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Methotrexate increases expression of cell cycle checkpoint genes via Jun-N-terminal kinase activation

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Abstract

Objective—To assess defects in expression of critical cell cycle checkpoint genes and proteins in subjects with rheumatoid arthritis relative to presence or absence of methotrexate medication and assess the role of Jun N-terminal kinase in methotrexate induction of these genes.

Methods—Flow cytometry analysis was used to quantify changes in intracellular proteins, measure reactive oxygen species (ROS), and determine apoptosis in different lymphoid populations. Quantitative reverse transcriptase polymerase chain reaction (Q-RT-PCR) was employed to determine changes in cell cycle checkpoint target genes.

Results—RA subjects express lower baseline levels of *MAPK9*, *TP53*, *CDKN1A*, *CDKN1B*, *CHEK2*, and *RANGAP1* messenger RNA (mRNA) and total JNK protein. *MAPK9*, *TP53*, *CDKN1A*, and *CDKN1B* mRNA expression, but not *CHEK2*, and *RANGAP1*, is higher in patients on low-dose MTX therapy. Further, JNK levels inversely correlate with CRP levels in RA patients. In tissue culture, MTX induces expression of both p53 and p21 by JNK2 and JNK1-dependent mechanisms, respectively, while *CHEK2* and *RANGAP1* are not induced by MTX. MTX also induces ROS production, JNK activation, and sensitivity to apoptosis in activated T cells. Supplementation with tetrahydrobiopterin blocks these MTX-mediated effects.

Conclusions—Our findings support the notion that MTX restores some, but not all of the proteins contributing to cell cycle checkpoint deficiencies in RA T cells by a JNK dependent pathway.

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Conflict of interest statement

TMA and NJO are co-owners of ArthroChip. Other authors claim no conflicts of interest.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting and revising the manuscript and all authors approved the final version to be published. Dr. Aune had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study Design: CFS, JTT, NJO, TMA

Sample recruitment and clinical characteristics: HAF, NJO

Experimentation and analysis: All authors contributed to experimentation or analysis of the data.

Keywords

Rheumatoid Arthritis (RA); Disease-Modifying Antirheumatic Drugs (DMARDs); Methotrexate (MTX); Apoptosis; p53; p21; Jun N-terminal kinase (JNK); Tetrahydrobiopterin (BH4); Reactive Oxygen Species (ROS)

INTRODUCTION

Rheumatoid arthritis is the most common serious autoimmune disease affecting 1.3 million people in the United States (1). Often characterized by bone erosion and cartilage destruction through chronic inflammation, RA is a multisystem disorder affecting synovial spaces between small and large joints. Accounting for 250,000 hospitalizations and 9 million clinic visits each year, costs associated with RA represent approximately 1% of the U.S. gross domestic product (2).

While initially developed as a chemotherapeutic, methotrexate (MTX) has been the mainstay for RA treatment since the 1980s (3-7). Once-weekly administration of 7.5 to 25 milligrams yields optimal clinical outcomes, compared to the 5000 mg/week dosage used in the treatment of malignancy (7, 8). MTX is a potent, competitive inhibitor of dihydrofolate reductase (DHFR) (9-11) resulting in decreased tetrahydrofolate levels, and inhibition of de novo purine and pyrimidine synthesis leading to cell cycle arrest (8, 12). However, mechanisms of action surrounding low-dose, once weekly MTX may differ significantly from high dose therapy with MTX. Since supplementation with low doses of folic acid, 1-5 mg/day, in RA patients does not attenuate clinical efficacy of MTX, the anti-inflammatory actions of low-dose MTX may stem from alternative pathways (8, 13, 14).

Although the etiology of RA is incompletely understood, T lymphocytes from subjects with RA exhibit loss of genomic integrity and deficiencies in specific proteins that repair DNA damage and induce cell cycle arrest and apoptosis. Specifically, reduced expression of ataxia telangiectasia (AT) mutated (ATM), a critical component of DNA damage repair and activation of p53-dependent cell cycle arrest and apoptosis, p53 itself, checkpoint kinase 2, which also phosphorylates p53, and cyclin kinase inhibitors, p21 and p27, contribute to these defects in RA (15-19).

We recently found that Jun-N-terminal kinase (JNK), a MAP kinase, is activated by MTX through production of reactive oxygen species (ROS) due to uncoupling of nitric oxide synthase (NOS) arising from MTX-dependent inhibition of DHFR, which blocks reduction of dihydrobiopterin (BH2) to tetrahydrobiopterin (BH4). MTX-mediated JNK activation results in induction of pro-apoptotic target genes and increased sensitivity to apoptosis (20). Since JNKs, members of the MAP kinase family of proteins, also directly phosphorylate p53 leading to its increased accumulation and activity (21-23) we hypothesized that JNKs, or MAPKs in general, may also be deficient in RA and that MTX therapy may correct, not only JNK deficiency, but also deficiencies in critical regulators of cell cycle checkpoints. Here, we show that RA lymphocytes exhibit a selective deficiency in *MAPK9* (JNK2), but not other MAPK transcripts. *MAPK9*, *TP53*, *CDKN1A*, and *CDKN1B* transcript levels, along with total JNK protein levels, are significantly lower in RA subjects compared to healthy control subjects (CTRL). Further, *MAPK9*, *TP53*, *CDKN1A*, and *CDKN1B* transcript levels, but not *CHEK2* and *RANGAP1*, are elevated in RA subjects taking MTX compared to RA subjects not taking MTX. In cell culture models, MTX directly induces increased expression of p53, p21, and p27, but not *CHEK2* and *RANGAP1*. We hypothesize that therapeutic activity of MTX may arise, in part, from its ability to restore expression levels of key proteins required for cell cycle checkpoint arrest and that defects in the cell cycle and

DNA damage-response pathway may not only contribute to disease pathogenesis but may also serve as important markers of RA disease progression and may represent novel therapeutic targets for disease management.

PATIENTS AND METHODS

Patient Populations

Our study group contained 43 CTRL subjects with no current chronic or acute infection and no family history of autoimmune disease, and 36 subjects meeting the American College of Rheumatology (ACR) clinical criteria for RA (24). Demographic characteristics of the different disease and therapy groups were not statistically different (Table 1). Blood samples were also obtained from subjects with the following autoimmune diseases: multiple sclerosis (MS), ulcerative colitis (UC), Crohn's disease, and systemic lupus erythematosus (SLE). These samples were collected from three different sites in the U.S. Age, race and gender were not statistically different among the study groups. Relevant institutional review board approval from all participating sites was obtained.

Drugs and Reagents

MTX, BH4, caffeine, theophylline, folic acid, and N-acetyl-L-cysteine (NAC) were from Sigma. CM-H2DCFDA was obtained from Invitrogen.

Cell Culture

Cells were cultured in RPMI 1640 media (1 μ g/ml folic acid) supplemented with 10% (V/V) FBS, 1% (V/V) penicillin-streptomycin, and 1% (V/V) L-glutamine at 37°C in 5% CO₂. The Jurkat human T cell line was obtained from the American Type Culture Collection (ATCC). Peripheral blood mononuclear cells (PBMC) were purified by Ficoll-Hypaque centrifugation or by using a cell preparation tube with sodium heparin following the manufacturer's published protocol (BD Biosciences #362753). For T cell activation, PBMC were cultured for 72 hours with anti-CD3 antibody (OKT3 Clone ATCC) in complete media containing 30 units/mL interleukin-2 (IL-2). Concentrations of MTX ranged from 0.1 μ M to 1 μ M, and culture periods ranged from 24 hours to 48 hours of continuous exposure to MTX. Pharmacokinetic analysis indicates that ingestion of a 20-mg tablet of MTX produces plasma MTX concentrations of ~0.5 μ M after 1 hour and ~0.1 μ M after 10 hours (25).

RNA isolation and quantitative real time polymerase chain reaction (Q-RT-PCR)

Total RNA was purified from blood collected in PAXgene tubes according to manufacturer's instructions (Qiagen) or from cell cultures using Tri-Reagent (Molecular Research Center) and quantified using a NanoDrop-1000 spectrophotometer. cDNA was synthesized from 5 μ g total RNA (SuperScript III First-Strand Synthesis Kit, Invitrogen) with Oligo dT as the primer. Q-RT-PCR reactions were prepared in duplicate in volumes of 25 μ L with 50ng cDNA, TaqMan assay mix, and TaqMan gene expression assay. *GAPDH* was used as a housekeeping gene and control. Q-RT-PCR was performed using the ABI-7300 Real Time PCR System (Applied Biosystems).

C reactive protein (CRP) & JNK assay

Whole blood samples were obtained from RA subjects. From these blood samples, PBMC were isolated via Ficoll-Hypaque centrifugation. Isolated PBMC were fixed and permeabilized prior to flow cytometry analysis to quantify total JNK protein levels, ex vivo. Plasma from each blood sample was retained for CRP analysis via enzyme linked immunosorbent assay (ELISA) following the manufacturer's supplied protocol (R&D Systems).

Flow cytometry

Cells were suspended in PBS with 10% FBS and 0.1% sodium azide. For intracellular protein determinations, cells were fixed with paraformaldehyde, permeabilized (triton X-100 and NP-40) using Perm/Wash Buffer (BD Biosciences), and labeled with primary antibodies for 24 hours at 0-4°C followed by incubation with fluorescent-labeled secondary antibodies for 1 hour at 0-4°C. The following primary antibodies were used: rabbit anti-JNK (Santa Cruz, sc-571), polyclonal rabbit anti-P-JNK (pT183/pY185) (BD Pharmingen 558268), polyclonal rabbit anti-p38 (Cell Signaling, 9212), polyclonal rabbit anti-p53 (Novus Biologicals, NB200-171), polyclonal rabbit anti-p21 (Abcam, ab7960); secondary antibodies: fluorescein isothiocyanate (FITC) – labeled goat anti-rabbit Ig (BD Pharmingen, 554020), and phycoerythrin (PE) - labeled goat anti-rabbit IgG (Southern Biotech, 4050-09). The following surface stains were used: Pacific Blue mouse anti-human CD4 (BD Pharmingen, 558116), Alexa Fluor 700 mouse anti-human CD8 (BD Pharmingen, 557945), and APC mouse anti-human CD19 (BD Pharmingen 555335). Apoptosis determinations utilized the PE Annexin-V Apoptosis Detection Kit I from BD Pharmingen. Cells were analyzed using a 3-laser BD LSRII flow cytometer in the Vanderbilt Medical Center Flow Core or a BD FACSCantoII in the Hershey Medical Center Flow Cytometry Core.

Plasmids and Cell transfection

JNK1 (MAPK8) and JNK2 (MAPK9) dominant-negative (DN) mutants (Addgene) were transfected into Jurkat T Cells using the Amaxa Cell Line Nucleofector Kit V (Amaxa, Koeln, Germany) according to the manufacturer's protocol.

Statistical Analysis

Statistically significant differences between groups were determined by Student's t-test. P values less than 0.05 were considered significant.

RESULTS

Reduced *MAPK9* expression, *in vivo*, in RA

Previously, we demonstrated that MTX stimulates increased JNK activity and expression levels in tissue culture models (20). Therefore, we sought to determine transcript levels of MAPK family members in RA and CTRL subjects by analyzing gene expression levels in subject blood samples collected in PAXgene tubes. Expression levels, determined by Q-RT-PCR, were calculated as the ratio to *GAPDH* for normalization. *MAPK8* and *MAPK9* genes correspond to JNK1 and JNK2 proteins, respectively. RA subjects exhibited significantly lower *MAPK9* mRNA levels. Transcript levels of other MAP Kinases, including genes encoding p38 and extracellular signal regulated kinase (ERKs) proteins, were not statistically different between the RA cohort and CTRL (Supplementary Table 1).

We next measured JNK expression levels by flow cytometry. Total JNK protein levels in RA subjects not on MTX were diminished in CD4 and CD8 T cells, as well as CD19+ B cells relative to CTRL subjects (Fig. 1 A). In contrast, p38 protein levels, another member of the MAP kinase family, often activated by a variety of different cellular stresses including inflammatory cytokines, UV exposure, and growth factors (26, 27), were not significantly different between the RA and CTRL cohorts in any of the lymphocyte subsets examined.

JNK expression correlates with decreased C reactive protein, *in vivo*

C reactive protein (CRP), found in plasma, is a common marker of inflammation often used during the treatment of rheumatoid arthritis to gauge disease activity or therapeutic efficacy (28, 29). Building upon our findings that JNK levels are diminished in RA patients not on

MTX, we sought to better understand the relationship between JNK and inflammation. Plasma and PBMC were isolated from RA patients not on current MTX therapy. JNK levels were determined by flow cytometry and CRP levels were measured by ELISA (R&D Systems). We found an inverse correlation between lymphocyte JNK levels and CRP concentrations, *in vivo* (Figure 1B). Thus, independent of MTX therapy, low JNK levels were associated with higher levels of inflammation and high JNK levels were associated with decreased levels of inflammation in RA.

MTX therapy restores *MAPK9* expression levels, *in vivo*

We next determined expression levels of *MAPK9* in RA subjects on or not on current MTX therapy relative to CTRL subjects. *MAPK9* levels in RA subjects on MTX therapy were significantly higher than those without MTX (Figure 1C). However, the mean *MAPK9* expression level in the RA + MTX cohort was still lower than in the CTRL cohort. Given these results, we analyzed additional autoimmune diseases to determine if *MAPK9* deficiency was unique to RA. Among the diseases examined, multiple sclerosis (MS), a neurologic autoimmune disease, also exhibited decreased transcript levels of *MAPK9* relative to CTRL (Figure 1D). In contrast, subjects with the autoimmune diseases, ulcerative colitis (UC), Crohn's, or systemic lupus erythematosus (SLE) did not exhibit decreased *MAPK9* transcript levels. Thus, we conclude that *MAPK9* deficiency exists in MS but is not a general feature of all human autoimmune diseases.

MTX therapy restores *TP53*, *CDKN1A*, and *CDKN1B* levels, *in vivo*

We next examined expression levels of genes encoding proteins necessary for cell cycle checkpoint arrest including: *TP53* (p53), *CDKN1B* (p27), *CDKN1A* (p21), *CHEK2* (CHK2), and *RANGAP1* (RANGAP1). Transcript levels of each gene were significantly under-expressed in lymphocytes from subjects with RA not on current MTX therapy (Figure 2). We compared expression levels of these additional genes in RA subjects on or not on current MTX therapy to expression levels of CTRL subjects. As with *MAPK9* deficiency, we found that *TP53*, *CDKN1A*, and *CDKN1B* levels were significantly increased in RA subjects on MTX therapy relative to those not on MTX, but levels were not restored to mean levels of the CTRL population. Transcript levels of *CHEK2* and *RANGAP1* were also decreased in RA subjects compared to CTRL. In contrast to expression levels of *TP53*, *CDKN1A*, and *CDKN1B*, there were no differences in transcript levels of either *CHEK2* or *RANGAP1* in the RA groups with or without MTX therapy. These studies suggest that MTX may restore deficiencies in expression of cell cycle checkpoint proteins, p53, p21, and p27, but not deficiencies in expression of cell cycle checkpoint proteins, CHK2 and RANGAP1.

Induction of *TP53* and *CDKN1A* by MTX in Jurkat T cells

Given our findings that expression levels of *TP53*, *CDKN1A*, and *CDKN1B*, but not *RANGAP1* or *CHEK2*, were elevated in RA subjects taking MTX compared to those not taking MTX, we sought to better understand mechanistic underpinnings of these differences. Previously we have shown that sub-micromolar concentrations of MTX induces transcription of *JUN* in a homogeneous T cell population (Jurkat T cells) by DHFR-dependent depletion of BH4 resulting in uncoupling of NOS and corresponding increased production of reactive oxygen species (ROS) and JNK activation (20). Therefore, we employed this model system to investigate MTX-induction of *TP53*, *CDKN1A*, *RANGAP1* and *CHEK2*. In these expression studies, we found that MTX directly stimulated an increase in transcript levels of *TP53* and *CDKN1A* (Figure 3A). In contrast, MTX did not significantly alter expression levels of *CHEK2* and *RANGAP1*. Since MTX stimulates adenosine release and activation of adenosine receptors, we determined if the broad-spectrum adenosine receptor antagonists, caffeine and theophylline, at pharmacologic

concentrations, significantly altered the transcriptional profile of these genes. Treatment with caffeine, theophylline, or the combination, did not alter induction of *TP53* or *CDKN1A* transcripts by MTX (Figure 3B). In contrast, supplementation with either the free radical scavenger N-acetyl-L-cysteine (NAC) or folic acid prevented induction of *TP53* and *CDKN1A* by MTX (Figure 3C). NAC supplementation of MTX-treated cultures prevents ROS production, JNK activation, and induction of JUN, a JNK target gene (20). Folic acid, at very high concentrations (100 μ M), also increases intracellular BH4 levels through its active form, 5-methyltetrahydrofolate, and restores the amount of available nitric oxide in the cell (30, 31). We also treated Jurkat cells with MTX and assayed intracellular protein concentrations by flow cytometry. MTX treatment significantly increased levels of JNK, p-JNK, p53, and p21. Supplementation with BH4 significantly reduced expression of these proteins (Figure 3D). MTX alters the transcriptional profile of cells by increasing expression of a number of genes that encode proteins with pro-apoptotic function (20). This shift depends upon ROS-stimulated phosphorylation of JNK. Therefore, we performed transient transfection experiments with JNK1- and JNK2-DN mutants to determine if either JNK1 or JNK2 activity was necessary for MTX-mediated induction of p53 or p21. We found that the JNK1 DN mutant reduced MTX dependent p21 expression while the JNK2 DN mutant decreased MTX-dependent p53 expression (Figure 3E). Therefore, we conclude from these studies that induction of p53 by MTX is mediated by JNK2, whereas induction of p21 by MTX is mediated by JNK1.

BH4 supplementation blocks MTX-mediated JNK induction, ROS production, and apoptosis priming in activated T cells

MTX increases sensitivity of Jurkat cells to apoptosis by a JNK dependent pathway (20). Isolated PBMC were stimulated by anti-CD3 for 72 hours and treated with MTX (0.1 μ M) for an additional 24 hours. Cultures were supplemented with IL-2 to promote T cell proliferation. Changes in apoptosis were determined by flow cytometry after labeling cells with Annexin V. Mitogen-activated T cells exhibited slightly increased sensitivity to apoptosis in response to MTX alone, but this was enhanced by exposure to anti-Fas, similar to that observed in Jurkat cells (Figure 4A). Supplementation of cell cultures with BH4 (30 μ M) prevented MTX mediated increases in sensitivity to apoptosis. Another form of apoptosis induced in activated T cells is via T cell receptor stimulation, often referred to as activation induced cell death (AICD) (32). Activated T cells were cultured with MTX for 24 hours and re-stimulated with anti-CD3. Apoptosis measurements by Annexin V labeling were performed after an additional one-day incubation. Treatment with MTX markedly increased the level of AICD in activated T cells (Figure 4B). We also measured JNK expression levels in activated T cells treated with MTX by flow cytometry. As in Jurkat cells, JNK expression levels were increased following stimulation by MTX (Figure 4C). Increases in JNK levels were prevented by supplementation with BH4. We also determined MTX stimulated ROS production in activated T cells by flow cytometry using the CM-H2DCFDA dye. MTX induced ROS production in activated T cells (Figure 4D). ROS production by activated T cells was also inhibited by supplementation with BH4. Our results support the notion that MTX inhibition of DHFR depletes intracellular stores of BH4 in activated T cells increasing ROS production leading to JNK activation and alteration in sensitivity to apoptosis. Addition of BH4 reversed these methotrexate-mediated effects.

DISCUSSION

RA T cells exhibit functional defects in cell survival, DNA damage responses, and apoptosis (15, 33-35). MAPKs also play key roles in these fundamental cellular processes (36) and our recent work demonstrates that MTX, one of the most frequently prescribed pharmacologies in the management of RA, activates JNK and prototypical downstream targets, c-JUN and c-

FOS, components of the AP-1 complex and increases sensitivity of cells to apoptosis, raising the question of whether JNK or other MAPK deficiencies may exist in RA T cells (20). Our results demonstrate that MAPK9 transcript levels, but not other MAPK transcripts, and JNK protein levels in lymphocytes, are markedly reduced in RA patients not on MTX therapy. RA subjects treated with once weekly MTX, exhibit increased MAPK9 transcript levels relative to RA subjects not on MTX.

Transcript levels of other genes encoding proteins critical to DNA damage induced cell cycle checkpoint arrest, *TP53*, *CDKN1A*, *CDKN1B*, *RANGAP1*, and *CHEK2*, are also diminished in RA subjects. Expression levels of *TP53*, *CDKN1A*, and, *CDKN1B*, but not *CHEK2* or *RANGAP1* are elevated relative to RA subjects not on MTX. Our results are consistent with a model whereby defects are present at each checkpoint along the cell cycle; G1: *CDKN1B* (p27), S: *CDKN1A* (p21), G2: *CHEK2* (CHK2), and M: *RANGAP1* (RANGAP1) (37-40) and that RA T cells respond to MTX therapy by restoring a portion, but not all of these cell cycle checkpoints (Figure 5). In a cell model, MTX induces protein expression of JNK and subsequent induction of p21 and p53 transcripts and protein through JNK1 and JNK2 mediated pathways, respectively. MTX fails to increase *RANGAP1* and *CHEK2* transcripts. Further, MTX stimulates activated T cells to increase ROS production, JNK protein levels, and to increase apoptosis sensitivity by a BH4 reversible mechanism. The general view is that checkpoints at each stage of the cell cycle exist to maintain fidelity of the genome through the process of DNA replication and cell division. Loss of genomic integrity in hematopoietic cells in RA may arise from combined defects in DNA repair machinery, e.g. ATM, MRE11, NBS1, RAD50, and defects in expression of proteins required to establish each cell cycle checkpoint. One hypothesis is that deficient DNA repair machinery exerts replicative stress upon T cells in RA increasing apoptosis and promoting proliferation and selection of autoreactive T cells in response to lymphopenia (33). Failure of cell cycle checkpoints may also contribute to RA by multiple mechanisms, including loss of immunologic tolerance to self-antigen, significant enhancement of T cell activation in response to external stimuli, or by maintenance of effector cells in a proliferative state for excessive periods of time. Defects in cell cycle arrest may also produce a pro-inflammatory state. For example, loss of genomic integrity leads to activation of the transcription factor NF- κ B via an ATM-dependent mechanism (41). Deficiencies in p53 may also contribute to NF- κ B activation (42). Thus, these defects in cell cycle checkpoints and repair of DNA damage may be critical to establish and maintain the chronic inflammation responsible for much of the pathogenesis of RA.

Therapeutic efficacy of MTX may arise, in part, via its ability to restore JNK and p53 pathways leading to apoptosis of activated RA T cells in the face of pervasive DNA damage. In addition, MTX may promote autoreactive T cells to undergo apoptosis upon secondary stimulation with self-antigen. Further, MTX treatment shifts the cytokine balance from a pro-inflammatory state to an anti-inflammatory state. Depletion of BH4 in activated T cells via inhibition of guanosine triphosphate cyclohydrolase-1 (GTPCH-1), the rate limiting enzyme in the synthesis of BH4, similarly shifts the cytokine balance from a pro-inflammatory to anti-inflammatory state by uncoupling iNOS (43). Thus, MTX may exert multiple effects on T cells to reduce the inflammatory state in RA.

Synovial fibroblast-like cells or synoviocytes in RA also exhibit defects in p53 function and exhibit defects in cell cycle control. Synoviocytes also contribute to extracellular matrix destruction and joint and cartilage destruction in RA (44, 45). Thus, MTX may also act on RA synoviocytes to restore p53 function and cell cycle control, thus reducing synovial hyperplasia and its manifestations. Future studies are planned to test this hypothesis.

Defects in cell cycle control and apoptosis play well-established roles in malignancy. An emerging view is that defects in these pathways have broader ramifications for human disease. For example, ATM-CHK2-p53 defects also exist in lymphocytes from patients with MS that confer an inability to properly undergo apoptosis due to under-expression of ATM and impaired ability to stabilize p53, which may contribute to perpetuation and progression of this disease (46). Our finding of decreased *MAPK9* mRNA expression in MS represents an additional connection between RA and MS. In the broad sense, defects in DNA damage responses have been linked to infertility, cardiovascular disease, and metabolic syndrome (47). Patients diagnosed with RA frequently suffer from inflammation-associated coronary artery atherosclerosis and increased insulin resistance, which may lead to increased mortality (48, 49).

MTX, the standard of care in the treatment of RA, restores a portion of these defects in cell cycle checkpoints and DNA damage responses in RA. However, as our data show, MTX still falls short in correcting the DNA damage response pathway and cell cycle checkpoint deficiencies to those of CTRL. These results highlight the need for additional pharmacologic agents to specifically target these additional cell cycle checkpoints.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. *MAPK9* (JNK2) under-expression in RA is corrected by MTX therapy. (A) Total JNK protein concentrations were determined in RA (N=18) patients and CTRL (N=18) by flow cytometry. Cells were fixed, permeabilized, and stained for CD4, CD8, and CD19 cell surface markers and intracellular proteins, (top panel) JNK and (lower panel) p38 using a secondary fluorescent labeled antibody. Values are expressed as relative fluorescence. (B) Increased JNK expression correlates with decreased CRP levels, in vivo. Serum and PBMC were isolated from RA patient (N=7). Intracellular JNK levels were determined as in (A) CRP levels were determined by ELISA (R&D Systems). (C) *MAPK9* expression levels in RA. Whole blood samples were obtained in PAXgene tubes; CTRL (N=43), RA subjects not on MTX (RA-MTX, N=18), and RA subjects on MTX (RA+MTX, N=18). Gene expression levels are expressed relative to GAPDH $\pm$ S.D. (D) *MAPK9* levels in MS (N=45), UC (N=20), Crohn's (N=23), and SLE (N=23) were determined as in (C). P values are relative to control groups. * $P<0.05$; *** $P<0.001$.

Figure 2. Deficiencies in expression of genes encoding p53, p27, and p21 are restored by MTX, in vivo. *TP53*, *CDKN1B*, *CDKN1A*, *CHEK2*, and *RANGAP1* transcript levels are relative to GAPDH transcript levels in CTRL (N=43), RA + MTX (N=18), and RA-MTX (n=18) subjects.

Figure 3.

Free radical scavengers, folic acid, or BH₄ block MTX-mediated induction of p53 and p21 by JNK. (A-C) JKT cells were cultured with indicated concentrations of MTX for 48 hours. Target gene transcript levels were normalized to GAPDH and calculated as fold-increase relative to untreated samples. JKT cells were cultured with (B) adenosine receptor antagonists, caffeine and/or theophylline, or (C) NAC, or folic acid. Results are expressed as in (A). (D) JKT cells were cultured with MTX $\pm$ BH₄. JNK, p-JNK, p53 or p21 levels were determined by flow cytometry. Left panel: representative flow diagram; background fluorescence (green), untreated cells (gray), MTX-treated cells (blue) and MTX-treated cells with BH₄ (red). Results are expressed as fold increase relative to untreated cells $\pm$ S.D. (E) JNK1-DN, JNK2-DN, or empty vector plasmids with GFP plasmid were transfected into JKT cells. Cells were cultured with MTX for 48 hours and assayed for p53 and p21 gating on GFP+ and - cells. Results are representative of three experiments. * $P < 0.05$ versus unstimulated cultures (A-E); ** $P < 0.05$ versus MTX- and NAC- or folic acid-treated cultures (C) MTX and BH₄ cultures (D) or MTX- and JNK1/2 DN plasmid transfected culture (E).

Figure 4.

BH4 supplementation reverses MTX-mediated apoptosis priming, JNK activation, and ROS production in activated T cells. (A) PBMC were isolated from whole blood, activated with anti-CD3, supplemented with IL-2, and treated with 0.1 μ M MTX for 48 hours with or without addition of BH4 (30 μ M). After 72 hr., cultures were stimulated with anti-Fas for 6 hours to induce apoptosis. (B) Activated T cells were treated with MTX for 48 hours and stimulated with anti-CD3 for an additional 6 hours. (A, B) Results are expressed as % Annexin V+ cells $\pm$ S.D. (C, D) Activated T cells were cultured with 0.1 μ M MTX for 48 hours with or without BH4 (30 μ M) and assayed for (C) JNK protein gating on CD4+ T cells. (D) Activated T cells were cultured with the CM-H2DCFDA dye for 1 hr. prior to flow cytometry measurement. ROS measurements are expressed as fluorescence relative to untreated cultures $\pm$ S.D gating on the lymphocyte population. (A) P values <0.05 are relative to unstimulated cultures (*) or stimulated cultures without the addition of BH4 (**)

Figure 5.
Summary model of cell cycle checkpoint gene expression deficiencies and MTX targets found in RA.

Table 1

Demographic and clinical characteristics of study populations

Study Population Characteristics			
Demographics	CTRL	RA – MTX	RA + MTX
Number of subjects	43	18	18
% female	74	78	100
Age (mean $\pm$ S.D. years)	44 $\pm$ 11	46 $\pm$ 11	48 $\pm$ 15
Ethnicity (%)			
White	74	94	80
African American	15	6	15
Hispanic	6	0	5
Asian	6	0	0
Clinical Characteristics		RA – MTX	RA + MTX
Disease duration (mean $\pm$ S.D. years)		10 $\pm$ 9	9 $\pm$ 7
% with active disease		72	68
% EORA (DD < 1 yr)		22	17
Medications (%)			
Hydroxychloroquine		44	39
Steroids		39	61
TNF inhibitors		44	28

Active disease defined by FDA criteria (presence of three or more of the following: morning stiffness, >45 min., swollen joints, > 3, tender joints, >6, and sedimentation rate, > 28 mm. EORA, early onset RA; DD, disease duration.