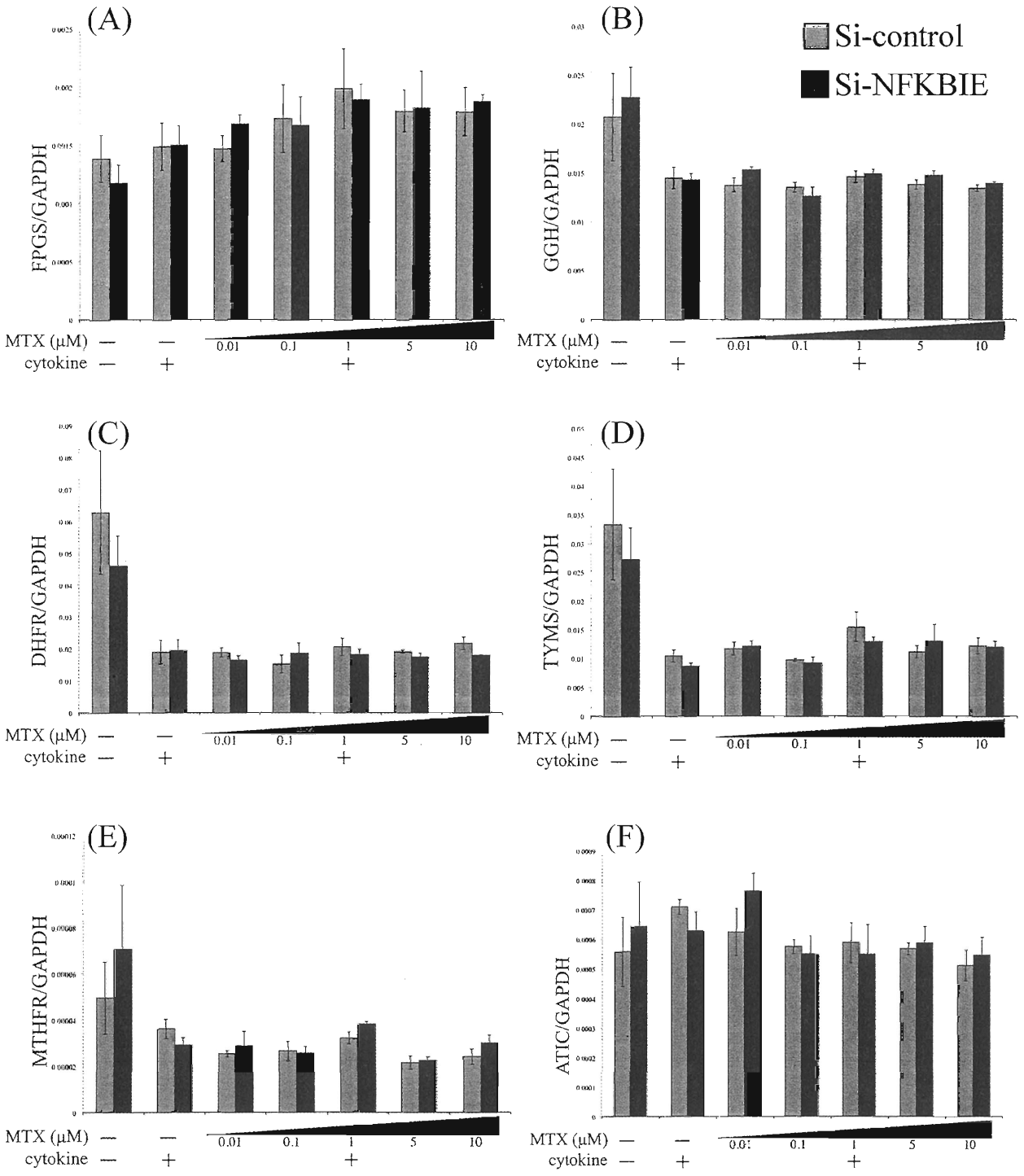
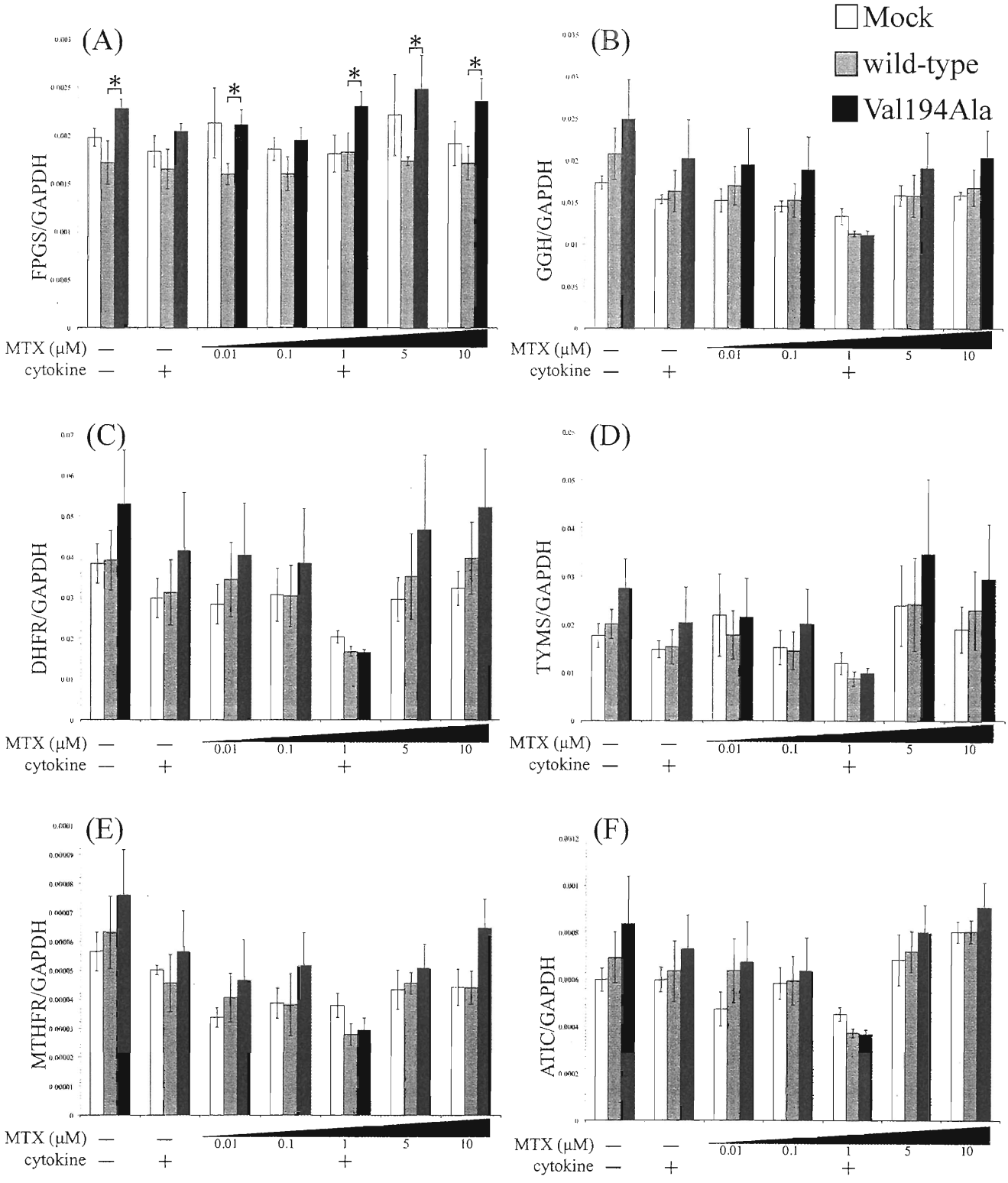

(A)
FPGS/GAPDH
MTX (μM) — — 0.01 0.1 1 5 10
cytokine — + +
(B)
Si-control
Si-NFKBIE
GGH/GAPDH
MTX (μM) — — 0.01 0.1 1 5 10
cytokine — + +
(C)
DHFR/GAPDH
MTX (μM) — — 0.01 0.1 1 5 10
cytokine — + +
(D)
TYMS/GAPDH
MTX (μM) — — 0.01 0.1 1 5 10
cytokine — + +
(E)
MTHFR/GAPDH
MTX (μM) — — 0.01 0.1 1 5 10
cytokine — + +
(F)
ATIC/GAPDH
MTX (μM) — — 0.01 0.1 1 5 10
cytokine — + +

Figure 5


(A)
FPGS/GAPDH
(B)
GGH/GAPDH
Mock
wild-type
Val194Ala
(C)
DHFR/GAPDH
(D)
TYMS/GAPDH
(E)
MTHFR/GAPDH
(F)
ATIC/GAPDH
MTX (μM) — — 0.01 0.1 1 5 10
cytokine — + +
*

Appendix 1

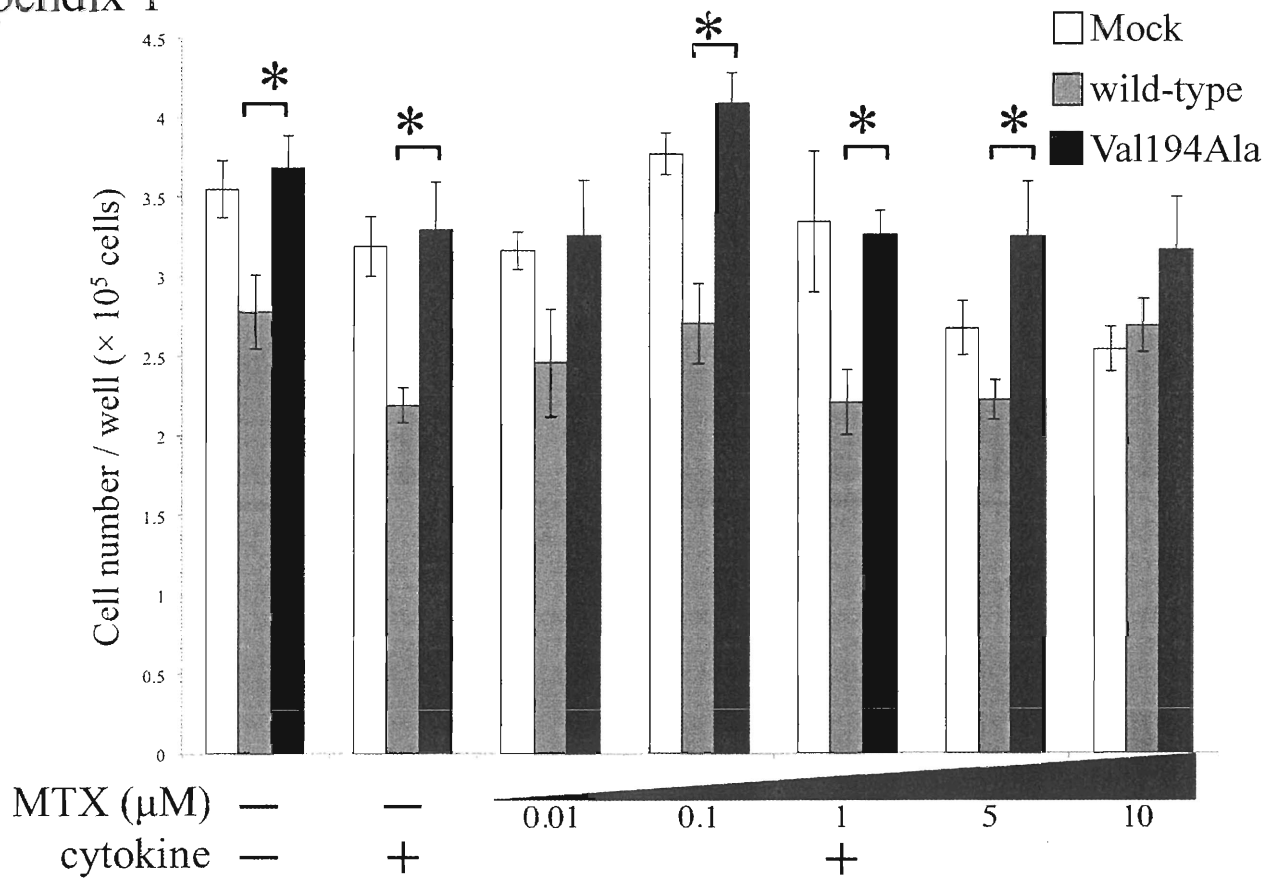

Mock
wild-type
Val194Ala
Cell number / well (× 10^5 cells)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
*
*
*
*
MTX (μM) — — 0.01 0.1 1 5 10
cytokine — + +

Table 1.

Primers for quantitative real-time PCR (qPCR).

| Genes name | GenBank accession number | Primer (5' →3' ) | Amplicon length (bp) |
|---|---|---|---|
| GAPDH<br>glyceraldehyde-3-phosphate<br>dehydrogenase | NG_007073 | F: GAA GGT GAA GGT CGG AGT C<br>R: GAA GAT GGT GAT GGG ATT TC | 226 |
| NFKBIE<br>nuclear factor of kappa light polypeptide<br>gene enhancer in B-cells inhibitor, epsilon | NM_004556 | F: GCC AGG CAG AGG AAC ATC TC<br>R: GCA GCG CTG TCT TAC CAC TG | 188 |
| SLC19A1<br>solute carrier family 19 (folate<br>transporter), member 1 | NG_028278 | F: TTC ATG GCG CAG ATA CGG<br>R: ACG GCC AGG TAG GAG TAC GA | 128 |
| ABCC1<br>ATP-binding cassette, sub-family C<br>(CFTR/MRP), member 1 | NG_028268 | F: TCA CCA CGT ACT TGA ACT GGC TG<br>R: GCA GGC AGT AGT TCC GGA ATT C | 183 |
| ABCC5<br>ATP-binding cassette, sub-family C<br>(CFTR/MRP), member 5 | NM_005688 | F: ACT CTC TAT CAT TCC TCA AGA GCC G<br>R: TAC TGG TTG AAG GGG TCC AAA TTT G | 75 |
| ABCG2 | NG_032067 | F: ACT GGG ACT GGT TAT AGG TGC C | 138 |

| | | | |
|---|---|---|---|
| ATP-binding cassette, sub-family G (WHITE), member 2 | | R: ACC ACA AAG AGT TCC ACG GC | |
| FPGS folylpolyglutamate synthase | NG_023245 | F: CCA GTT TGA CTA TGC CGT CTT C<br>R: TGT CAC TGT GAA GTT CTG TTG G | 85 |
| GGH gamma-glutamyl hydrolase | NG_028126 | F: ATT TCC CAT GCA CCT AAT GC<br>R: TGC TTT CTC CTC TTC AGA TTC AG | 114 |
| DHFR dihydrofolate reductase | NG_023304 | F: ATC ACC CAG GCC ATC TTA AAC<br>R: TGG ACA TCA GAG AGA ACA CCT G | 133 |
| TYMS thymidylate synthetase | NG_028255 | F: GGA GTT GAC CAA CTG CAA AGA G<br>R: CAC CAC ATA GAA CTG GCA GAG G | 144 |
| MTHFR methylenetetrahydrofolate reductase | NG_013351 | F: GGA GGA GGG AGG CTT CAA CTA C<br>R: CTT CAG GTC AGC CTC AAA GCT C | 140 |
| ATIC 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase | NG_013002 | F: GAG AGC AGA AAT CTC CAA TGC<br>R: AAC CCA TTC CTT CTT CTC TGC | 136 |