METHOTREXATE AND RHEUMATOID ARTHRITIS:
AT THE CROSSROADS BETWEEN INFLAMMATION AND DEFECTS IN
CELL CYCLE CHECKPOINTS

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LIST OF ABBREVIATIONS

ADOR- adenosine receptor

AICD- activation-induced cell death

ATM- ataxia telangiectasia mutated

CTRL- control

DHFR- dihydrofolate reductase

DNA-PKcs- DNA-dependent (DN) protein kinase, catalytic subunit

FLS- fibroblast-like synoviocytes

HLA- human leukocyte antigen

JNK- c-Jun-N-terminal kinase

lincRNA- long intergenic non-coding RNA

lncRNA- long/large non-coding RNA

MAPK- mitogen activated protein kinase

MFI- mean fluorescence intensity

MS- multiple sclerosis

MTX- methotrexate

PBMC- peripheral blood mononuclear cells

qPCR- quantitative polymerase chain reaction

RA- rheumatoid arthritis

ROS- reactive oxygen species

RT-PCR- reverse transcriptase-polymerase chain reaction

SLE- systemic lupus erythematosus

CHAPTER I

INTRODUCTION

Overview

Modern therapies to treat human disease are the product of thousands of years of empirical research with origins in the earliest of recorded human history. These early texts often recorded symptoms and prescribed treatments based upon clinical observations. These early prescriptions were entirely composed of naturally occurring products such as plants (herbalism), animals, and minerals. The practice of compounding these different elements into a single suspension or tablet established the basis of modern pharmacology. With the increasing ability for scientists to uncover the molecular basis of human diseases, so too has increased our desire to develop treatments to address these findings. Coinciding with the birth of molecular medicine in the mid-twentieth century, we witnessed the emergence of intelligent or rational drug design.[1] Most of the drugs developed during this early period sought to treat cancer. While radiation was an effective therapy to treat local tumors, additional pharmacologics were needed to reach every organ of the body to treat metastatic disease.[2] Many of these drugs have since been replaced by more targeted therapies in the treatment of cancer. However, a few of these early drugs have since been applied to the treatment

of other human conditions, particularly the rheumatic diseases.[2] Developed more than half a century ago, methotrexate is the standard of care for the treatment of rheumatoid arthritis (RA), but its use in the management of this disease was unintended and discovered largely by serendipity.[1,3-5] Here we will explore the history of this anchor drug and its proposed mechanism of action in the treatment of neoplastic and non-neoplastic disease.

Origins of Methotrexate

Folates are critical components of cellular division, and DNA and RNA synthesis. The synthetic form of folate, folic acid, was first isolated in the early 1940s and was found to exacerbate acute forms of leukemia when added to a patient's diet.[1,3,4] Conversely, additional studies found that decreasing dietary amounts of folic acid decreased the leukemia cell counts in patients. From these early observations, work began to design analogues of folic acid, which could be used to treat cancer, particularly leukemia. Aminopterin was designed to reduce proliferation of cancerous cells via the inhibition of folate. Seminal work by Sidney Farber, a pathologist at Harvard Medical School and Boston Children's hospital, demonstrated that aminopterin produced remission in children diagnosed with acute lymphoblastic leukemia (ALL).[1,2,6] Even though this only produced brief remissions, it was proof of concept that folate antagonism could suppress the proliferation of malignant cells. Thus,

the clinical efficacy of aminopterin in the treatment of ALL cemented aminopterin as one of the world's first chemotherapeutics.[1]

Work that followed nearly a decade later by Sidney Futterman, Michael Osborn, and Frank Heunnekens identified dihydrofolate reductase (DHFR) from chicken liver as the enzyme responsible for the reduction of folic acid to metabolically active forms.[1,7] Thus, blockade of DHFR was implicated as a therapeutic target of chemotherapeutic doses of aminopterin. Isolation of this enzyme allowed for the creation of potent inhibitors of DHFR. Specifically, another folate analog, methotrexate, was identified in a study of leukemia-bearing mice and when compared to aminopterin increased survival in these mice.[1] The chemical structures of these two compounds are highlighted in Figure 1-1. From these initial data in mice, two reports found that methotrexate at very high doses cured women diagnosed with choriocarcinoma, a malignant trophoblastic cancer of the placenta.[2] This was the first solid tumor to be cured by a drug in humans and stimulated interest in investigating the effects of methotrexate in additional forms of cancer.[1,8] Of particular interest was the reduced side effect profile observed in the methotrexate-treated cohort. Compared to radiation or alkylating agents that can lead to infertility or additional malignancies, methotrexate monotherapy did not produce these deleterious effects.[1] Today, methotrexate is currently used in the treatment of large cell or high grade lymphomas, head and neck cancer, breast cancer, bladder cancer, and osteogenic sarcoma.[1] It is often
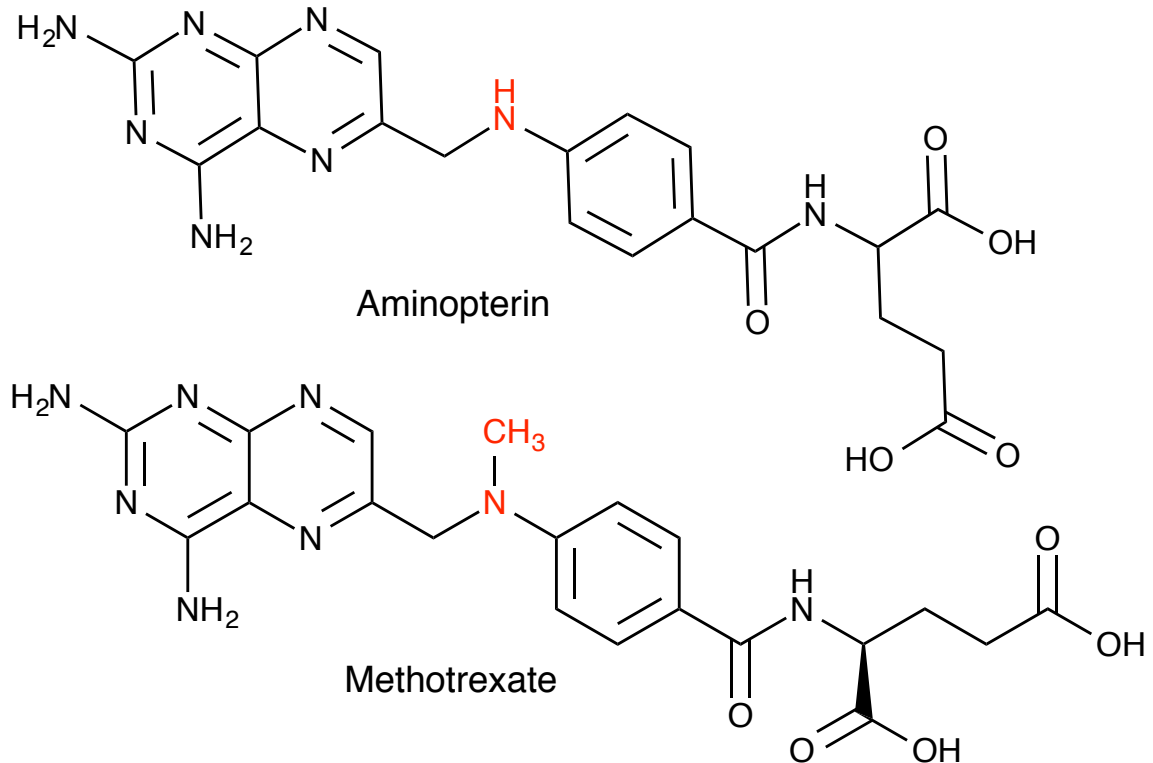
Figure 1-1. Chemical structures of aminopterin and methotrexate. In contrast to aminopterin, methotrexate contains an added methyl group in place of a hydrogen atom at the 10 position of the paraaminobenzoic acid moiety shown in red. *Adapted from: Berg et al. Biochemistry. 2012. p750a. and St. Clair et al. Rheumatoid Arthritis. 2004. p303-305.*

used in combination with other therapies including 6-mercaptopurine (6MP). Studies have shown that the combination of methotrexate, 6MP, vincristine, and prednisone improve patient outcomes in the treatment of ALL.[1] In particular, a treatment regiment first prescribing methotrexate and following with 6MP in sequence improves cure rates.[1]

Given the immunosuppressive potential of aminopterin and methotrexate in the treatment of malignancy, Gubner et al reported in 1951 that proliferative responses of formalin injection in rat paws was abrogated

with aminopterin treatment.[1,9] Further, in a small population of patients with active rheumatoid arthritis, Gubner and colleagues showed that the overwhelming majority of patients treated with aminopterin developed reduced indices of disease activity. When the therapy was stopped, the patients experienced relapse. The toxicities reported included nausea and diarrhea, even at low doses (1-2mg/day).[1] Due to these discomforts, methotrexate, which closely resembles aminopterin, was substituted.[1] Patients were able to tolerate methotrexate reasonably well at low doses. The role of aminopterin, and later methotrexate, was also investigated in other non-neoplastic diseases including psoriasis, a chronic skin condition producing thick patches of irritated skin that manifest as red or white scales and similar therapeutic benefits were observed.[1] It is interesting to note that this early report describing the therapeutic potential of methotrexate or other folate analogs was largely ignored for a quarter century. It would not be until the late 1980s that methotrexate is approved for the treatment of rheumatoid arthritis.[10]

Mechanism of Action of Methotrexate

Given the therapeutic potential for methotrexate in the treatment of these forms of cancer and even autoimmune disease, significant resources have been expended to investigate its mechanism of action. Bertino et al provided significant insight demonstrating that methotrexate is actively

transported into cells through reduced folate transporter 1 (RFT-1).[11] Methotrexate, like naturally occurring folates, is polyglutamated once taken up by the cell. Folates exist in cells as polyglutamates through the addition of 6 glutamyl groups in a gamma peptide linkage to the folate substrate using the enzyme folylpolyglutamate synthase (FPGS).[12] These long-lived methotrexate polyglutamates remain in the liver of patients for a long period as well as in the bone marrow myeloid precursors.[12] Polyglutamation of methotrexate occurs within 12-24 hours after treatment and polyglutamates constitute the active form of the drug.[12,13] Thus, methotrexate is commonly referred to as a pro-drug, a compound that undergoes a biochemical modification to become its active form. Summarized in Figure 1-2, inhibition of DHFR, at pharmacologically relevant doses of MTX required for the treatment of malignancy, inhibits purine, pyrimidine, and thymidylate biosynthesis through reduced levels of tetrahydrofolate (FH_4) in the cell. Blockade of these enzymes, which are critical for nucleotide generation, halts rapid division of tumor cells through induction of apoptosis. Thus, one goal of methotrexate therapy is to increase the cellular cytotoxicity profile. Alterations to this pathway in the form of mutated RFT-1 or DHFR can lead to methotrexate resistance in cancer patients.[2,12] Interestingly, cancer subjects resistant to methotrexate often exhibit increased levels of DHFR protein.
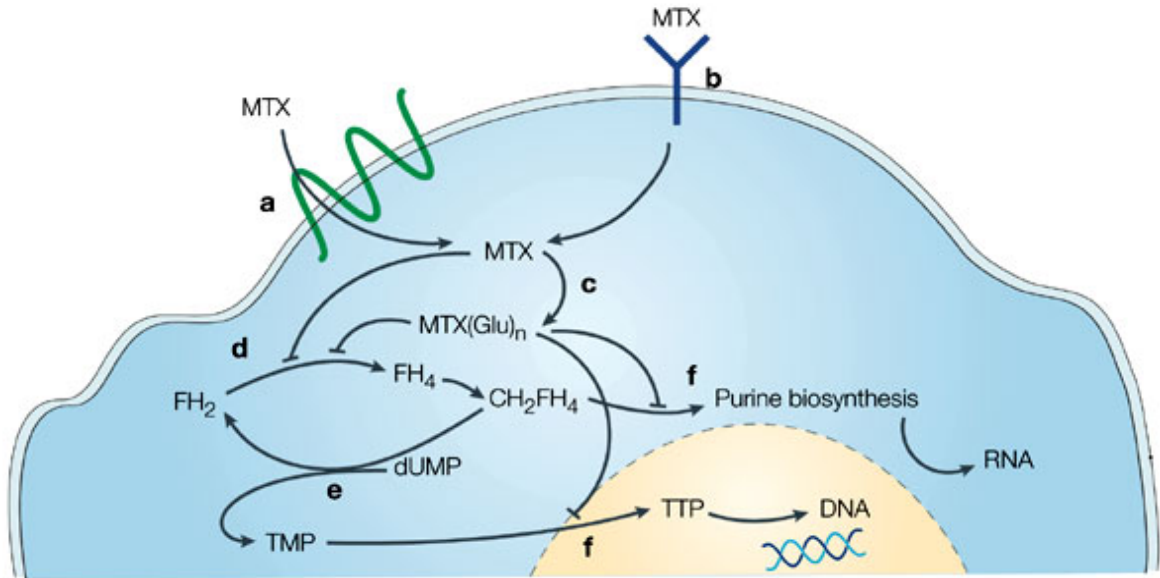
Figure 1-2. Mechanism of action of methotrexate. Methotrexate (MTX) enters the cell through the reduced folate carrier (**a**) using an endocytic pathway activated by a folate receptor (**b**). After entering the cell, methotrexate is polyglutamated (Glu) by the enzyme folylpolyglutamate synthase (**c**). Methotrexate and its polyglutamates inhibit the enzyme dihydrofolate reductase (**d**), thereby blocking the conversion of dihydrofolate (FH_2) to tetrahydrofolate (FH_4). As tetrahydrofolate stores are depleted, thymidylate (TMP) synthesis (**e**) is reduced,which ultimately inhibits DNA synthesis (**f**). Long-chain polyglutamates of MTX have the same affinity as MTX for the target enzyme dihydrofolate reductase, but have markedly increased inhibitory effects on both thymidylate synthesis (**e**) and purine biosynthesis (**f**), which is required for RNA production.
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It is hypothesized that gene amplification events may take place that are long-lived in tumor cells or that amplification occurs through extrachromosomal elements, called amplisomes, that contain DHFR genes.[12] This is currently an area of active exploration and future studies are required to determine the exact mechanisms.

While methotrexate is still used in the modern treatment of cancer, it is in the treatment of rheumatoid arthritis that physicians have observed methotrexate's greatest, long-term effectiveness. Often heralded as the drug

that revolutionized the field of rheumatology, low-dose, once-weekly methotrexate differs by approximately three orders of magnitude (milligrams versus grams) compared to dosing schemes required for the treatment of malignancies. When the FDA first approved methotrexate in 1988 for the treatment of rheumatoid arthritis, it was assumed that the mechanism of action by which methotrexate exerts its anti-inflammatory effects in rheumatoid arthritis would closely resemble the mechanism of action found in the treatment of cancer. Rheumatoid arthritis is a chronic, inflammatory condition of the small and large joints characterized by inflammation of the synovium, or lining of the joint (Figure 1-3). While the precise etiology of this disease remains unknown, the growing appreciation for the molecular basis of this disease has provided several clues. At the heart of these analyses are the cell types found in the joint spaces of patients with active disease.
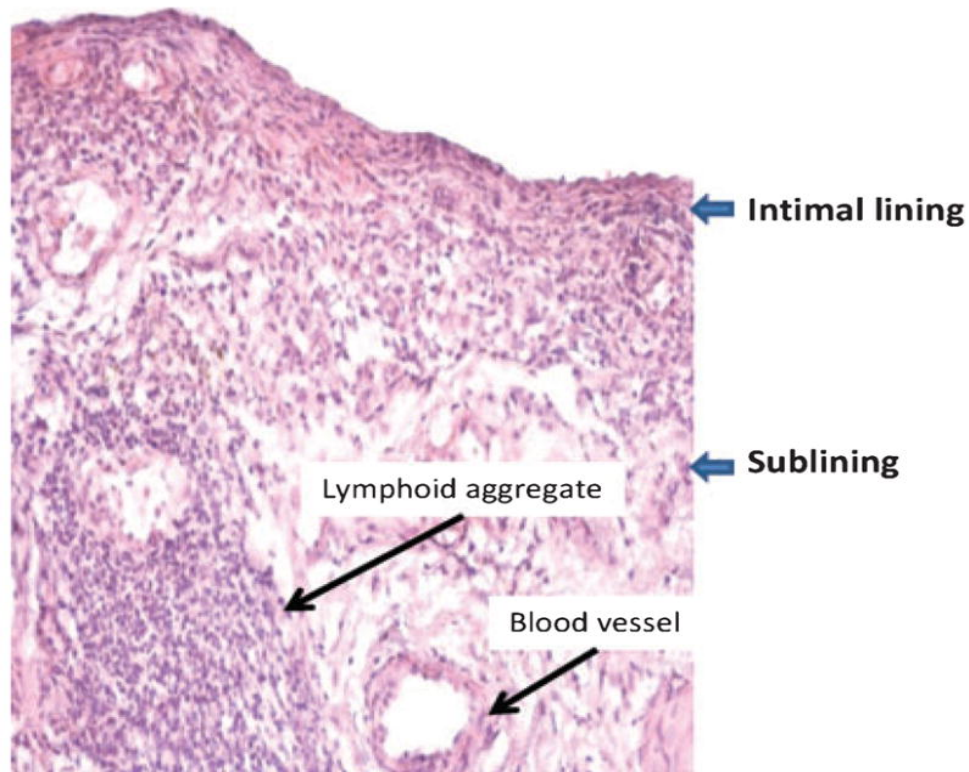
Figure 1-3. Histopathologic appearance of rheumatoid arthritis (RA) synovial tissue. The synovium in RA is marked by intimal lining hyperplasia, infiltrating mononuclear cells in the sublining, and occasional lymphoid aggregates.
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Lymphocytes are the most common cell infiltrate found in the synovial space. In fact, of these lymphocytes, the majority are T lymphocytes making up approximately 30-50% of all cell types. Of the T lymphocytes found in the RA joint, it has been reported that the majority are CD4^{+} CD45RO^{+} memory cells.[14] B cells constitute about 5% of the sublining synovial cells.[14] Clonal expansion of the B cells in the joint spaces of RA subjects suggests a maturation process driven by an antigen, which still remains unidentified. In normal tissue, the synovial space is only 1 or 2 cells in depth and is

comprised of both Type A (macrophage-like) and Type B (fibroblast-like) cells.[14] However, in active rheumatoid arthritis this number increases ten-fold and is primarily thought to be the consequence of hypercellularlity due to the increase of both Type A and Type B cells.[14,15] Many studies have suggested that Type A cells in RA display an activated phenotype and due to circulation are constantly replenished from the bone marrow. Locally, while in the joint spaces, these Type A, macrophage-like cells produce "pro-inflammatory cytokines, chemokines, and growth factors" that in turn activate fibroblast-like synoviocytes and induce these cells to produce additional pro-inflammatory mediators including "IL-6, prostanoids, and matrix metalloproteinases." [14] This process can create both paracrine and autocrine signaling networks that give rise to the chronic synovitis and recruitment of additional immune cells to the joint, which eventually erodes the extracellular matrix and destroys the joint space. This phenomenon is referred to as the 'pannus', an expansive synovial tissue.[14] Phenotypically, this pannus closely resembles a tumor. Nuclear factor κB (NF-κB), a transcription factor that is ubiquitously expressed and functions as a critical regulator of cell proliferation, differentiation, and inflammation, is also overexpressed in the RA synovium. Briefly, nuclear factor κB consists of five proteins, c-Rel, RelA (p65), RelB, p50/p105, and p52/p100 that form either a homodimer or heterodimer.[14] c-Rel, RelA, and RelB function as the major transactivation subunits. Unless activated, these subunits reside in the

cytoplasm along with their inhibitor, IκB.[14] Phosphorylation of IκB causes IκB to be degraded by the proteasome, thus releasing NF-κB dimers to migrate to the nucleus where they will target the promoter regions of target genes.[14] Electromobility shift assays show constitutively high levels of p50 and p65 expression in the synovium of rheumatoid arthritis subjects and induction of pro-inflammatory cytokines such as IL-1, IL-6, and TNF-α through IKK signaling pathways.[16] Depletion of p65 or the IKK family member, IKKβ, in the synovial tissue with siRNAs or introduction of dominant negative mutants reduces levels of these pro-inflammatory cytokines.[17] In addition to increased levels of NF-κB, both synoviocytes and T cells in RA exhibit defects in expression of the guardian protein p53 leading to inability for these cells to undergo apoptosis and loss of genomic integrity.[15,18] p53 is a critical regulator of cell cycle progression and reduced p53 levels have also been found in a number of cancers including leukemia. Linking the contribution of these observations to the pervasive, non-resolving inflammation found in RA is a common goal in the management of the disease. Without this understanding, most therapeutics lack the specificity to precisely target the underlying defects contributing to disease progression. As such, most newly developed biologic agents attempt to disrupt the downstream, NF-κB activation-pro-inflammatory cytokine loop, by using drugs like Enbrel (etanercept), which selectively blocks the inflammatory cytokine, TNF-α.[19] These newer biologic therapies have added to the ability of

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physicians to improve outcomes and decrease disability. However, despite these advances excess mortality observed in patients with RA continues and recent data suggest that the mortality gap between RA patients and the rest of the population continues to widen.[20,21]

Given the pro-inflammatory, anti-apoptotic phenotype exhibited by both synoviocytes and T cells in RA, and the ability of methotrexate to mitigate indices of inflammation it is logical to question if methotrexate may exert its anti-inflammatory properties through modulation of these pathways. For the past 30 years, the precise mechanism employed by methotrexate to exert its anti-inflammatory effects in RA has been the focus of thorough investigation.[1,11,22-35] In the treatment of cancer, methotrexate induces apoptosis by blocking the folate-dependent processes involved with DNA and RNA synthesis ultimately leading to cell death. Curiously, however, folic or folinic acid supplementation in RA patients receiving methotrexate did not reverse its anti-inflammatory effects in randomized, blinded trials.[1,3,34,36] Thus, other mechanisms have been proposed. A prevailing theory is that methotrexate exerts its mechanism of action through a number of different mechanisms including release of adenosine that function in parallel to blockade of nucleotide synthesis.[3] Reduced levels of methyl donors including tetrahydrofolate (FH_4) and methyltetrahydrofolate through inhibition of DHFR blocks generation of lymphotoxic polyamines through methionine and S-adenosylmethionine (SAM).[4,5,13,30,34,36] Polyamine reduction

has been posited as one anti-inflammatory mechanism since polyamines can be converted to lymphotoxins.[36] However, use of 3-deazaadensoine, a transmethylation inhibitor, did not demonstrate a significant clinical benefit in RA patients.[36] Yet, low-doses of methotrexate also inhibit chemotaxis in monocytes through a process reversed by S-adenosylmethionine supporting the contribution of this pathway in RA.[37] The retention of methotrexate polyglutamates in cells exceeds its half-life in plasma, suggesting that the methotrexate metabolites persist in tissues. These polyglutamates have also been shown to inhibit aminoimidazolecarboxamidoribonucleotide (AICAR) transformylase resulting in elevated intracellular AICAR levels. RA subjects exhibit high levels of AICAR in their urine during the course of methotrexate therapy.[3,36] Increased AICAR levels are strong inhibitors of adenosine monophosphate (AMP) and adenosine deaminase, involved in the consumption of AMP and adenosine to IMP and inosine. Accumulation of adenosine in tissues has anti-inflammatory effects and AICARriboside, which also inhibits adenosine deaminase, is increased in RA.[36,38] Methotrexate has also been shown to enhance vasodilation leading to increased blood flow through inhibition of adenosine deamination in whole blood in humans.[39] The direct quantification of methotrexate-mediated adenosine release in humans receiving methotrexate has been unsuccessful largely because the half life of adenosine in blood and tissue is very brief making these measurements technically challenging.[36,40] In animal models, however, the

anti-inflammatory effects of methotrexate have been shown to be mediated by adenosine using the carrageenanan-induced air pouch model of inflammation and reversals with A2A adenosine receptor antagonists and supplementation of adenosine deaminase.[36,41] Figure 1-4 summarizes this pathway.
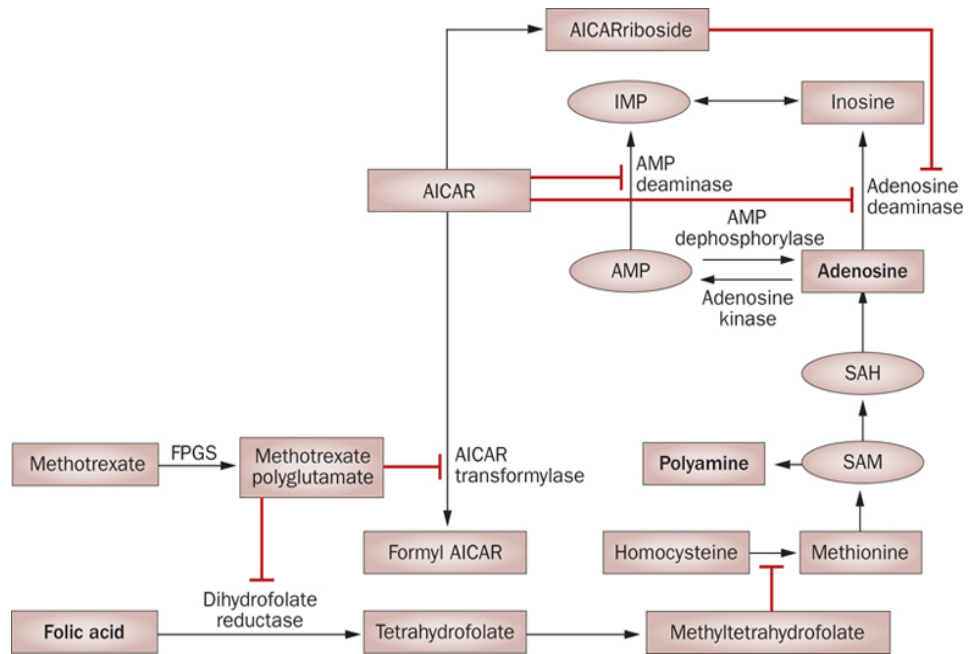
Figure 1-4. Proposed mechanisms of the anti-inflammatory actions of methotrexate. Methotrexate exerts its anti-inflammatory actions through a number of cellular mechanisms. Competitive inhibition of dihydrofolate reductase diminishes the *de novo* synthesis of purines and pyrimidines by preventing the regeneration from dihydrofolate of tetrahydrofolate, which is essential for the generation of folate cofactors required for purine and pyrimidine synthesis. Reduction of the levels of methyl donors, such as tetrahydrofolate and methyltetrahydrofolate, by the inhibition of dihydrofolate reductase results in the inhibition of the generation of lymphotoxic polyamines through methionine and SAM. Inhibition of AICAR transformylase results in an increase in intracellular AICAR levels. This increase has potent inhibitory effects on AMP deaminase and adenosine deaminase, which are involved in the catabolism of AMP and adenosine to IMP and inosine, respectively. The consequent accumulation of adenosine confers anti-inflammatory effects. Levels of AICARriboside, a metabolite of AICAR, also accumulate and inhibit adenosine deaminase.
Abbreviations: AICAR, aminoimidazolecarboxamidoribonucleotide; AICARriboside, aminoimidazolecarboxamidoribonucleoside; AMP, adenosine monophosphate; FPGS, folyl polyglutamate synthase; IMP, inosine monophosphate; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine.
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Other studies have also shown that MTX inhibits T cell activation, induces apoptosis, and alters expression of T cell cytokines and adhesion molecules.[22,26,42,43] Additional work by Phillips et al posit that the anti-inflammatory properties of MTX are critically dependent upon the ability to produce reactive oxygen species in both T cells and monocytes, which ultimately lead to apoptosis.[25] Given the pronounced anti-inflammatory properties of low-dose MTX therapy in RA, it is unclear how the known biochemical pathways affected by MTX, e.g. inhibition of DHFR, activation of adenosine synthesis and release, should produce this anti-inflammatory profile. Our work has sought to explore the question of whether either additional biochemical pathways are targeted by methotrexate or additional biochemical consequences of DHFR inhibition by methotrexate may produce these anti-inflammatory properties observed in subjects with RA receiving low-dose MTX as therapy.

The Cell Cycle and Cell Cycle Checkpoints

The process by which eukaryotic cells reproduce is known as the cell cycle. This process leads to precise duplication of the organism's genome and segregation of chromosomes into daughter cells.[44] It is important to study how the cell cycle is regulated and organized since proliferation is one of the most fundamental activities of cells and completion of cell division is essential for the development of all organisms.[45] It is also required for the

production of gametes and hence propagates survival of species.[44] Identification and understanding of the molecular mechanisms of cell cycle control provides potential targets that can be manipulated to control cell growth in a variety of complex diseases such as cancer.[46,47]

Mitosis was the first cell cycle stage observed in salamander cells by Walther Flemming in 1878.[48] Later, in the 1950s, DNA was shown to be the genetic material of all cells, the crystal structure was determined, and DNA was subsequently shown to be synthesized during a discrete period of interphase, termed the S phase, flanked by gap phases.[44,45] Today, we appreciate that the cell cycle has four phases: the G1 phase, S phase, G2 phase, and M phase.[44] Cells can exit the cell cycle between the M and G1 phase through cell death or apoptosis, or quiescence termed G0, and subsequently re-enter at G1.[44] This process is summarized in Figure 1-5.
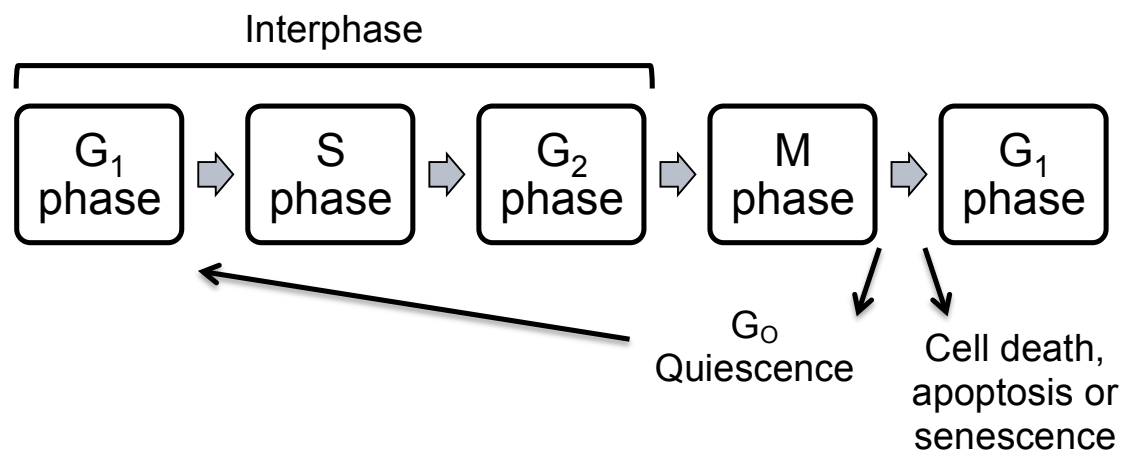
Figure 1-5. The basic stages of the cell cycle. *Adapted from: Alberts et al. Molecular Biology of the Cell. 2012*

These phases last for varying amounts of time, but in a typical mammalian cell, such as a cultured fibroblast, the total cell-cycle length is approximately 24 hours in length. The G1 phase lasts between 12-15hrs, the S phase lasts between 6-8hrs, G2 lasts 5hrs, and mitosis (M) lasts for approximately 1hr.[44] Understanding the signals and mechanisms responsible for triggering cell-cycle transitions remains an area of active investigation.[46]

Major advancements in the field were accomplished through a series of experiments utilizing the power of yeast genetics and amphibian and marine invertebrate biological systems.[45,49] Maturation promoting factor (MPF), also referred to as mitosis promoting factor, which drives the cell into mitosis, was identified in oocytes and subsequently biochemically purified by Lohka and Maller.[50] Prior to MPF isolation, through a series of yeast studies by Leland Hartwell and Paul Nurse, temperature-sensitive cell division cycle (cdc) mutants were isolated in two strains of yeast and it was found that cdc2 was required for cell cycle progression.[45] Cdc2 was later identified as a principal component of MPF.[45,51-56] Concurrent with this observation, it was noticed that proteins accumulate cyclically in eggs and embryos of marine invertebrates and these proteins were named cyclins. In these experiments, cyclin concentration correlates with maximal MPF activity and one of these cyclins, Cyclin B, was isolated from sea urchin eggs by Tim Hunt and identified as another critical component of MPF.[45,57,58] Collectively, these findings led to the 2001 Nobel Prize in Physiology or Medicine for discoveries

of these key regulators of the cell cycle and form the basis for today's fundamental understanding of the cell cycle.[49]

Cyclins are cyclin dependent kinase or CDK regulator subunits that are essential for kinase activity of the Cdk.[44] They help to recruit substrates and target the complex to subcellular locations and govern duration of the CDK active state.[44] Cdc2 was the first recognized CDK, and was subsequently renamed Cdk1 or cyclin-dependent kinase 1. Yeast have only 1 Cdk, but contain multiple cyclins.[44] Higher organisms have multiple cyclins and multiple Cdks.[44] Major Cdks and cyclins of higher eukaryotes for the different cell-cycle phases are G1: Cdk4, Cdk6, and Cyclin D; G1/S: Cdk2 and Cyclin E; S: Cdk2 and Cyclin A; and M: Cdk1 and Cyclin B.[44] Cyclin levels oscillate throughout the cell cycle regulating Cdk activity such that cyclical activation of Cdks drive cell-cycle transitions.[46,59]

Since Cdk-cyclin complexes drive the cell cycle forward, a major question moving forward was what controls the activity of these complexes?[45] Methods for regulating CDK-cyclin activity include phosphorylation and dephosphorylation of Cdk, addition of subunits (inhibitors), control of intracellular localization, and degradation of cyclin and inhibitor subunits.[44,60,61] One example of such regulation includes the phosphorylation event on Cdk1.[46] It was found that Wee1 kinase inhibits mitotic entry resulting in a 'stop' event in the cell cycle whereas Cdc25 phosphatase promotes mitotic entry.[62] Cdk inhibitors (CKIs) are also key negative

regulators of G1/S phase transition.[63-65] There are two classes of CKIs: INKs which are inhibitors of kinases and cyclin/kinase inhibitory proteins (CIPs/KIPs).[49] INKs when bound to the Cdk/Cyclin molecule trigger release of cyclin thus inactivating the cell cycle at the G1/S phase. CIPs/KIPs inhibit kinase activity by binding to the Cdk-cyclin complex. Two examples of this particular class of inhibitor are p21 (CIP) and p27 (KIP1). In humans, these two proteins are transcribed by the genes *CDKN1A* and *CDKN1B*, respectively.[46,63,65] Nuclear accumulation of Cdk1-cyclin B1 complexes and Cdc25C in late prophase is required for mitosis. Phosphorylation of Cyclin B1 and Cdc25 leads to their nuclear accumulation. Lastly, degradation of Cyclin B at various stages during the cell cycle in *Xenopus laevis* embryos led to the discovery that cyclins are proteolyzed by the ubiquitin (Ub)-proteasome system.[57,59,66,67] Ubiquitination of Cyclin B leads to degradation of Cyclin B by the proteasome.[64,65,67] Thus, the cell cycle is both a protease-driven and kinase-driven process. Deregulation of various Cdk inhibitors has been documented in a variety of tumors.[46] Typically, oncogenic changes are associated with decreased levels of Cdk inhibitors in tumors. Thus, an area of intense focus is understanding the regulation of these Cdk inhibitors in the context of malignancy.[46]

It is currently estimated that 10,000 trillion cell replication events will have occurred in the lifetime of a human being.[68] There are also greater than 3-3.5 billion base pairs of DNA in a cell.[68] Abnormalities in the process of cell

division can occur as the product of a variety of stresses, both internal and external.[68,69] In fact, a cell typically accumulates in excess of 10,000 DNA lesions per cell in a given day.[68] Checkpoints exist to prevent damaged cells from further progress in the cell cycle.[69,70] Checkpoints also ensure the completion of one cell cycle phase prior to the onset of another phase.[44] They also respond to DNA or microtubule stress to halt cell cycle progression to provide time for repair.[44] Early evidence for cell cycle checkpoints was found in patients suffering from Ataxia Telangiectasia (AT). In normal eukaryotic cells, DNA synthesis is slowed and stopped before mitosis in response to ionizing radiation.[71-73] However, patients suffering from AT continue from the S-phase and into mitosis.[74] Patients present with the disease as early as 4-5 years of age and often have a predisposition to immune deficiencies, cancer, neuro-degeneration, and often succumb to the disease by 20 years of age.[74,75] Molecularly, these patients exhibit a loss of G1, S, and G2/M checkpoints. Through a series of positional cloning experiments, a gene, *ATM* (ataxia telangiectasia mutated) was identified as the gene that was mutated in subjects with AT.[76] Thus in the context of DNA damage-inducing agents, such as ionizing radiation or even normal cellular biochemistry, subjects with AT are unable to repair these damaged cells because of reduced function of ATM. [77]

Other sentinel regulators of the DNA damage response have been identified in addition to ATM and include ataxia-telangiectasia and Rad3-

related (ATR), and checkpoint kinases CHK1 and CHK2. These pathways are activated in response to DNA damage and result in increased levels of p21 or blockade of CDK activators, such as Cdc25 phosphatase. These proteins in the DNA damage response are thought to have anticancer properties through maintenance of genome integrity via DNA repair and cell cycle arrest when necessary.[46] In certain forms of cancer, these pathways are activated as measured by constitutive expression of phosphorylated H2AX and the tumor suppressor, p53.[78,79] Often these markers are at their highest expression in "pre-invasive stages of human tumors and correlate with the presence of senescence markers".[46] This is often followed by a period of reduced DNA damage response through reduction in ATM, CHK2, and p53 expression leading to reduced expression of p21.[46,80,81] These defects contribute to progression in the cell cycle despite damaged DNA thus leading to genomic instability and tumorigenesis.[69,81,82]

Previous work conducted in our laboratory and the laboratory of others demonstrates that similar defects exist in human autoimmune disease. Specifically, ATM, CHK2, p53 and p21 are under-expressed in lymphocytes obtained from subjects with rheumatoid arthritis.[18,83] These lymphocytes also accumulate DNA damage.[84] It has been suggested that these defects contribute to increased genomic instability and entry into a phase of early senescence.[85] In synovial tissue, which is the major site of inflammation and tissue destruction in rheumatoid arthritis, loss of function mutation have

been described that may also contribute to the hyperproliferative state of the synovium in rheumatoid arthritis.[14,86] If or how these deficiencies may contribute to the chronic inflammatory state of rheumatoid arthritis is not known.

The following four chapters will examine contributions of methotrexate in treating defects in apoptosis and cell cycle checkpoints and further investigate molecular mechanisms responsible for anti-inflammatory properties of methotrexate in the treatment of RA. These studies extend to both lymphocytes and fibroblast-like synoviocytes, key effector cells in RA pathogenesis. The first chapter examines the ability of methotrexate to induce apoptosis and demonstrates that increased JNK activation alters apoptosis sensitivity in cells treated with methotrexate. Methotrexate blocks the reduction of dihydrobiopterin (BH_2) to tetrahydrobiopterin (BH_4), which results in increased reactive oxygen species in lymphocytes leading to activated JNK. In the second chapter, we demonstrate that methotrexate treatment increases expression of cell cycle checkpoints, p53 and p21, through activation of JNK via a BH_4 and ROS-dependent mechanism. The third chapter examines the cell type specificity of this BH_4 pathway and finds that reduced activity of NF-κB in T cells is dependent upon MTX-mediated blockade of BH_4 and activation of JNK and induction of p53. This is in contrast to synoviocytes where the adenosine pathway appears to be responsible for MTX-mediated inhibition of NF-κB. In the final chapter, we

further examine the link between p53 and NF-κB in T cells by analyzing the downstream target of p53 activation, lincRNA-p21. This molecule belongs to a relatively new class of non-coding RNAs. We find that lincRNA-p21, which is underexpressed in RA, is induced by MTX both *in vitro* and *in vivo* and inhibits NF-κB activity in T cells. This process is independent of both the adenosine pathway and the BH_4 pathway. Rather, induction of lincRNA-p21 is critically dependent upon phosphorylation and activation of DNA-PKcs by methotrexate (Gene symbol: *PRKDC*), a sentinel of the DNA damage response.

CHAPTER II

INCREASED SENSITIVITY TO APOPTOSIS INDUCED BY METHOTREXATE IS MEDIATED BY JNK

Abstract

Objective. Low-dose methotrexate is an effective therapy for rheumatoid arthritis (RA), yet its mechanism of action is incompletely understood. The aim of this study was to explore the induction of apoptosis by MTX.

Methods. Flow cytometry was performed to assess changes in the levels of intracellular proteins, reactive oxygen species (ROS), and apoptosis. Quantitative polymerase chain reaction was performed to assess changes in the transcript levels of select target genes in response to MTX.

Results. MTX did not directly induce apoptosis but rather "primed" cells for markedly increased sensitivity to apoptosis via either mitochondrial or death receptor pathways, by a JNK-dependent mechanism. Increased sensitivity to apoptosis was mediated, at least in part, by MTX-dependent production of ROS, JNK activation, and JNK-dependent induction of genes whose protein products promote apoptosis. Supplementation with tetrahydrobiopterin blocked these MTX-induced effects. Patients with RA

who were receiving low-dose MTX therapy expressed elevated levels of the JNK target genes, *JUN*.

Conclusion. Our results support a model whereby MTX inhibits reduction of dihydrobiopterin to tetrahydrobiopterin, resulting in increased production of ROS, increased JNK activity, and increased sensitivity to apoptosis. The finding of increased *JUN* levels in patients with RA receiving low-dose MTX supports the notion that this pathway is activated by MTX *in vivo* and may contribute to the efficacy of MTX in inflammatory disease.

Introduction

The folic acid antagonist, methotrexate [MTX], was initially developed during the 1940s to inhibit dihydrofolate reductase [DHFR] for treatment of malignancies.[87-89] The clinical potential of MTX in treating rheumatoid arthritis [RA] was initially suggested by Gubner in 1951, after studying the effects of MTX in six patients diagnosed with RA and was confirmed by further studies conducted during the 1980s.[9,11,28,90,91] These studies had durations of 12 to 18 weeks, and dosages varied from 7.5 to 25 milligrams per week. MTX possessed anti-inflammatory effects in RA patients, as trial subjects demonstrated improved function, global assessments, joint scores and marked decreases in pain. Use of weekly low doses of MTX is not limited

to RA therapy. Over the years, this treatment option has expanded to include additional inflammatory and autoimmune diseases.[33,35,92,93]

In contrast to treatment of malignancy, much lower and more infrequent doses of MTX are used to treat inflammatory disease and basic mechanisms may differ greatly from those targeting cancer. Exact mechanisms underlying anti-inflammatory actions of MTX remain inconclusive in spite of its widespread application.[36] MTX, like natural folates, is polyglutamated once taken up by cells. MTX-polyglutamates are believed to represent the active form of the drug and levels of MTX-polyglutamates correlate with clinical efficacy in patients with RA.[31,94] MTX stimulated synthesis of adenosine and its release by cells and subsequent activation of adenosine receptors may be one mediating factor contributing to anti-inflammatory actions of MTX.[13,23,36] MTX also inhibits T cell activation, induces T cell apoptosis, and alters expression of T cell cytokines and adhesion molecules.[22,26,42,43] Such actions may be partly mediated by synthesis and release of adenosine. MTX action may also be dependent, in part, upon its ability to stimulate production of reactive oxygen species [ROS].[25]

From these studies, underlying mechanisms behind induction of apoptosis by low concentrations of MTX are not immediately apparent. In general, cells undergo apoptosis via their ability to activate intrinsic [mitochondrial] or extrinsic [death receptor] pathways.[95-101] For example, the

stress-inducible p53 protein plays a central role in transducing DNA damage signals to cause cellular apoptosis. Death receptors, such as Fas or the family of TNF receptors transmit signals through adapter molecules such as Fas-associated death domain, TNFR-associated death domain or death domain-associated protein. These adaptor proteins activate death caspases causing apoptotic cell death. Both intrinsic and extrinsic apoptosis pathways are thought to involve JNK signaling.[101] Here, we sought to investigate induction of apoptosis by low concentrations of MTX in a transformed human T cell line. We find that low concentrations of MTX do not directly induce apoptosis. Rather, MTX markedly increases sensitivity of cells to apoptosis mediated via either death receptor or mitochondrial pathways, in part, by increasing expression of genes whose protein products play key roles in induction of apoptosis. This alteration in the transcriptional profile of cells treated with MTX is dependent upon induction and activation of JNK by ROS. Apoptosis sensitivity and ROS production are prevented by supplementation with tetrahydrobiopterin [BH$_4$] suggesting that inhibition of reduction of dihydrobiopterin [BH$_2$] by DHFR initiates this pathway. Studies of subjects with RA who are on current MTX therapy support the notion that these pathways may be activated, *in vivo*, by MTX and contribute to therapeutic efficacy of MTX.

Materials and Methods

Drugs and Reagents. MTX, adenosine, caffeine, theophylline, NAC, H_2O_2, and BI-78D3 were from Sigma. pepJIP1 was from Enzo Life Sciences. Anti-Fas antibody was obtained from Medical and Biological Laboratories, LTD. Caspase 3 activity was determined by enzymatic assay from Promega (CaspACE Colorimetric Assay System). 384-well apoptosis TLDA plates were from Applied Biosystems. Plasmids containing JNK1 [MAPK8] and JNK2 [MAPK9] dominant negative mutants were obtained from Addgene.

Cell Preparation and Culture. The human Jurkat T cell leukemia line was from ATCC. PBMC were purified by Ficoll-Hypaque gradient centrifugation. Cells were cultured in RPMI media with 10% FCS, Penn-Strep, and L-glutamine in a 37°C atmosphere of 5% CO_2 in air. Cells were plated at 0.8×10^6 cells per ml in 5 ml cultures. Cell viability and numbers were determined microscopically after staining with trypan blue.

Cell Transfection. Cells were transfected using the Amaxa Cell Line Nucleofector Kit V (Amaxa, Koeln, Germany). Briefly, 1×10^6 Jurkat T cells were resuspended in 100uL Cell Line Nucleofector Solution V containing 2μg of plasmid DNA and 2μg pmaxGFP vector. The cell suspension was transferred to the provided cuvette and nucleofected using program X-001 on the Amaxa Nucleofector apparatus (Amaxa). Cells were incubated at room

temperature for 10 minutes then transferred to pre-warmed culture medium in 6-well plates.

Quantitative 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 and quantified using a NanoDrop-1000 spectrophotometer. Five mg total RNA was used for cDNA synthesis [SuperScript III First-Strand Synthesis Kit, Invitrogen] with Oligo dT as the primer. RT-PCR reactions were prepared in duplicate in reaction volumes of 25 ml with 50 ng cDNA, TaqMan assay mix, and TaqMan gene expression assay. *GAPDH* was used as a housekeeping gene and control. RT-PCR was performed using the ABI-7300 Real Time PCR System [Applied Biosystems].

Western Blotting. Whole cell lysates were prepared in PBS containing 1% NP-40 (Igepal CA 630), 50mm Tris HCl, 150mm NaCl, 2mm EDTA, 0.1% SDS, plus a cocktail of protease inhibitors [Roche] and sodium orthovanadate. Equal amounts of protein were resolved by SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes. Membranes were blocked in 5% nonfat milk, 0.1% Tween-20 in PBS. Rabbit polyclonal antibodies to JNK1 [ab10664] and phosphorylated JNK1 [cross-reactive with P-JNK2; P-JNK 1/2, ab4821] were from Abcam. The ECL Plus Chemiluminescent Kit [Applied Biosystems] was used to visualize protein bands.

Flow cytometry. For apoptosis determinations, cells were labeled with the PE Annexin-V Apoptosis Detection Kit I from BD-Pharmingen. For intracellular protein determinations, cells were fixed [paraformaldehyde], permeabilized [triton X-100 and NP-40], and labeled with primary antibodies for 24 hr at 0-4° C as described in the text followed by incubation with fluorescent-labeled secondary antibodies for 1 hr at 0-4°C. The following antibodies were employed: primary antibodies, rabbit anti-JNK [Santa Cruz, sc-571], polyclonal rabbit anti-p-JNK [pT183/pY185] [BD Pharmingen 558268], rabbit monoclonal to PUMA [abcam 33906], c-JUN [abcam, ChIP Grade [ab31419], TRAILR1 [DR4] [abcam 18362], and c-Fos antibody [abcam 7963]. FITC goat anti-rabbit Ig [BD Pharmingen, 554020] was used as the secondary. Cells were analyzed using the 3-laser BD LSRII flow cytometer.

Patient Populations. The study group was composed of 36 control subjects who had no current chronic or acute infections and no family history of autoimmune diseases and 70 subjects meeting the American College of Rheumatology clinical criteria for RA. No other exclusion or inclusion criteria were employed except for the ability to provide informed consent. The Committees for the Protection of Human Subjects of Vanderbilt University and UT Southwestern Medical Center approved these studies. The approximate female-to-male ratio in all study groups was 3:1. Age ranges [36-58 years] and racial distributions in all groups were similar. Current

therapies were determined by questionnaire and confirmed by chart review. Patients on MTX therapy were receiving doses of 15-25 mg per week.

Statistics. Statistical significance was determined by the unpaired T test with Welch's correction. $P < 0.05$ was considered significant.

Results

Apoptosis "priming" by MTX. Various studies have demonstrated the ability of MTX to induce apoptosis or alter cell viability. We cultured Jurkat T cells with MTX and monitored apoptosis by measuring the activity of caspase 3. Jurkat cells cultured with MTX, low concentrations of either H_2O_2 or anti-Fas for 24 hours, or combinations of MTX and H_2O_2 or MTX and anti-Fas exhibited minimal activation of caspase 3 relative to cells cultured with high concentrations of H_2O_2 (Figure 2-1A). We next cultured cells for 48 hours with MTX and then exposed them to either anti-Fas antibody or H_2O_2 for an additional 24 hours to activate death receptor or mitochondrial apoptosis pathways, respectively. Pretreatment of Jurkat cells with MTX at concentrations of 0.1μM resulted in a marked increase in activity of caspase 3 following culture with either H_2O_2 or anti-Fas (Figure 2-1B). As a second measure of apoptosis, we used flow cytometry to investigate changes in annexin V labeling. We used JNK1-DN and JNK2-DN mutants to assess the relative contributions of JNK1 and JNK2 to increased apoptosis sensitivity.

A

caspase 3 activation

1.0

0.5

0.0

MTX, µM	-	0.1	1.0	-	0.1	1.0	-	0.1	1.0	-
H_2O_2, mM	-	-	-	0.1	0.1	0.1	-	-	-	1.0
anti-Fas	-	-	-	-	-	-	+	+	+	-

B

caspase 3 activation

1.0

0.5

0.0

MTX, 0.1 µM	-	-	-	+	+	+
H_2O_2, 100 µM	-	+	-	-	+	-
anti-Fas	-	-	+	-	-	+

C

% Annexin +

40

20

0

MTX	-	+	-	-	+	+	+	+	+
H_2O_2	-	-	+	-	+	-	-	-	-
anti-Fas	-	-	-	+	-	+	+	+	+
JNK1-DN	-	-	-	-	-	-	+	-	+
JNK2-DN	-	-	-	-	-	-	-	+	+

D

-
BI-78D3
pepJIP1

% Annexin V +

40

20

0

- MTX, anti-Fas

E

-
NAc

% Annexin V +

30

20

10

0

- MTX, anti-Fas
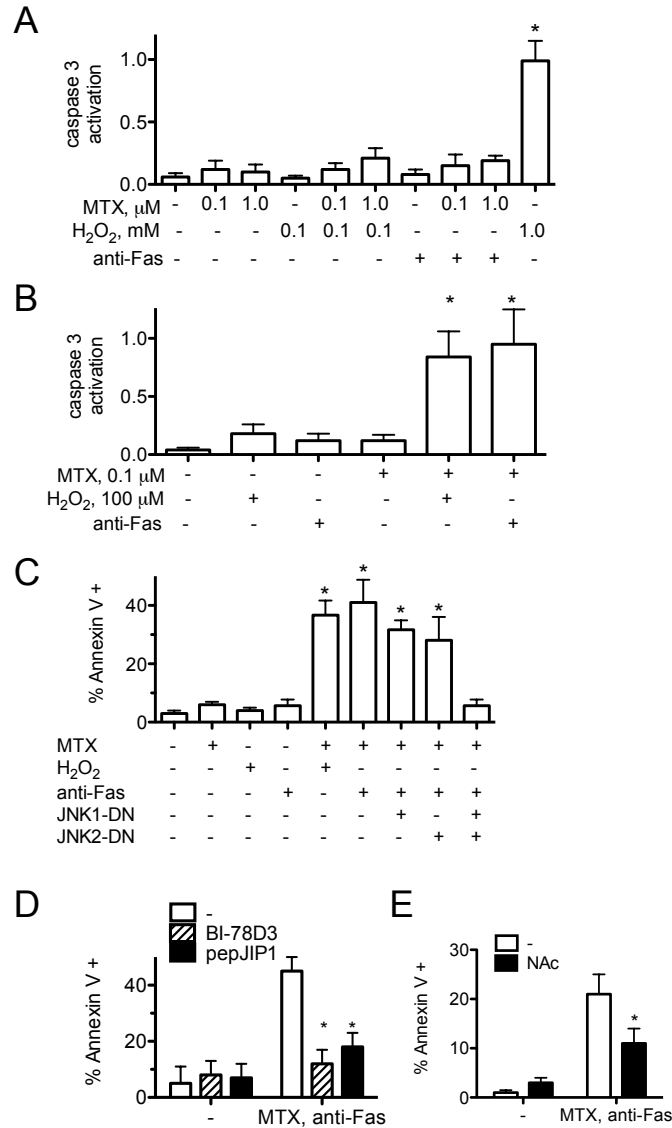
Figure 2-1. **Methotrexate (MTX) primes Jurkat cells for increased sensitivity to apoptosis. A** and **B**, Jurkat cells were cultured for 48 hours with MTX, H_2O_2, or 50ng/ml anti-Fas (**A**) or were cultured for 48 hours with the indicated concentrations of MTX, followed by culture with H_2O_2 or anti-Fas for 24 hours (**B**), and the levels of caspase 3 were determined. **C**, JNK1-dominant negative (DN), JNK2-DN, or empty-vector plasmids with a green fluorescent protein (GFP) plasmid were introduced by transient transfection. Cells were cultured for 48 hours with or without MTX 10^{-7} M and treated with anti-Fas for an additional 6 hours. The percentage of annexin V-positive cells was determined by flow cytometry after gating on GFP-positive and GFP-negative cells. **D** and **E**, Jurkat cells were cultured for 48 hours with MTX (10^{-7} M) in the presence or absence of BI-78D3 or pepJIP1 (**D**) or in the presence or absence of N-acetyl-L-cysteine (NAC) (**E**), followed by culture with anti-Fas for 6 hours. The percentage of annexin V-positive cells was determined by flow cytometry. Bars show the mean ± SD. * = P < 0.05 versus untreated cells.

Jurkat cells, either untreated or cultured with MTX for 48 hours, exhibited low percentages of annexin-V positive cells. Treatment with H_2O_2 or anti-Fas only slightly increased the percentages of annexin-V positive cells. However, treatment of MTX-cultured cells with H_2O_2 or anti-Fas resulted in a marked increase in the percentage of annexin-V positive cells (Figure 2-1C). This increase in apoptosis (annexin-V-positive cells) was slightly abrogated by the presence of either JNK1-DN or JNK2-DN mutants but was substantially abrogated by the presence of both JNK1-DN and JNK2-DN mutants.

We also tested the ability of the specific JNK inhibitors BI-78D3 and pepJIP1, which target JNK-JNK interacting protein 1 interaction sites, to prevent apoptosis.[102,103] Both inhibitors blocked MTX-dependent increases in sensitivity to apoptosis (Figure 2-1D). NAC, a free radical scavenger and reducing agents, also blocked MTX-dependent apoptosis (Figure 2-1E). Based on these results, we concluded that MTX primes cells for markedly increased sensitivity to apoptosis via death receptor and mitochondrial pathways, which is dependent on JNK1 and JNK2 enzymes. Additionally, ROS may contribute to increased apoptosis sensitivity.

We next sought to determine whether MTX treatment of Jurkat cells altered the expression levels of a panel of genes who protein products are known to have activating or inhibitor effects on apoptosis. Jurkat cells were cultured with MTX for 48 hours prior to RNA isolation, cDNA synthesis, and analysis by quantitative PCR. Although we did not observe reduced

expression of genes such as *BCL2*, whose protein products protect cells from apoptosis (Supplementary Fig. 2-1), we observed increased expression of several genes, *BBC3* (PUMA, Bcl-2 binding component 3), *BCL3* (B cell CLL/lymphoma 3), *HRK* (hara-kiri), *LTB* (lymphotoxin β), *TNFRSF10A* (cytotoxic TRAIL receptor, TRAILR-1), *TNFSF10* (TRAIL, APO2L), *TNFRSF1B* (TNFR2), and *TNFRSF25* (TRAMP, APO-3), whose protein products are known to play critical roles in promoting sensitivity to apoptosis (Figure 2-2A). We also investigated changes in expression of the prototypical JNK target genes, *JUN* and *FOS*. These genes also had increased expression levels following MTX treatment.

To assess changes in the protein levels of target genes and to assess the relative contributions of JNK1 and JNK2 to these changes, we measured intracellular protein levels by flow cytometry with specific antibodies and used JNK1- or JNK2-DN mutants to specifically inhibit JNK1 or JNK2. MTX treatment resulted in increased protein expression of the known JNK target, c-Jun, and this increase was inhibited by the JNK1-DN mutant but not the JNK2-DN mutant (Figure 2-2B). We also determined changes in TRAILR-1, c-Fos, and PUMA protein levels after MTX treatment in the presence of either JNK1- or JNK2-DN mutants, using flow cytometry.
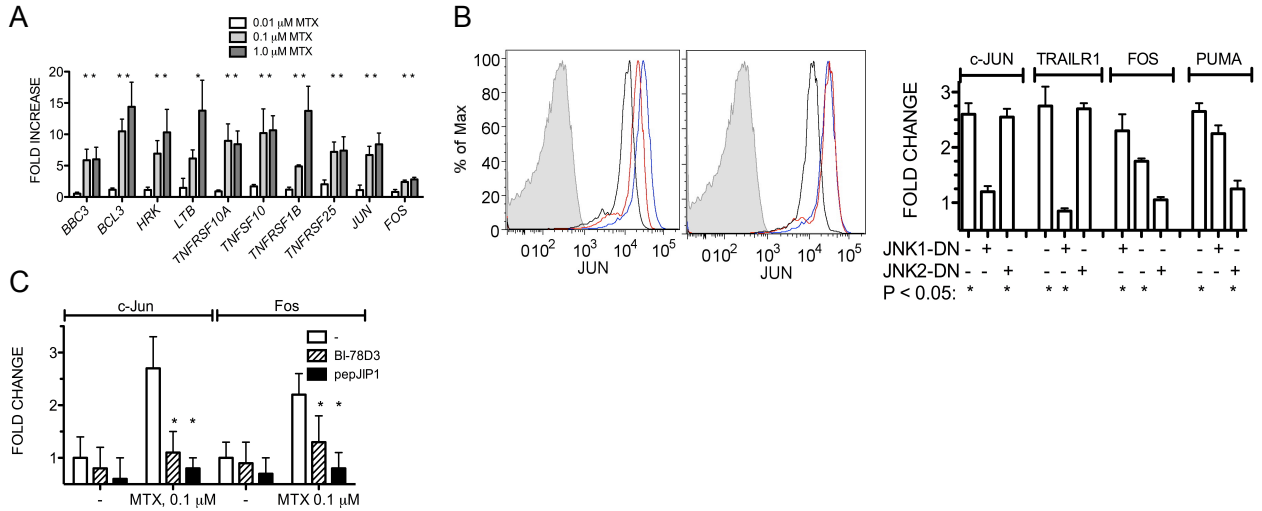
Figure 2-2. **MTX induces expression of genes whose protein products promote apoptosis. A,** Jurkat cells were cultured with MTX for 48 hours, and transcript levels of genes whose protein products are known to influence apoptosis were determined. Results are expressed as the fold increase compared with untreated cells after normalization to *GAPDH*. **B,** JNK1-DN, JNK2-DN, or empty-vector plasmids with a GFP expression plasmid were introduced into Jurkat cells by nucleofection. Left and middle, C-Jun levels in untreated cells (black lines), MTX-treated cells (blue lines), and MTX-treated cells transfected with JNK1-DN (red lines; left) or MTX-treated cells transfected with JNK2-DN (red lines; middle) are shown. Right, Changes in the expression levels of c-Jun, Fos, PUMA, and TRAILR-1 in MTX-treated Jurkat cells were determined by flow cytometry and are expressed as the fold change relative to untreated cells. **C,** MTX-mediated increases in c-Jun and Fos protein levels were inhibited by the specific JNK inhibitors BI-78D3 and pepJIP1. Bars show the mean ± SD. * = P < 0.05 versus untreated cells. Max = maximum (see Figure 2-1 for other definitions).

Induction of TRAILR-1 by MTX was also abrogated by the JNK1-DN mutant, whereas the induction of c-Fos and PUMA was relatively unaffected by the JNK1-DN mutant but was almost completely abrogated by the JNK2-DN mutant (Figure 2-2B). The JNK-specific inhibitors BI-78D3 and pepJIP1 also inhibited induction of c-Jun and c-Fos expression by MTX (Figure 2-2C).

Taken together, these results provided evidence that MTX increased sensitivity to apoptosis, at least in part, by inducing expression of genes whose protein products promote apoptosis. The results observed with the JNK-DN mutants implicated both JNK1 and JNK2 as regulators of this gene family.

Induction of *JUN* and *FOS* by MTX. *JUN* and *FOS* are prototypical JNK target genes.[104,105] Thus, we further explored induction of these genes by MTX. We also examined the effects of adenosine, because MTX is known to induce adenosine production.[36] MTX at concentrations of 0.1-1.0μM stimulated a gradual increase in *JUN* levels over the course of 24-72 hours (Figure 2-3A). In contrast, adenosine at concentrations of 0.01-100μM failed to induce changes in *JUN* expression levels over this time period (Figure 2-3B). This range of adenosine concentrations should be sufficient to activate known human adenosine receptors, which have 50% maximum response concentrations for adenosine of ~0.3μM to 30μM.[106-108]
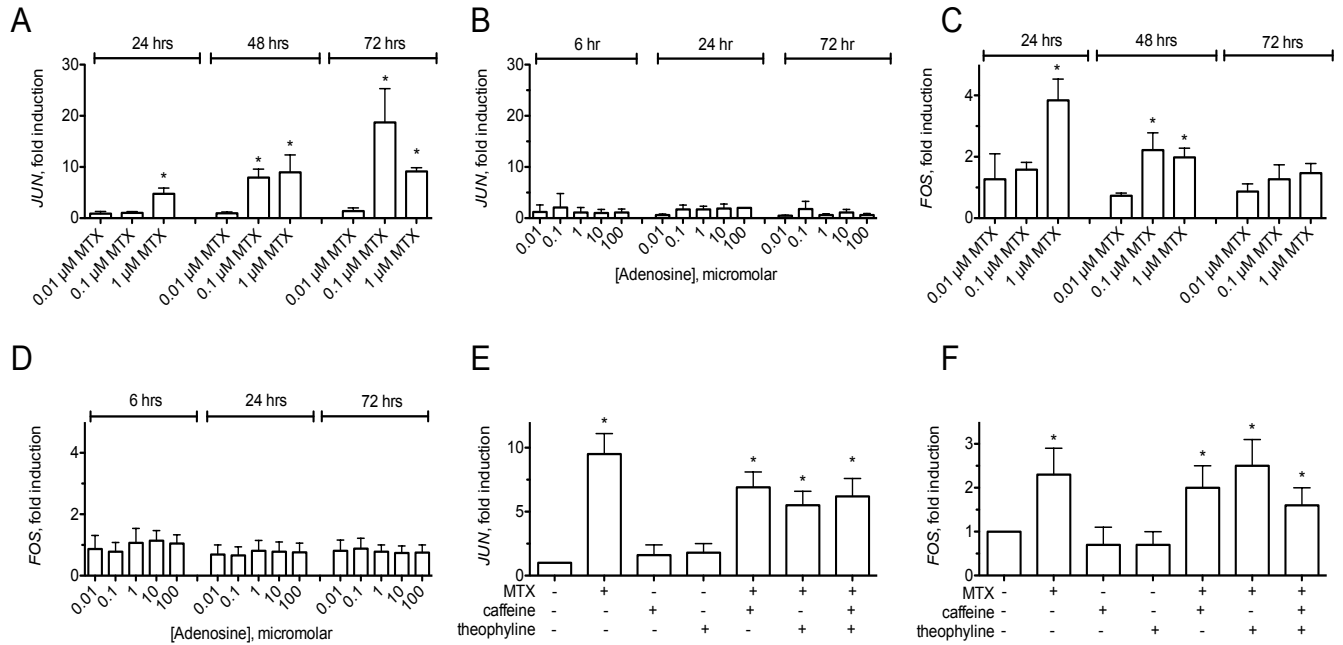
Figure 2-3. **Induction of *JUN* and *FOS* expression by methotrexate (MTX) in Jurkat T cells. A-D,** Jurkat T cells were cultured with the indicated concentrations of MTX (**A** and **C**) or adenosine (**B** and **D**). At the indicated times, cell cultures were harvested, total RNA was purified, and cDNA was synthesized. The relative expression of *JUN* and *FOS* was normalized to *GAPDH* levels and expressed as the fold induction relative to untreated cultures. **E** and **F**, Jurkat cells were also cultured with MTX and the adenosine receptor antagonists caffeine (20 μM) and/or theophylline (20 μM) for 48 hours. The expression levels of *JUN* and *FOS* were determined by polymerase chain reaction. Bars show the mean ± SD. * = $P < 0.05$ versus unstimulated cultures.

We also determined the expression levels of *JUN* family members, *JUNB* and *JUND*. Treatment of Jurkat cells with MTX or adenosine did not change expression levels of these *JUN* family members. Culture with MTX or adenosine did not affect cell numbers or viability, as determined microscopically. Thus, exposure to MTX, but not adenosine, directly increased *JUN* expression levels in a uniform T cell population but did not change expression levels of the Jun family members, *JUNB* and *JUND*.

Because the transcription factor activator protein 1 (AP-1) is a heterodimer composed of a Jun family member and a Fos family member[109], we determined the impact of MTX treatment on levels of Fos family member transcripts. Similar to what was observed for *JUN*, *FOS* messenger RNA (mRNA) expression levels increased in Jurkat cells after stimulation by MTX (Figure 2-3C). In contrast, stimulation by adenosine did not alter *FOS* mRNA expression (Figure 2-3D). We examined changes in the mRNA levels of additional *FOS* family members *FOSL1* and *FOSB*. We did not detect changes in transcript levels of these 2 genes following exposure to MTX or adenosine. The kinetics of *FOS* induction were different from the kinetics of *JUN* induction following MTX stimulation. *FOS* mRNA levels reached a peak after 24-48 hours, depending on the concentration of MTX, and declined by 72 hours, whereas *JUN* mRNA levels reached a peak at 72 hours. Taken together, these results demonstrated that MTX induced increased expression of both the *JUN* and *FOS* components of the AP-1 transcriptional complex in

Jurkat cells. They also showed that MTX specifically induced *JUN* and *FOS* mRNA but not mRNA encoding other Jun and Fos family members.

As an alternate approach, we sought to determine whether the adenosine receptor antagonists with broad specificity, caffeine and/or theophylline, interfered with the induction of *JUN* and *FOS* mRNA. Caffeine, theophylline, or their combination, at pharmacologically active concentrations, did not interfere with the induction of *JUN* and *FOS* mRNA. Caffeine, theophylline, or their combination, at pharmacologically active concentrations, did not interfere with the induction of *JUN* and *FOS* mRNA by MTX (Figure 2-3E and 2-3F). Taken together, these results do not provide evidence against the notion that the anti-inflammatory effects of MTX may be mediated, in part, via stimulation of adenosine release and activation of adenosine receptors. Rather, the results presented here clearly demonstrated that adenosine alone is not sufficient to increase the expression of *FOS* and *JUN* mRNA in T cells, and that adenosine receptor antagonists with broad specificity do not interfere with the induction of *FOS* and *JUN* mRNA by MTX.

Blockade of MTX-induced apoptosis by free radical scavengers and tetrahydrobiopterin (BH4). MTX, at concentrations similar to those inducing *JUN* and *FOS* expression, leads to the production of ROS and priming for apoptosis.[24] Furthermore, ROS are known to activate JNK, leading to increased *JUN* expression.[101,110,111] We cultured Jurkat cells with

MTX and the free radical scavenger N-acetyl-L-cysteine (NAC) and examined changes in apoptosis sensitivity and *JUN* expression levels. The addition of NAC inhibited MTX-mediated changes in apoptosis sensitivity (Figure 2-4A) and MTX-mediated increases in *JUN* expression in Jurkat cells but did not alter baseline *JUN* levels (Figure 2-4B). Therefore, we attempted to determine whether MTX increased the production of ROS, using flow cytometry after labeling cells with CM-H$_2$DCFDA. MTX caused a marked increase in ROS production (Figure 2-4C) that was effectively reduced by NAC (Figure 2-4D). We concluded that the JNK-dependent increases in apoptosis sensitivity and *JUN* transcript levels induced by MTX were mediated, at least in part, through ROS production. MTX inhibits the reduction of dihydrofolate to tetrahydrofolate and the reduction of dihydrobiopterin (BH$_2$) to BH$_4$ catalyzed by DHFR.[112-114] BH$_4$ is a necessary cofactor for nitric oxide synthases (NOS) including endothelial NOS (eNOS), which is expressed in T lymphocytes.[115,116] The loss of BH$_4$ uncouples eNOS from NO synthesis, leading to excess production of ROS. Thus, we tested whether supplementation with BH$_4$ could reverse MTX-mediated increased apoptosis and ROS production. We observed that BH$_4$ supplementation effectively reduced MTX-dependent apoptosis (Figure 2-4E), and MTX-dependent increased ROS production (Figure 2-4F).
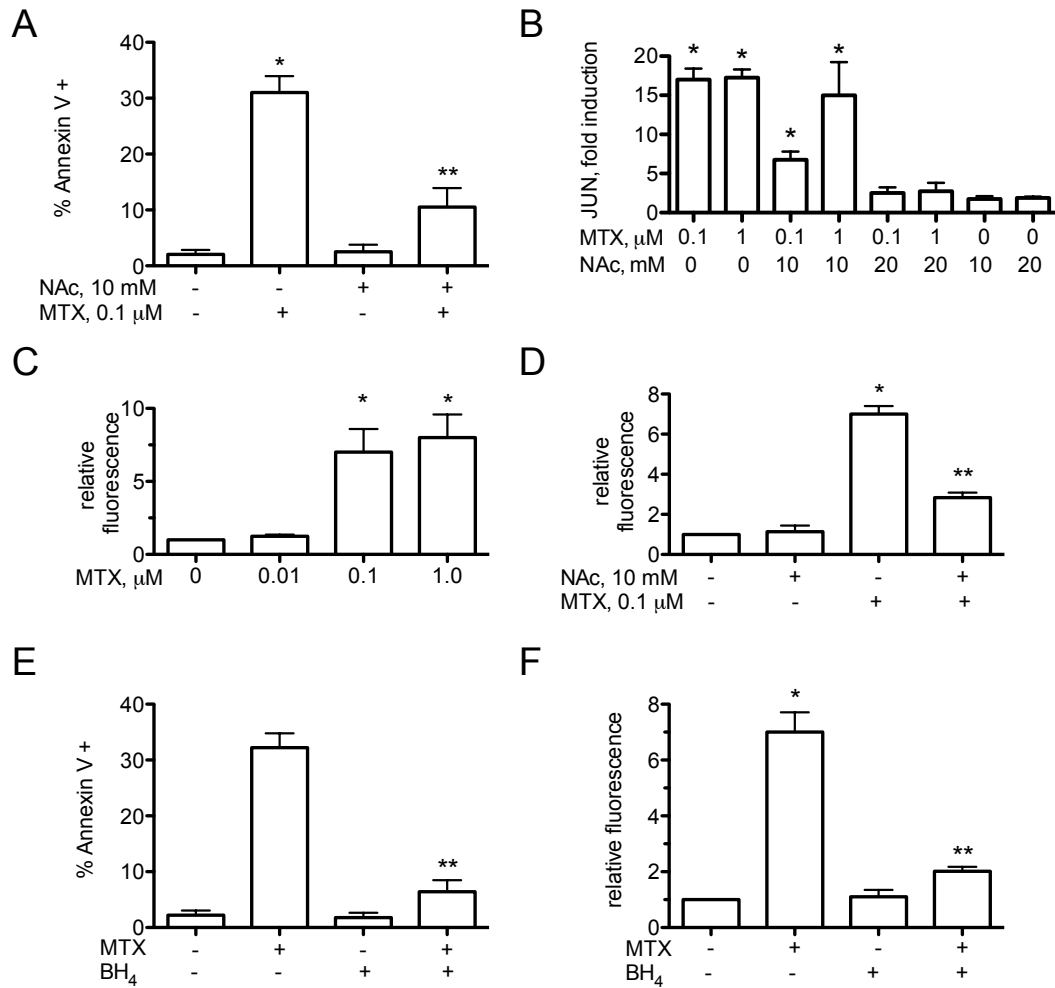
Figure 2-4. Free radical scavengers or tetrahydrobiopterin (BH₄) supplementation blocks methotrexate (MTX)-mediated increases in apoptosis sensitivity. A and **B**, Jurkat cells were cultured for 48 hours with MTX and/or the free radical scavenger *N*-acetyl-L-cysteine (NAC), stimulated with anti-Fas for 6 hours, and the percentage of annexin-V-positive cells was determined (**A**) or the cultures were harvested and processed, and the *jun* transcript levels were determined (**B**). **C** and **D**, Jurkat cells were treated for 48 hours with MTX alone (**C**) or with MTX in the presence or absence of NAC, and reactive oxygen species (ROS) production was determined by labeling cells with CM-H₂DCFDA. **E** and **F**, Jurkat cells were cultured for 48 hours with MTX (0.1 μM) with or without BH₄ supplementation (30 μM). The percentage of annexin V-positive cells after anti-Fas treatment was determined by flow cytometry (**E**), and ROS levels were determined after loading Jurkat cells with CM-H₂DCFDA. Bars show the mean ± SD. * = *P* < 0.05 versus unstimulated cultures; ** = *P* < 0.05 versus MTX- and NAC-treated cultures (**A** and **D**) or MTX- and BH₄-treated cultures (**E** and **F**).

These results are consistent with a model whereby inhibition of DHFR by MTX reduces intracellular levels of BH_4, resulting in NOS-catalyzed ROS overproduction, leading to activation of JNK and increased apoptosis sensitivity.

Induction of JNK expression and activity by MTX. Inhibition of MTX-mediated increased *JUN* expression, priming for apoptosis, and expression of genes whose protein products are proapoptotic, by JNK inhibitors and by JNK1- and JNK2-DN mutants, suggest that MTX may increase JNK expression or activity. JNK is a member of the MAP kinase family and is activated by phosphorylation on threonine and tyrosine residues by JNK kinases, also termed MAP kinase kinases, in response to an array of stimuli.[117,118] There are 3 JNK genes that produce multiple mRNA and protein isoforms. We used Western blotting to determine whether stimulation by MTX changed JNK1 total protein levels and phosphorylated JNK1/2 levels. Jurkat cells were cultured with different concentrations of MTX for 2 days. Whole cell lysates were prepared and analyzed by SDS-PAGE and Western blotting with antibodies specific for JNK1 total protein or antibodies specific for the phosphorylated forms of JNK1 and JNK2. Culturing cells with MTX resulted in an increase in total JNK1 protein levels and levels of phosphorylated JNK1/2 (Figure 2-5A).
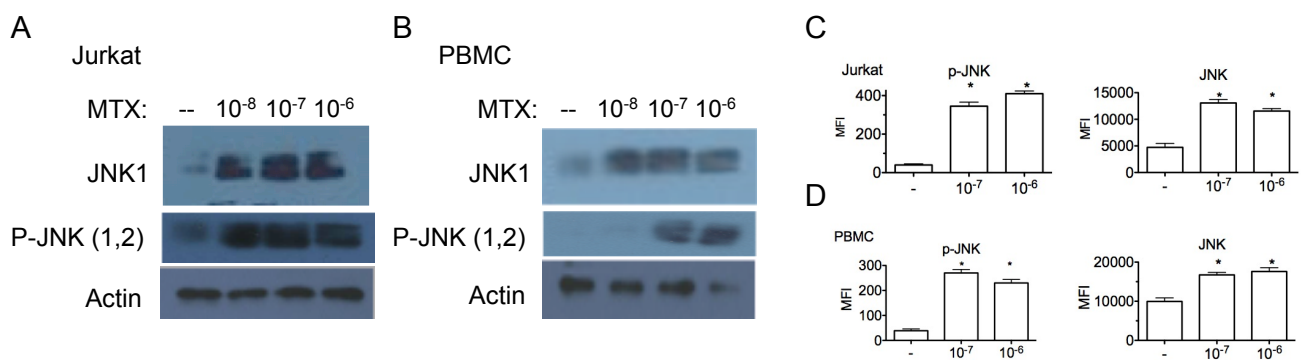
Figure 2-5. **Methotrexate (MTX) induces increased levels of JNK and p-JNK. A** and **B**, Jurkat T cells (**A**) or human peripheral blood mononuclear cells (PBMCs) (**B**) were treated with the indicated concentrations of MTX. After 48 hours, cells were harvested, whole cell lysates were prepared, and JNK1 and p-JNK levels were determined by Western blotting with specific antibodies for either JNK1 or p-JNK1/2. **C** and **D**, Jurkat cells (**C**) and PBMCs (**D**) were treated with or without MTX, labeled with specific antibodies for p-JNK or JNK, and analyzed by flow cytometry. Bars show the mean ± SD mean fluorescence intensity (MFI). * = $P < 0.05$ versus untreated cells.

We also cultured human PBMC with MTX for 2 days and analyzed whole cell lysates by SDS-PAGE and Western blotting. Similar to what was observed in Jurkat cells, culturing PBMCs with MTX resulted in concentration-dependent increases in total JNK1 protein and phosphorylated JNK1/2 (Figure 2-5B). We also examined changes in total phosphorylated JNK and total JNK levels by flow cytometry. These experiments confirmed changes in phosphorylated JNK and total JNK levels in Jurkat cells (Figure 2-5C) and PBMCs (Figure 2-5D) following MTX treatment. We concluded that MTX induced an increase in the cellular levels of phosphorylated JNK protein and total JNK protein in both Jurkat cells and human PBMCs.

JUN expression in RA patients receiving MTX. Given that MTX increased p-JNK levels in both Jurkat cells and PBMCs and expression of the known JNK target gene, _JUN_, we sought evidence for similar effects of MTX _in vivo_. To test this hypothesis, we determined expression levels of _JUN_ in control subjects relative to patients with RA who were or were not currently receiving low-dose weekly MTX therapy. Blood samples were collected in PAXgene tubes. The expression levels of target genes were determined relative to _GAPDH_. We observed increased expression of _JUN_ in the blood of patients with RA who were currently receiving MTX therapy relative to patients who were not receiving MTX therapy and control subjects (Figure 2-6, far left).
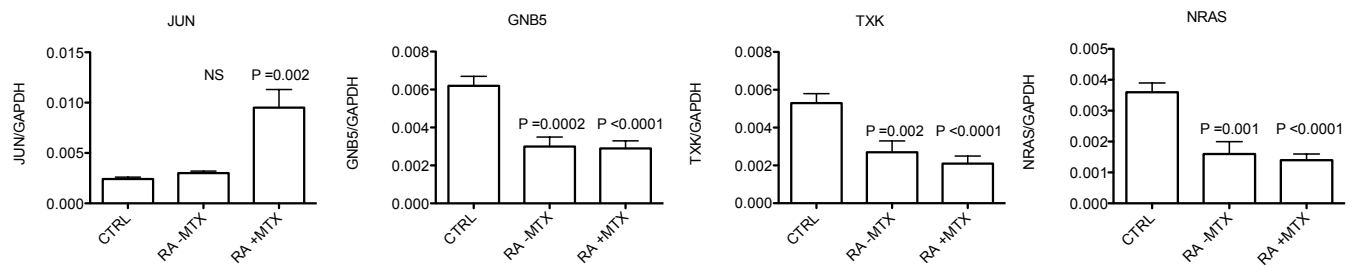
Figure 2-6. **Expression levels of the JNK target gene *JUN in vivo* in patients with rheumatoid arthritis (RA) as a function of current methotrexate (MTX) therapy.** Blood was obtained in PAXgene tubes from control subjects (CTRL; n = 36), patients with RA not receiving MTX (RA – MTX; n = 28), and patients with RA receiving MTX (RA + MTX; n = 22). Bars show the mean ± SD gene expression levels relative to *GAPDH*. *P* values are versus control. NS = not significant.

In contrast, expression levels of 3 other genes, *GNB5*, *TXK*, and *NRAS*, were reduced in patients with RA compared with control subjects, independent of MTX therapy (or other therapies). We concluded that low-dose MTX therapy increased expression levels of *JUN* in whole blood, while the expression levels of other target genes that are reduced in patients with RA did not change in response to MTX therapy.

Discussion

MTX was developed in the 1940's as an antagonist of DHFR, and since that time, the therapeutic efficacy of MTX has been attributed to inhibiting reduction of dihydrofolate to tetrahydrofolate resulting in inhibition of purine synthesis necessary for DNA and RNA synthesis.[9,91] However, folate supplementation does not interfere with anti-inflammatory effects of MTX suggesting the presence of additional mechanisms of action of low-dose MTX for treatment of this class of disease.[119] In the 1990's, it was proposed that production of adenosine, as a by-product of DHFR inhibition, stimulates adenosine receptors, thus contributing to the anti-inflammatory effects of MTX.[13,36] An additional possible mechanism by which MTX may exert its anti-inflammatory effects is by induction of apoptosis of inflammatory cells, such as lymphocytes, yet it is not immediately apparent how these

biochemical pathways, which are activated or inhibited by MTX, may induce apoptosis.

Our new model suggests that MTX inhibits DHFR-catalyzed BH_2 reduction to BH_4. The downstream biochemical effector pathways stimulated by loss of BH_4 lead to altered cell sensitivity to apoptosis, thus shifting the decades-long focus of MTX action away from inhibition of dihydrofolate reduction to inhibition of BH_2 reduction to BH_4 [Supplementary Fig. 2-2]. Our results support a model whereby submicromolar concentrations of methotrexate stimulate increased sensitivity to apoptosis via the death receptor and mitochondrial pathways. Increased apoptosis sensitivity depends upon increased expression and activity of JNK and subsequent increased expression of JNK target genes. JNK target genes include those whose protein products increase sensitivity of cells to apoptosis. JNK activation is mediated in part by ROS, which we propose is induced by MTX-dependent depletion of BH_4 levels uncoupling eNOS from NO synthesis, resulting in overproduction of ROS. Specific JNK inhibitors prevent both MTX-mediated effects on gene and protein induction and changes in sensitivity to apoptosis. Both JNK1-DN and JNK2-DN mutants interfere with these MTX-mediated effects. Thus, both JNK enzymes contribute to MTX-mediated changes in the transcriptional program in Jurkat cells and to alterations in sensitivity to apoptosis by regulating expression of different target genes. Furthermore, patients with RA receiving low-dose weekly MTX

exhibit increased expression of *JUN*, the prototypical JNK target gene, thus, supporting the notion that this pathway is activated by MTX *in vivo* and may contribute to efficacy of MTX in inflammatory disease.

It is well established that JNK enzymes play both positive and negative roles in both death receptor and mitochondrial pathways of apoptosis.[104] These roles can be mediated via JNK translocation to the nucleus and phosphorylation of transcription factors. c-Jun is the prototypical transcription factor phosphorylated by JNK, but JNK also phosphorylates additional Jun family members, ATF and Elk family members, p53, c-Myc and others.[120] JNK can also translocate to mitochondria and regulate activity of proteins involved in apoptosis, such as BAD, Bim, and 14-3-3, via phosphorylation. In large part, these studies have examined roles of JNK in stimulating or preventing apoptosis in response to either external or internal stimuli. Our results support a new role for JNK in regulating apoptosis. The treatment of Jurkat T cells with MTX induces increased expression and activity of JNK, increased expression of *JUN* mRNA, and increased expression of a number of genes whose protein products are proapoptotic. However, MTX does not directly stimulate apoptosis under these conditions. Rather, MTX-treated Jurkat cells are 'primed' to exhibit markedly increased sensitivity to apoptosis when exposed to stimuli activating death receptor and mitochondrial pathways via a JNK-mediated pathway. Further experimentation will be required to address

whether JNK also 'primes' Jurkat cells for apoptosis by modulating activity of proapoptotic or antiapoptotic proteins via direct phosphorylation.

In many cell types, activation of JNK is mediated by its phosphorylation in response to stimuli, including cellular stress and inflammatory cytokines. In contrast, T lymphocytes exhibit a second level of regulation. Resting or naïve T lymphocytes express low quantities of JNK enzymes.[121] Activation via T cell receptor ligation leads to markedly increased expression of JNK genes and protein, whereas JNK phosphorylation requires CD28-mediated co-stimulatory signals. Our results demonstrate that MTX treatment of Jurkat cells or human PBMC (70% T lymphocytes) leads to increased levels of phosphorylated JNK and total JNK protein. Whether MTX treatment of non-T cells or non-lymphoid cells induces increased levels of phosphorylated JNK and total JNK protein remains to be determined.

The potential of JNK inhibitors as therapeutics for inflammatory disease has also attracted considerable interest.[104,117,122-126] Modulation of JNK enzymes, either by genetic means or with small molecule inhibitors, interferes with a number of processes linked to inflammatory disease. For example, many genes linked to inflammation, such as those encoding TNF-α, matrix metalloproteinases, and adhesion molecules, possess AP-1-response elements in their promoters and are regulated by JNK enzymes through activation of AP-1 and ATF-2 transcription factors. Our results suggest a

second paradigm shift. Our model suggests that correctly targeted activation of JNK, rather than its inhibition, represents a therapeutic goal for the treatment of inflammatory disease, especially RA. In lymphocytes, increased JNK activity and subsequent increased sensitivity to apoptosis may also have therapeutic benefits in inflammatory diseases that are dependent upon T lymphocyte function by eliminating self-reactive T cells. For example, activated T cells undergo apoptosis if exposed to secondary T cell receptor ligation in a process termed activation-induced cell death.[95]

We speculate that MTX therapy may 'prime' self-reactive T cells to undergo increased activation-induced cell death in response to secondary exposure to self-antigen and thus produce its therapeutic benefit. Future studies will be required to determine if this novel MTX-induced pathway can be exploited for therapeutic benefit.

CHAPTER III

METHOTREXATE INCREASES EXPRESSION OF CELL CYCLE CHECKPOINTS GENES VIA JNK ACTIVATION

Abstract

Objective. To assess defects in expression of critical cell cycle checkpoint genes and proteins in subjects with rheumatoid arthritis (RA) 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.

Introduction

Rheumatoid arthritis is the most common serious autoimmune disease affecting ~1.3 million people in the United States.[127] Often characterized by bone erosion and cartilage destruction through chronic inflammation, RA is a multisystem disorder affecting synovial spaces between small and large joints. It accounts for ~250,000 hospitalizations and ~9 million clinic visits each year, and its associated costs represent approximately ~1% of the U.S. gross domestic product.[128]

Though initially developed as a chemotherapeutic agent, methotrexate (MTX) has been the mainstay for RA treatment since the 1980s.[3,9,11,28,90] Once-weekly administration of 7.5-25 mg yields optimal clinical outcomes in RA, compared to the 5,000 mg/week dosage used in the treatment of malignancy.[3,25] MTX is a potent, competitive inhibitor of dihydrofolate reductase (DHFR)[6,87,88] resulting in decreased tetrahydrofolate levels, and

inhibition of de novo purine and pyrimidine synthesis, leading to cell cycle arrest.[25,34] 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) does not attenuate clinical efficacy of MTX, the anti-inflammatory actions of low-dose MTX may stem from alternative pathways.[25,36,119]

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.[18,84,129-131]

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 (BH_2) to tetrahydrobiopterin (BH_4). MTX-mediated JNK activation results in induction of pro-apoptotic target genes and increased sensitivity to apoptosis.[132] Since JNKs, members of the MAP kinase family of proteins, also directly phosphorylate p53 leading to its increased accumulation and

activity[133-135] 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.

Materials 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.[136] Demographic characteristics of the different disease and therapy groups were not statistically different (Supplementary Table 3-1). 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, BH_4, caffeine, theophylline, folic acid, and N-acetyl-L-cysteine (NAC) were from Sigma. CM-H_2DCFDA was obtained from Invitrogen.

Cell Culture. Cells were cultured in RPMI 1640 media (1 μg/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% CO_2. 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 0.1μM to 1μM, 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 ~0.5μM after 1 hour and ~0.1 μM after 10 hours.[137]

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 μg 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 μL 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.[132]   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 (Supplementary Table 3-2). 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 3-2).

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. 3-1A). 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[138,139], 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.[140,141] 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 3-1B). 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.
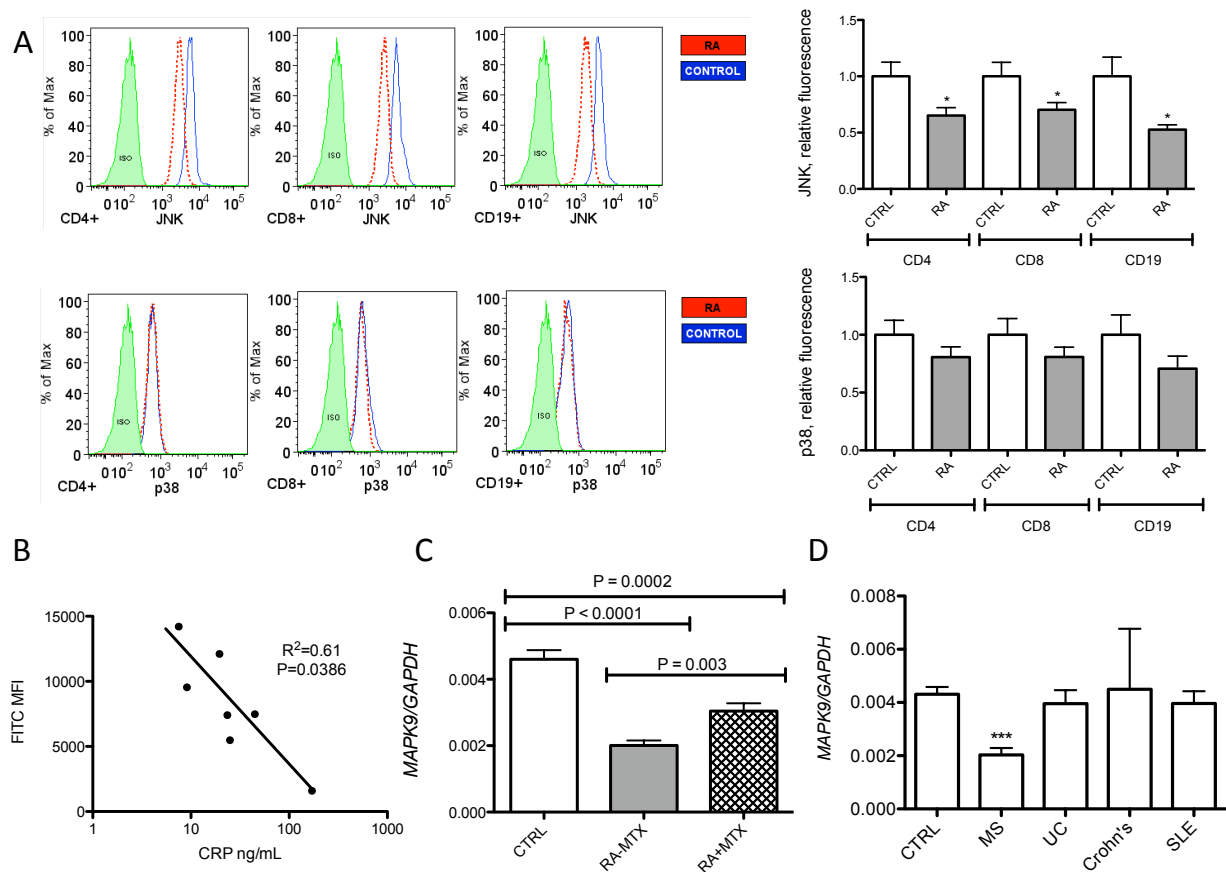
Figure 3-1. *MAPK9* (JNK-2) underexpression in rheumatoid arthritis (RA) is corrected by methotrexate (MTX) therapy. A, JNK total protein concentrations in RA patients ($n = 18$) and controls ($n = 18$) were determined by flow cytometry. Cells were fixed, permeabilized, and stained for CD4, CD8, and CD19 cell surface markers and intracellular proteins JNK and p38 using fluorescence-labeled secondary antibody. $* = P < 0.05$ versus controls. **B,** The relationship between JNK expression levels and C-reactive protein (CRP) levels was determined. Serum and peripheral blood mononuclear cells were isolated from RA patients ($n = 7$). Intracellular JNK levels were determined as described in **A,** and CRP levels were determined by enzyme-linked immunosorbent assay. Increased JNK expression correlated with decreased CRP levels in vivo. **C**, *MAPK9* expression levels relative to expression of *GAPDH* were determined. Whole blood samples from control subjects ($n = 43$), RA patients not receiving MTX (RA − MTX) ($n = 18$), and RA patients receiving MTX (RA + MTX) ($n = 18$) were obtained in PAXgene tubes. **D**, *MAPK9* levels in samples from patients with multiple sclerosis (MS) ($n = 45$), ulcerative colitis (UC) ($n = 20$), Crohn's disease ($n = 23$), and systemic lupus erythematosus (SLE) ($n = 23$) were determined as described in **C**. $*** = P < 0.001$ versus controls. Values in **A** (right panels), **C**, and **D** are the mean ± SD. ISO = isotype control; FITC = fluorescein isothiocyanate; MFI = mean fluorescence intensity.

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 3-1C). 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 3-1D). 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 3-2). 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.
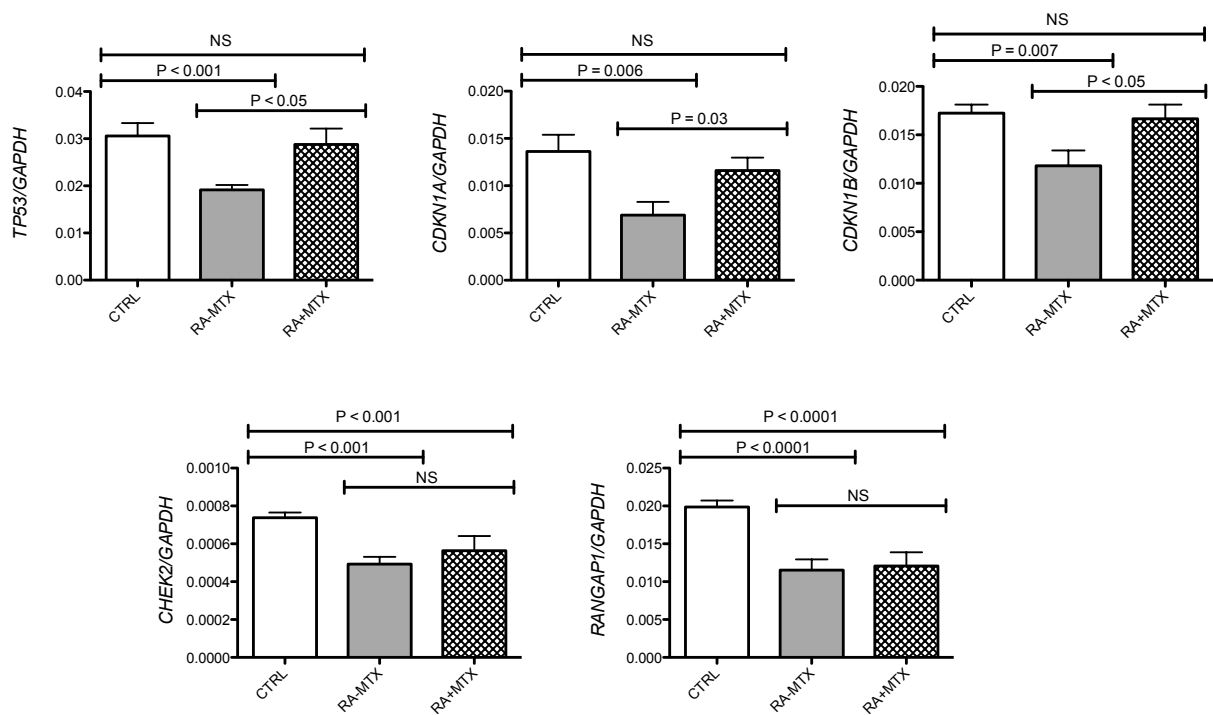
Figure 3-2. Deficiencies in expression of genes encoding p53, p27, and p21 are restored by MTX in vivo. *TP53*, *CDKN1B*, *CDKN1A*, *CHEK2*, and *RANGAP1* transcript levels relative to *GAPDH* transcript levels in control subjects (n = 43), RA patients not receiving MTX (n = 18), and RA patients receiving MTX are shown. Values are the mean ± SD. NS = not significant (see Figure 1 for other definitions).

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.[132] 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 3-3A).   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 3-3B). 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. 3-3C). 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 mM), also increases intracellular BH4 levels through its active form, 5-methyltetrahydrofolate, and restores the amount of available nitric oxide in the cell.[142,143] 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 3-3D). MTX alters the transcriptional profile of cells by increasing expression of a number of genes that encode proteins with pro-apoptotic function.[132] 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 3-3E). 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.
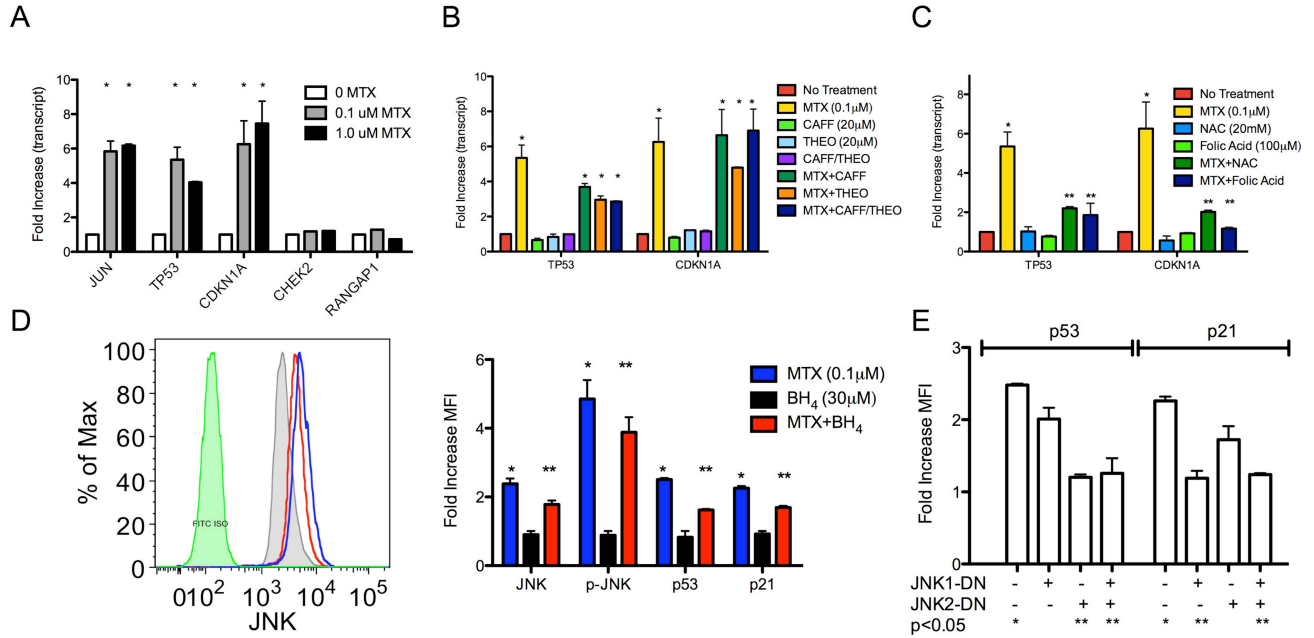
Figure 3-3. Free radical scavengers, folic acid, and tetrahydrobiopterin (BH₄) block MTX-mediated induction of p53 and p21 by JNK. A, JKT cells were cultured with MTX at 0.1 μ*M* or 1.0 μ*M* for 48 hours. Target gene transcript levels were normalized to *GAPDH* and calculated as the fold increase relative to levels in untreated samples (set at 1). ∗ = *P* < 0.05 versus unstimulated cultures. **B** and **C,** JKT cells were cultured with the adenosine receptor antagonists caffeine (CAFF) and/or theophylline (THEO) (**B**) or with *N*-acetyl-l-cysteine (NAC) or folic acid (**C**). Results are expressed as described in **A.** ∗ = *P* < 0.05 versus unstimulated cultures; ∗∗ = *P* < 0.05 versus cultures stimulated with MTX alone. **D,** JKT cells were cultured with MTX with or without BH₄, and levels of JNK, p-JNK, p53, and p21 were determined by flow cytometry. Representative flow diagram shows background fluorescence (green) and results obtained with untreated cells (gray), MTX-treated cells (blue), and MTX plus BH₄–treated cells (red). ∗ = *P* < 0.05 versus unstimulated cultures; ∗∗ = *P* < 0.05 versus cultures stimulated with MTX or BH₄ alone. **E,** JNK-1–dominant-negative (DN), JNK-2–DN, or empty vector plasmids with green fluorescent protein (GFP) marker were transfected into JKT cells. Cells were cultured with MTX for 48 hours and assayed for p53 and p21 with gating on GFP-positive and - negative cells. Results are representative of 3 experiments. ∗ = *P* < 0.05 versus unstimulated cultures; ∗∗ = *P* < 0.05 versus MTX alone or MTX and JNK-1–DN plasmid–transfected culture (p53), or versus MTX alone or MTX and JNK-2–DN plasmid–transfected culture (p21). Values are the mean ± SD. See Figure (3-1) for other definitions.

BH$_4$ 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.[132] Isolated PBMC were stimulated by anti-CD3 for 72 hours and treated with MTX (0.1μM) 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 3-4A). Supplementation of cell cultures with BH4 (30 mM) 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).[144] 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 3-4B). 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 3-4C). Increases in JNK levels were prevented by supplementation with BH4.
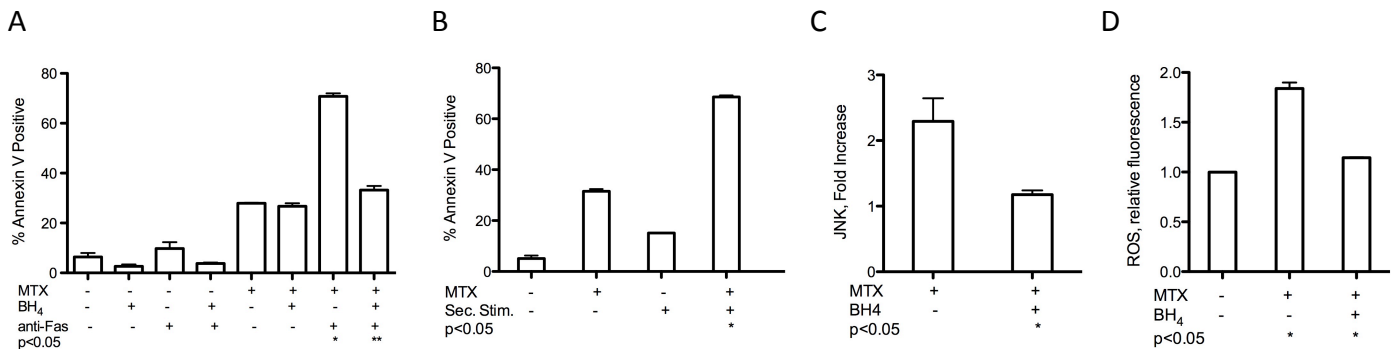
Figure 3-4. Tetrahydrobiopterin (BH₄) reverses methotrexate (MTX)–mediated apoptosis priming, JNK activation, and reactive oxygen species (ROS) production in activated T cells. A, Activated peripheral blood mononuclear cells were treated with 0.1 μM MTX for 24 hours with or without addition of BH₄ (30 μM). After 24 hours, cultures were stimulated with anti-Fas for 6 hours to induce apoptosis. ∗ = P < 0.05 versus unstimulated cultures; ∗∗ = P < 0.05 versus stimulation with MTX and anti-Fas without BH₄. **B,** Activated T cells were treated with MTX for 24 hours and stimulated with anti-CD3 (secondary stimulation [Sec. Stim.]) for an additional 6 hours. ∗ = P < 0.05 versus unstimulated and MTX-treated cultures. **C,** Activated T cells were cultured with MTX for 24 hours with or without BH₄ (30 μM) and assayed for JNK protein with gating on CD4+ T cells. ∗ = P < 0.05 versus stimulation with MTX without BH₄. **D,** Activated T cells were treated as described in **C** and then cultured with 5,6-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate dye for 1 hour prior to flow cytometry. ROS production was measured with gating on the lymphocyte population, with measurements expressed as fluorescence relative to that obtained with untreated cultures without addition of BH₄. ∗ = P < 0.05 versus unstimulated cultures. Values are the mean ± SD.

We also determined MTX stimulated ROS production in activated T cells by flow cytometry using the CM-H$_2$DCFDA dye. MTX induced ROS production in activated T cells (Figure 3-4D). ROS production by activated T cells was also inhibited by supplementation with BH$_4$. Our results support the notion that MTX inhibition of DHFR depletes intracellular stores of BH$_4$ in activated T cells increasing ROS production leading to JNK activation and alteration in sensitivity to apoptosis. Addition of BH$_4$ reversed these methotrexate-mediated effects.

Discussion

RA T cells exhibit functional defects in cell survival, DNA damage responses, and apoptosis.[18,145-147] MAPKs also play key roles in these fundamental cellular processes[148] and our recent work demonstrates that MTX, one of the most frequently prescribed pharmacologics 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.[132] 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)[149-152] and that RA T cells respond to MTX therapy by restoring a portion, but not all of these cell cycle checkpoints (Figure 3-6). 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 BH_4 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.

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NOT INDUCED BY METHOTREXATE

CHEK2 – G2 Checkpoint
Activated by ATM

RAN – M Checkpoint
Decreased *RANGAP1*
expression inactivates RAN
abrogating mitotic checkpoint.

G2

M

S

G1

CDKN1A, p21 – S Checkpoint
Induced by p53
Induced by JNK1

CDKN1B, p27 – G1 Checkpoint
p27 is induced by p53
p53 induced by JNK2

INDUCED BY METHOTREXATE
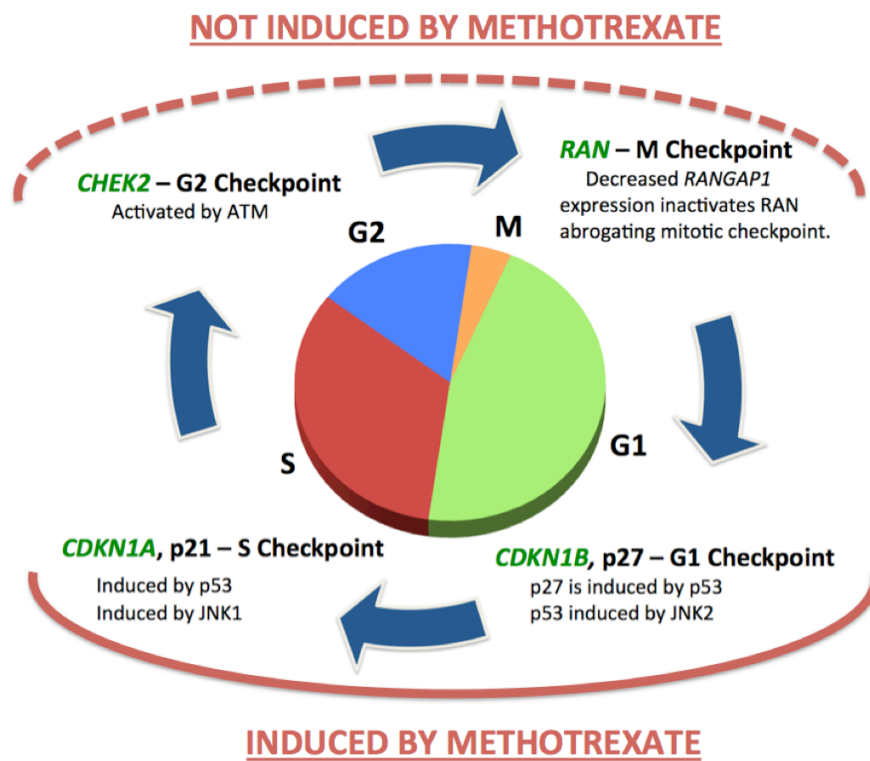
Figure 3-5. Summary model of cell cycle checkpoint gene expression deficiencies and methotrexate targets found in rheumatoid arthritis.

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.[145] 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-kB via an ATM-dependent mechanism.[153] Deficiencies in p53 may also contribute to NF-kB activation.[154] 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 BH_4 in activated T cells via inhibition of guanosine triphosphate cyclohydrolase-1 (GTPCH-1), the rate

limiting enzyme in the synthesis of BH_4, similarly shifts the cytokine balance from a pro-inflammatory to anti-inflammatory state by uncoupling iNOS.[155] 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.[15,86] 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.[156] 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.[157] Patients diagnosed with RA frequently suffer from inflammation-associated coronary artery atherosclerosis leading to increased mortality.[158,159]

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.

CHAPTER IV

METHOTREXATE-MEDIATED INHIBITION OF NF-κB ACTIVATION

BY DISTINCT PATHWAYS IN T CELLS AND SYNOVIOCYTES

Abstract

Introduction. Methotrexate (MTX) is one of the most commonly prescribed disease-modifying anti-rheumatic drugs for the treatment of rheumatoid arthritis (RA). Despite decades-long experience with MTX, the precise mechanisms underlying its anti-inflammatory effects remain incompletely understood. NF-κB is a critical regulator of inflammatory processes. Therefore, we sought to investigate mechanisms of methotrexate-mediated inhibition of NF-κB activation in lymphocytes and fibroblast-like synoviocytes.

Methods. An NF-κB luciferase reporter plasmid was used to measure NF-κB activation across experimental stimuli. Western blotting and flow cytometry were used to quantify changes in intracellular protein levels. Flow cytometry was performed to determine levels of reactive oxygen species and of apoptosis. Quantitative reverse transcription-polymerase chain reaction was used to identify changes in MTX target genes.

Results. In T cell lines, methotrexate (0.1 μM) inhibited activation of NF-κB via depletion of tetrahydrobiopterin and increased JNK-dependent p53 activity. Inhibitors of tetrahydrobiopterin activity or synthesis also inhibited NF-κB activation and, similar to methotrexate, increased JNK, p53, p21 and *JUN* activity. Patients with rheumatoid arthritis expressed increased levels of phosphorylated or active RelA (p65) compared to controls. Levels of phosphorylated RelA were reduced in patients receiving low-dose methotrexate therapy. In contrast, inhibition of NF-κB activation by methotrexate was not mediated via BH_4 depletion and JNK activation in synoviocytes but rather was completely prevented by adenosine receptor antagonists.

*Conclusion*s. Our findings support a model whereby distinct pathways are activated by methotrexate in lymphocytes and synoviocytes, respectively, to inhibit NF-κB activation.

Introduction

Rheumatoid arthritis (RA) is the most common, serious autoimmune disease affecting 1% of the world's population.[14] Methotrexate (MTX) is the standard of care for the treatment of RA, but the precise mechanism by which MTX exerts its anti-inflammatory effects remains incompletely understood. MTX was originally designed in the 1940s as a folic acid antagonist for the

treatment of malignancy. In cancer, folate antagonism via competitive inhibition of dihydrofolate reductase (DHFR) decreases de novo methyl donors tetrahydrofolate and methyltetrahydrofolate blocking purine and pyrimidine biosynthesis, effectively halting DNA replication and cell proliferation.[11] It wasn't until the late 1970s and early 1980s that MTX became widely used in RA, but it has since emerged as the basis by which all other therapies for RA are judged.[3,28] At the time, it was inferred that the anti-inflammatory and immunomodulatory effects of MTX stem from a similar biochemical pathway. However, work spanning the last three decades has indicated that there is still much to learn about the functional role of MTX in the management of RA.

MTX is polyglutamated once taken up by cells. MTX-polyglutamates are believed to represent its active form and levels of MTX-polyglutamates correlate with clinical efficacy in patients with RA.[160] A prevailing theory has been that anti-inflammatory effects of MTX stem from inhibition of aminoimidazolecarboxamidoribonucleotide (AICAR) transformylase causing increased intracellular AICAR levels. Increased AICAR levels inhibit adenosine monophosphate deaminase and adenosine deaminase leading to accumulation and release of adenosine and subsequent A2A and A3 adenosine receptor activation producing anti-inflammatory properties.[4,5,23,34,36,41,161] However, since folate supplementation does not reverse the anti-inflammatory effects of MTX, *in vivo*, the mechanism by

which MTX exerts its vulnerary effects in RA may stem from additional biochemical pathways.[119] DHFR also catalyzes reduction of dihydrobiopterin (BH_2) to tetrahydrobiopterin (BH_4), which is inhibited by MTX [112-114,162]. BH_4 is a necessary cofactor of all nitric oxide synthases (NOS) and loss of BH_4 'uncouples' NOS leading to loss of NO synthesis and gain of reactive oxygen species (ROS) synthesis, such as H_2O_2. MTX-mediated NOS 'uncoupling' and ROS production activates Jun-N-terminal kinase (JNK) and JNK-dependent induction of p53 and p21 and increased sensitivity to apoptosis via intrinsic and extrinsic pathways. Subjects with RA exhibit reduced levels of JNK, p21 and p53 in PBMC while subjects with RA receiving MTX possess normal levels of JNK, p21 and p53 in PBMC, suggesting that MTX-mediated inhibition of reduction of BH_2 to BH_4 also contributes to the therapeutic effects of MTX in RA, possibly by eliminating self-reactive T cells.[132,163]

Excess TNF-α production plays a central role in RA pathogenesis as evidenced by efficacy of therapies that selectively reduce TNF-α levels, *in vivo*. Activation of the transcription factor, NF-κB is a major cellular response to TNF-receptor signaling.[164-166] MTX is also thought to reduce inflammation by lowering levels of TNF-α and/or NF-κB activity.[167] However, mechanistically, it is not apparent if and how any of those pathways activated by concentrations of MTX achieved *in vivo* by standard low-dose therapy may inhibit NF-κB activity. Further, it is unclear if different cells involved in RA pathogenesis, e.g. T lymphocytes, synoviocytes, respond to

MTX by activating a single common pathway or multiple pathways. Since these pathways are similarly activated in both primary cells and cell lines, to address these questions, we determined if low concentrations of MTX inhibited NF-κB activation in tissue culture models in both Jurkat T lymphocytes and fibroblast-like synoviocytes (FLS) and *in vivo* in subjects with RA. To do so, we employed an NF-κB reporter construct in cell-based assays and measured phosphorylation of RelA (p65) as an indicator of NF-κB activity, *in vivo*. Our studies in Jurkat T cells demonstrate that MTX inhibits NF-κB activation via MTX-dependent depletion of BH_4, increased ROS synthesis, and JNK and p53 activation. Further, we find that a BH_4 antagonist or inhibition of BH4 synthesis also stimulates JNK phosphorylation and induces p53 leading to decreased NF-κB activation. *In vivo*, RA PBMC exhibit elevated levels of phosphorylated p65 (p-p65) relative to control (CTRL) PBMC. Levels of p-p65 are near those of CTRL PBMC in PBMC from RA subjects taking MTX. In contrast to these results, MTX fails to induce ROS synthesis, JNK activation and downstream effects, apparently because synoviocytes express extremely low levels of NOS enzymes. However, MTX also inhibits NF-κB in synoviocytes, which appears dependent upon adenosine release and activation of A2A and A3 adenosine receptors. Our data are consistent with the notion that two independent pathways activated by MTX target two distinct cell lineages to produce anti-inflammatory effects in RA.

Materials and Methods

Drugs and Reagents. Methotrexate, (6R)-5,6,7,8-tetrahydrobiopterin dihydrochloride (BH$_4$), caffeine, theophylline, BI-78D3, 2,4-diamino-6-hydroxypyrimidine (DAHP), and (3-[4,5-Dimethylthiazol-2-yl]-2,5-dipehyltetrazolium bromide) (MTT) were from Sigma-Aldrich (St. Louis, MO). 4-amino-7, 8-dihydro-L-biopterin (4-ABH$_4$) was from Schircks Laboratories (Jona, Switzerland). L-JNKi1 was from Enzo Life Sciences (Farmingdale, NY). Recombinant human tumor necrosis factor-alpha (TNF-α) was from Becton Dickinson (BD) Biosciences (Bedford, MA). The following primary antibodies were used: polyclonal rabbit anti-phospho-p65 (ab10684; Abcam, Cambridge, MA), polyclonal anti-phospho-JNK pT183/pY185 (558268; BD), polyclonal rabbit anti-p53 (NB200-171; Novus Biologicals, Littleton, CO), polyclonal rabbit anti-p21 (ab7960; Abcam), monoclonal mouse TRAILR1 (ab18362; Abcam), and monoclonal rabbit anti-PUMA (ab33906; Abcam). Fluorescein isothiocyanate-conjugated goat anti-rabbit Ig (554020; BD) was used as secondary antibody. The NF-κB-luciferase reporter (NF-κB-luc) construct containing five κB elements was a gift from Dr. Dean W. Ballard (Vanderbilt, Nashville, TN). Jun-N-terminal kinase (JNK) – 1 and JNK-2 dominant negative (DN) mutants were from the laboratory of Dr. Roger J. Davis (University of Massachusetts Medical School, Worcester, MA). The p53-DN construct was from the laboratory of Dr. William Kaelin, Jr.

(Harvard Medical School, Boston, MA). Plasmids were obtained from the Addgene repository.

Cell culture. Cells were cultured in RPMI 1640 medium (1μg/mL folic acid) supplemented with fetal bovine serum (FBS) at 10% (volume/volume; Jurkat) or 20% (v/v; fibroblast-like synoviocytes, FLS), 1% (v/v) penicillin/streptomycin, and 1% (v/v) L-glutamine at 37°C in a humidified atmosphere of 5% CO_2. Jurkat T cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Fibroblast-like synoviocytes (FLS) from patients with RA were a generous gift from Dr. James W. Thomas (Vanderbilt, Nashville, TN).

Transient transfections and luciferase measurements. Jurkat T cells were transfected using diethylaminoethyl (DEAE)-dextran. Cells were incubated for 10 minutes at room temperature with 1μg NF-κB-luc construct per 1.0×10^6 cells in a solution of 0.5mg/mL DEAE-dextran in Tris-buffered saline. Cells were resuspended in complete RPMI 1640 culture medium with 100μM chloroquine diphosphate and incubated at 37°C for 1 hour. Immediately following chloroquine treatment, cells were washed and resuspended in complete culture medium and incubated overnight prior to further experimental treatment. Synoviocytes were transfected using Lipofectamine-2000 (Life Technologies, Grand Island, NY) according to the manufacturer's protocol. Plasmid amounts were equalized across transfections. Luciferase was measured using Steady-Glo (E2510) from

Promega (Madison, WI) according to the manufacturer's supplied protocol on a TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA).

RNA isolation, cDNA synthesis, and real-time PCR. Total RNA was isolated using Tri-Reagent (Molecular Research Center, Cincinati, OH), purified with the RNeasy MinElute Cleanup Kit (Qiagen, Germantown, MD), and quantified using a Nano Drop 1000 spectrophotometer. Complementary DNA (cDNA) was reverse transcribed from 5μg total RNA using the SuperScript III First-Strand Synthesis Kit (Life Technologies) using oligo(dT) as the primer and purified using the Qiagen QiaQuick PCR purification kit. Real-time qPCR (ABI-7300 Real Time PCR System; Applied Biosystems) was performed in duplicate using TaqMan gene expression assays in volumes of 25μL with 50ng cDNA and TaqMan Gene Expression Master Mix. Fold change expression levels were determined by the ΔΔCT method comparing expression of test genes to *GAPDH*.

Flow Cytometry. Cells were suspended in phosphate buffered saline with 10% FBS and 0.1% sodium azide. For intracellular protein determinations, cells were fixed with BD Cytofix Buffer, permeabilized using BD Phospho Perm/Wash Buffer (BD Biosciences), and labeled with primary antibodies for 24 hours at 4°C followed by incubation with fluorescence-labeled secondary antibodies for 1 hour at 4°C. Cells were analyzed using a 3-laser BD LSRII flow cytometer at the Vanderbilt Medical Center Flow

Cytometry Core facility. Supplemental analysis was performed using FlowJo (Treestar, Ashland, OR).

Study Populations. The study group consisted of 9 control subjects (age: 48 ± 8, 7 females, 2 males, 8 Caucasian, 1 African American) with no current chronic or acute infection and no family history of autoimmune disease, and 8 patients meeting the American College of Rheumatology/European League Against Rheumatism classification criteria for RA (age: 41 ± 12, 7 females, 1 male, 6 Caucasian, 2 African American). Demographic characteristics among control and disease cohorts did not differ significantly. The sample collection protocol was approved by the Vanderbilt University Human Research Protection Program and the Vanderbilt Institutional Review Board. Written informed consent was obtained at the time of blood draw.

Western Blotting. Immunoblotting was performed as described previously [132]. Briefly, peripheral blood mononuclear cells were isolated from whole blood using BD Vacutainer Cell Preparation Tubes. Whole cell lystates were resolved by SDS polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes overnight at 4°C. Membranes were washed and blocked using Odyssey Blocking Buffer (Li-COR Biosciences, Lincoln, NE) for 1 hr at room temperature. Membranes were thoroughly rinsed and incubated with primary antibodies overnight at 4°C. Membranes were then washed and incubated with fluorescently labeled

IRDye 700/800 antibodies diluted in Odyssey blocking buffer in the dark. Blots were washed and resuspended in TBS prior to scanning and band quantification using the Li-COR Odyssey Infrared Imaging System (Li-COR Biosciences, Lincoln, NE).

Statistical analysis. Data are expressed as the mean ± SD of three or more independent experiments. Significance was determined by Student's *t*-test using GraphPad Prism Software (La Jolla, CA). *P* values < 0.05 were considered significant.

Results

Methotrexate reduces NF-κB activity in Jurkat cells.

To determine the effects of MTX on NF-κB activation, an NF-κB reporter construct was transfected into Jurkat T cells. Transfected cells were treated for 48 hours with 0.1μM MTX and stimulated with either phorbol 12-myristate 13-acetate (PMA, 50nM) and ionomycin (1μM) or 5ng TNF-α for 24 hours. We found that methotrexate markedly reduced stimulation of NF-κB activity in response to either PMA/ionomycin or TNF-α (Fig. 4-1 A). MTX-mediated inhibition of NF-κB activation by TNF-α was significantly reversed by supplementation of cultures with BH$_4$, the free radical scavenger, N-acetyl cysteine (NAc), JNK inhibitors, BI-78D3 or L-JNKi1, or by transient transfection of JNK1-, JNK2-, or p53-dominant negative (DN) expression

vectors (Fig. 4-1 B). JNK inhibitors BI-78D3 and pepJIP1 (L-JNKi1) target the JNK-JNK-interacting protein 1 (JIP1) binding site and prevent JNK phosphorylation.[102,168] Consistent with our previous studies, we conclude that MTX-mediated inhibition of NF-κB activation by TNF-α resulted from MTX-dependent BH_4 depletion leading to increased ROS production, JNK activation and JNK-dependent induction of p53, which is the final mediator of inhibition of NF-κB activation.

We also tested the ability of folic and folinic acid to reverse MTX-mediated inhibition of NF-κB activation by TNF-α. Supplementation of cultures with either folic acid or folinic acid blocked inhibition of NF-κB activation by MTX (Fig. 4-1 C). BH_2 and folate are converted to BH_4 through a salvage pathway regulated by DHFR expression.[169,170] Blockade of DHFR by MTX depletes tetrahydrofolate levels and decreases cellular amounts of BH_4. Supplementation of MTX-treated cultures with folic acid and/or folinic acid increases intracellular BH_4 bioavailability.[114] MTX also has been shown to stimulate the release of adenosine and activate adenosine (ADOR) receptors. Therefore, we examined the ability of two nonselective ADOR antagonists, caffeine and theophylline, to reverse the effects of MTX. Treatment of cells with MTX and either caffeine or theophylline alone at pharmacologic concentrations did not reverse MTX-mediated inhibition of NF-κB activation (Fig. 4-1 D).
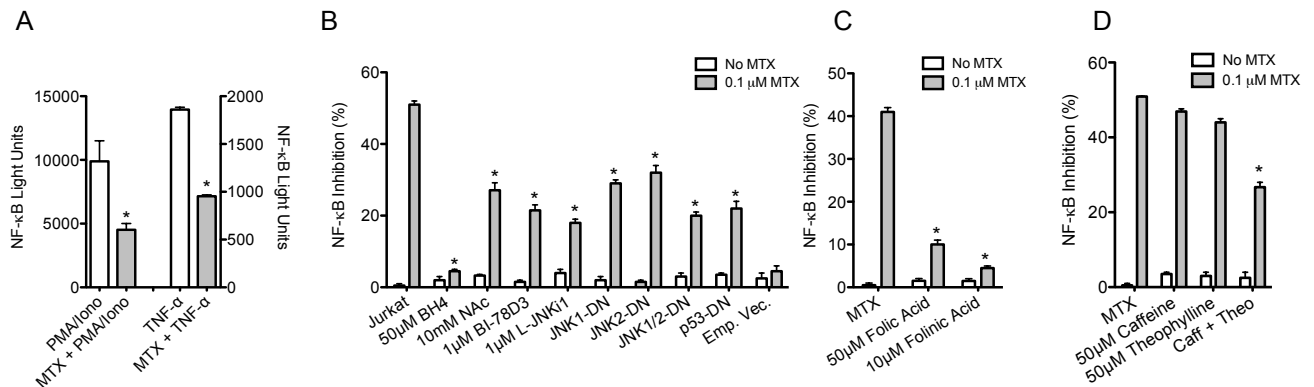
Figure 4-1. **Inhibition of NF-κB activation by MTX. A-D** JKT cells containing an NF-κB-luciferase reporter were cultured with 0.1 μM MTX for 48 hours and stimulated with PMA (50nM) and ionomycin (1μM) **(A)** or TNF-α (5ng) **(A-D)** 24 hours prior to luciferase measurements. **B**, MTX-treated JKT cells were cultured with or without BH₄, *N*-acetyl-L-cysteine (NAC), or JNK inhibitors, BI-78D3 or L-JNKi1. JNK1-DN, JNK2-DN, p53-DN or empty vector plasmids with a green fluorescent protein (GFP) plasmid were transiently transfected into JKT cells. **C** and **D** MTX-treated JKT cells were treated with folic or folinic acid **(C)** or adenosine receptor antagonists caffeine and/or theophylline **(D)**. Values are the mean ± SD. **A,** * = p <0.05 versus PMA/ionomycin or TNF-α treated cultures. **B-D,** * = p < 0.05 versus cultures stimulated with MTX alone.

However, incubation of cells with MTX and the combination of caffeine and theophylline significantly reduced inhibitory effects of MTX. We interpret these results to suggest that release of adenosine and ADOR activation also contributed to MTX-mediated inhibition of NF-κB activation.

Induction of *TP53*, *CDKN1A*, and *JUN* by the 4-amino analogue of tetrahydrobiopterin.

Given our findings that methotrexate inhibits NF-κB through blockade of tetrahydrobiopterin biosynthesis, we investigated if pterin-site inhibitors of NOS also inhibited NF-κB activation. One such inhibitor is 4-aminotetrahydrobiopterin (4-ABH$_4$). Jurkat cells treated for 48 hours with 4-ABH$_4$ show increased *TP53*, *CDKN1A*, and *JUN* expression levels and corresponding increases in phosphorylated JNK, p53, and p21 protein (Fig. 4-2A & 4-2B) closely mirroring the stimulatory activity of MTX. We also determined if 4-ABH$_4$ inhibited TNF-dependent NF-κB activation in T cells. We found that 4-ABH$_4$ decreased TNF-induced NF-κB activity to a level similar to MTX (Fig. 4-2 C). As an additional experimental comparator, we used diamino-hydroxypyrimidine (DAHP), which inhibits GTPCH-1 (GTP cyclohydrolase 1), the rate limiting enzyme in BH$_4$ synthesis [155], and found that DAHP also significantly abrogated NF-κB activation. Thus, both a BH$_4$ antagonist and an inhibitor of BH$_4$ synthesis stimulated a pathway in T cells similar to that stimulated by MTX leading to inhibition of NF-κB by TNF-α activation.
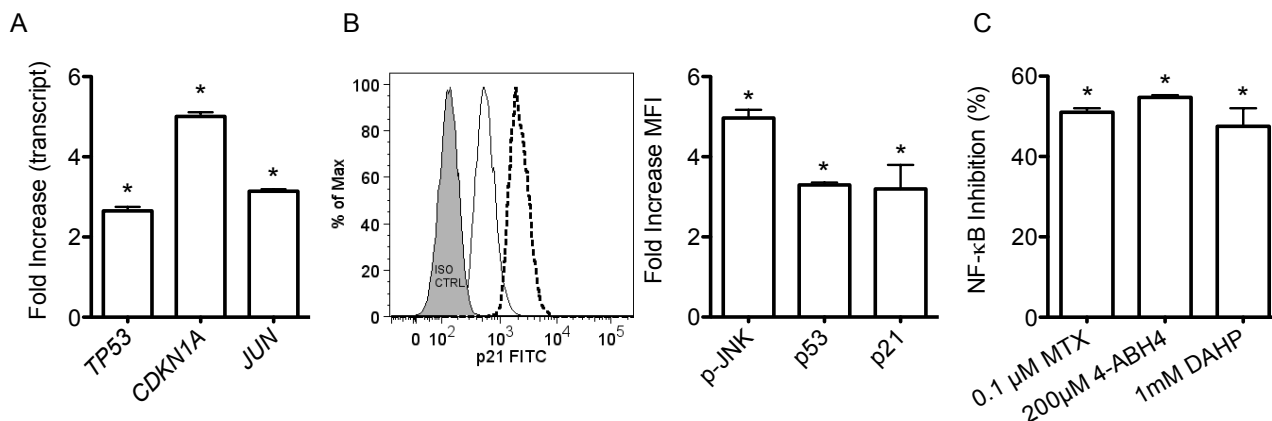
Figure 4-2. **Increased expression of p-JNK, p53, and p21, and inhibition of NF-κB activation by a BH₄ anatagonist or an inhibitor of BH₄ synthesis. A-C,** Jurkat cells were treated with the BH₄ antagonist, 4-ABH₄ (200μM), for 48 hours and **(A)** transcript levels of *TP53*, *CDKN1A*, and *JUN* were measured by quantitative PCR. Results are expressed as fold induction relative to *GAPDH*. **B,** Levels of p-JNK, p53, and p21 protein were determined by flow cytometry. A representative flow diagram shows background fluorescence (gray) and results obtained with untreated (solid line) or 4-ABH₄ (dashed line) treated Jurkat cells. **C,** MTX, 4-ABH₄, or DAHP treated Jurkat cells were stimulated with TNF-α for 24 hours prior to luciferase measurements. Results are expressed as % inhibition of TNF-α stimulated NF-κB activity in relative light units. Values are the mean ± SD. * = p < 0.05 versus untreated cells.

Methotrexate corrects elevated p-p65 levels in RA subjects, *in vivo.*

Since MTX reduced activation of NF-κB by TNF-α in T cells, we sought to examine the basal level of NF-κB activation in subjects with RA. For these studies, we prepared whole cell lysates from PBMC obtained from healthy control subjects and patients fulfilling the American College of Rheumatology/European League Against Rheumatism criteria for RA. Subjects with RA were divided into those receiving or not receiving MTX therapy. Cell lysates were prepared and analyzed by SDS-PAGE and Western blotting using a specific p-p65 antibody. In control subjects, we found that p-p65 was essentially undetectable by western blotting (Fig. 4-3 A & 4-3 B). In contrast, we detected increased p-p65 levels in RA subjects not receiving low-dose MTX as therapy. RA patients receiving once-weekly MTX exhibited decreased p-p65 levels, similar to controls. We conclude from these studies that RA subjects not receiving MTX exhibit chronic activation of NF-κB compared to controls. Chronic activation of NF-κB is markedly reduced in RA subjects on stable MTX therapy.
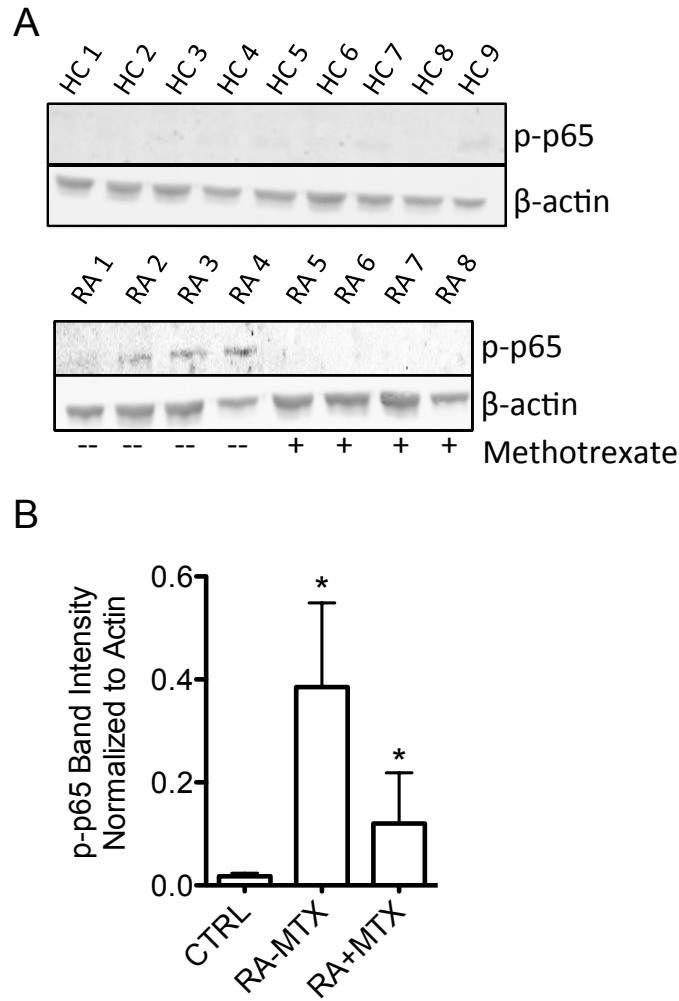
Figure 4-3. **Levels of p-p65 in PBMC from CTRL subjects or subjects with RA receiving or not receiving MTX therapy,** *in vivo.* **A.** Whole cell lysates were prepared and p-p65 levels were determined by Western blotting in healthy control subjects (HC), and in RA patients receiving (RA+MTX) and not receiving MTX (RA-MTX). **B** Quantification of band intensities. Values are the mean ± SD. * = p < 0.05.

MTX-mediated inhibition of NF-κB activation in FLS.

Both lymphocytes and fibroblast-like synoviocytes (FLS) are key effector cells in RA pathogenesis. Therefore, we determined if MTX also inhibited TNF-α mediated NF-κB activation low-passage FLS. FLS were transfected with the NF-κB luciferase reporter construct using lipofectamine and treated with 0.1μM MTX for 48 hours. As in T cells, MTX inhibited TNF-α dependent activation of NF-κB by about 50% (Fig. 4-4 A). However, in contrast to T cells, JNK inhibition did not attenuate inhibition of NF-κB activation by MTX. In T cells, MTX induces cell cycle checkpoints and increases sensitivity to apoptosis via a JNK-dependent pathway [132,163]. To examine these responses in FLS, we measured apoptosis by annexin-V labeling. FLS were treated with MTX for 48 hours and exposed to either anti-Fas or H_2O_2 for an additional 24 hours as extrinsic or intrinsic mediators of apoptosis, respectively. In contrast to T cells, FLS did not exhibit increased levels of apoptosis after treatment with MTX followed by either anti-Fas or H_2O_2 (Fig. 4-4 B). Further, treatment of FLS with MTX or 4-ABH$_4$ did not reduce cell number as measured by MTT assay (Fig. 4-4 C). Levels of ROS or pro-apoptotic proteins as assessed by flow cytometry were also not increased in FLS by either MTX or 4A-BH$_4$ (Fig. 4-4 D & 4-4E). Finally, we performed mRNA measurements of low-passage or activated FLS and found that MTX or 4-ABH$_4$ did not increase transcript levels of *TP53*, *CDKN1A*, or *JUN*, which is in marked contrast to T cells (Fig. 4-4 F, left panel). One possible explanation for the

failure of MTX to activate these ROS-JNK dependent pathways in FLS as is seen in both Jurkat and primary T cells is if FLS had significantly lower levels of NOS enzymes compared to T cells. To test this possibility we compared NOS2 and NOS3 transcript levels among Jurkat T cells, primary activated T cells and FLS and found that Jurkat T cells and primary T cells expressed much higher *NOS2* and *NOS3* transcripts compared to FLS (Fig. 4-4 F, right panel). Thus, failure to activate these ROS-JNK dependent pathways in FLS is probably due to low levels of NOS enzymes in FLS thus preventing sufficient NOS-dependent ROS synthesis to activate JNK dependent pathways.

Given these findings, we sought alternative explanations for decreased NF-κB activation in MTX-treated FLS. Therefore, we examined effects of ADOR antagonists, caffeine and/or theophylline at pharmacologic concentrations. We found that the combination of caffeine and theophylline markedly abrogated inhibition of NF-κB activation by MTX (Fig. 4-4 G). Thus, in contrast to T cells, MTX appears to mediate inhibition of NF-κB activation in FLS by stimulating adenosine release.
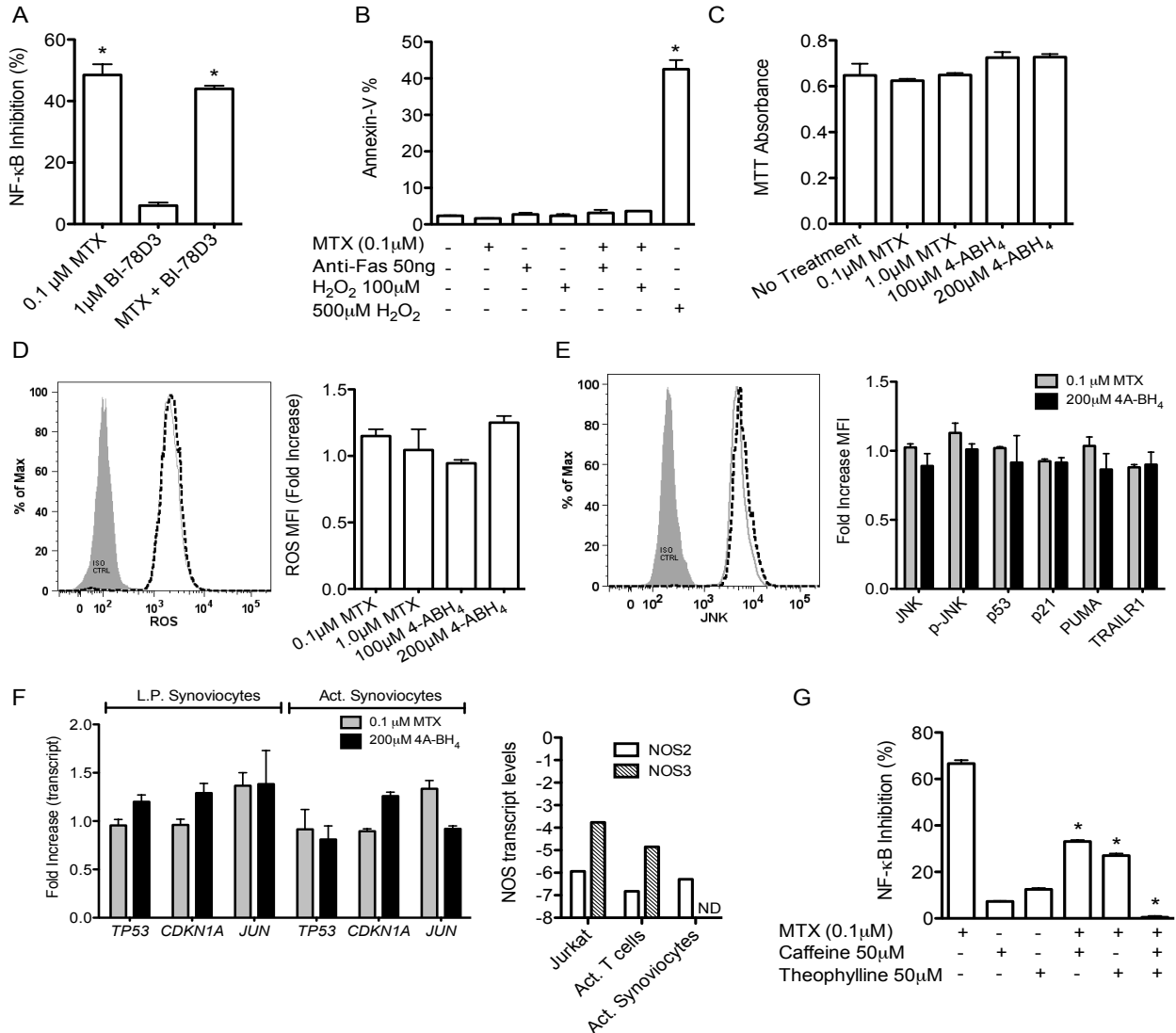
Figure 4-4. **Reversal of methotrexate-mediated inhibition of NF-κB activation by adenosine receptor antagonists in FLS. A-G** FLS were treated with indicated concentrations of MTX or 4-ABH₄ for 48 hours. **A**, FLS were transfected with an NF-κB luciferase reporter construct and treated with MTX ± BI-78D3. **B**, FLS were cultured with MTX followed by culture with anti-Fas antibody or H_2O_2 for an additional 6 hours. The percentage of annexin V-positive cells was determined by flow cytometry. **C,** FLS proliferation evaluated by MTT assay. **D**, Synthesis of ROS was determined by labeling FLS with CM-H_2DCFDA and flow cytometry. **E,** Levels of JNK, p-JNK, p53, p21, PUMA, and TRAILR1 in MTX and 4A-BH₄ treated FLS were determined by flow cytometry and are reported as the fold increase compared to untreated FLS. **F,** Transcript measurements of left panel: *TP53*, *CDKN1A*, and *JUN* in MTX or 4-ABH₄ treated low-passage FLS or activated FLS stimulated with IL-1β (2ng/mL), TNF-α (50ng/mL) and LPS (1μg/mL) for 24 hours or right panel: *NOS2* or *NOS3* mRNA levels for the indicated cell type. **G,** FLS transiently transfected with an NF-κB luciferase reporter were treated with MTX ± adenosine receptor antagonists caffeine and/or theophylline for 48 hours and stimulated with TNF-α 24 hours prior to luciferase measurements. Values are the mean ± SD. **A-F**, * = p < 0.05 versus untreated FLS. **G** * = p <0.05 versus MTX treated FLS.

Discussion

Our current studies coupled with previous studies indicate that MTX-mediated inhibition of DHFR initiates two parallel anti-inflammatory pathways: (1) inhibition of BH_2 reduction to BH_4 leading to iNOS 'uncoupling', ROS production, JNK activation and downstream effects and (2) AICAR-dependent adenosine release and activation of ADOR. Both pathways ultimately lead to inhibition of stimulus-dependent activation of NF-κB, which most likely is a major contributor to the activity of MTX in RA. Interestingly, these two pathways seem to be activated in a cell-type specific manner. The BH_2/BH_4 pathway seems to predominate in lymphocytes while the ADOR activation pathway is the predominant pathway in synoviocytes (Fig. 4-5). A major difference between the two cell lineages is that synoviocytes express extremely low levels of NOS enzymes compared to T cells which probably explains failure to activate the BH_2/BH_4 pathway in synoviocytes.

A BH_4 antagonist or inhibition of BH_4 synthesis also stimulates ROS production, JNK activation and downstream effector pathways similar to MTX. In activated T cells, inhibition of BH_4 synthesis also decreases production of the pro-inflammatory, TH_1 associated cytokine, IFN-γ and increases production of the anti-inflammatory TH_2 cytokine IL-4.[155,171] MTX induces a similar shift and reduces expression of pro-inflammatory cytokines,

IL-1, IL-2, IL-6 and IFN-γ and increases expression of anti-inflammatory cytokines such as IL-4 and IL-10 in subjects with RA.[172] Thus, we would argue that inhibition of BH_4 synthesis by MTX might explain the pro-inflammatory to anti-inflammatory shift found in RA.
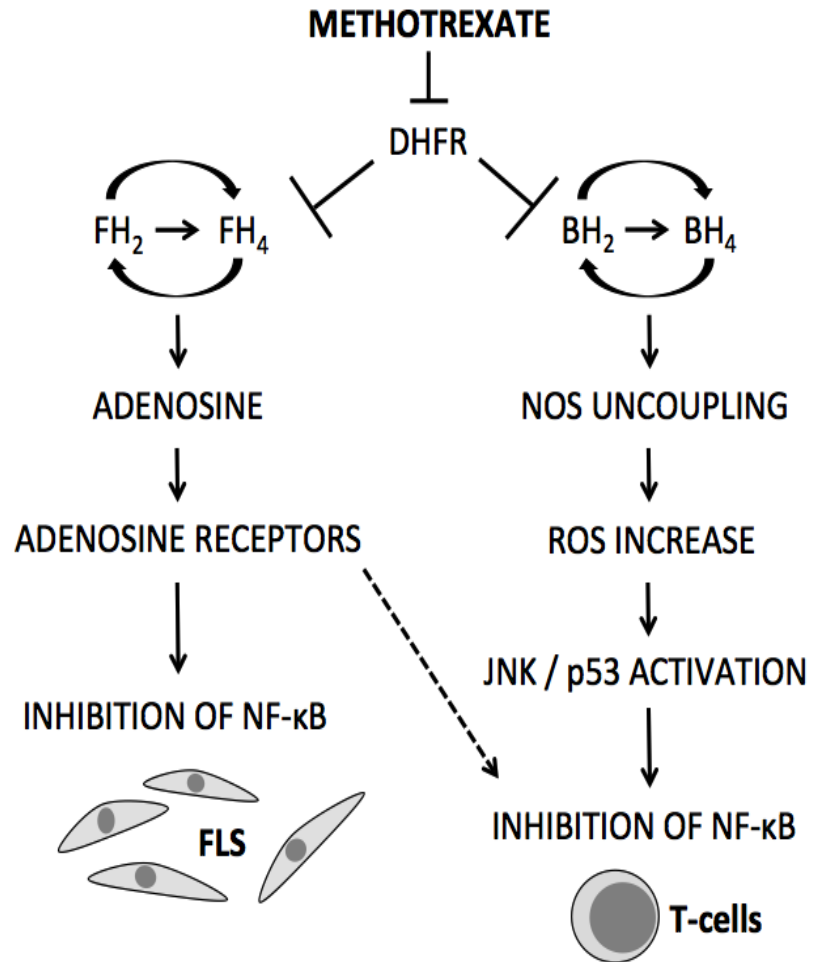
Figure 4-5. Summary model of MTX mediated inhibition of NF-κB activation in FLS and T cells.

Of particular interest is the association between MTX, p53, and NF-κB. While NF-κB governs cellular responses to external stimuli, p53, often referred to as the guardian of the genome, regulates endogenous or intrinsic processes eliminating cells with excess DNA damage through "cell cycle arrest, apoptosis, or senescence".[173]   Together with NF-κB, these master regulators of internal and external stimuli must achieve a balance. Each transcription factor responds to a different form of cellular stress, adopting two very different strategies that have evolved into mutually exclusive processes.[173] Importantly, NF-κB and p53 are known to have opposing functions. The interplay between these two factors and their response to therapy in RA has not been fully examined. In RA, defects in the p53 pathway exist in both PBMC and synovium.[15,18,86,174] Defects in these apoptosis and DNA damage control responses are blunted conferring genomic instability and aberrant cell function.[85,175]   We hypothesize that deficiencies in p53 in RA may also lead to increased activation of NF-κB, which we find in RA PBMC. MTX acts to restore levels of p53 via JNK activation. Therefore, MTX may act to restore the balance between these two critical transcription factors, allowing clearance of damaged cells in the periphery of RA patients and reducing the chronic inflammation found in the joint spaces. In fact, depressed levels of p53 in RA coupled with constitutive expression of NF-κB could elicit a pro-inflammatory cytokine loop that sustains disease pathogenesis.

Apart from RA, connections between p53 and NF-κB have been experimentally demonstrated in cancer models and in mouse endotoxemia. Overexpression of wild type p53 in human colon cancer cells reduces endogenous levels of NF-κB activity and restores the ability of these cells to undergo apoptosis.[176] Further, glucocorticoids, which inhibit inflammation, block NF-κB activity through a p53 dependent process. Mortality from LPS-induced endotoxemia, a process critically dependent upon NF-κB activity, is markedly increased in mice that lack p53. The conclusion from these studies, closely mirroring our own work, is that p53 mediates repression of NF-κB.[177]

CHAPTER V

METHOTREXATE INHIBITS NF-κB ACTIVITY VIA LINCRNA-P21

INDUCTION

Abstract

Objective. We sought to determine interrelationships among expression of lincRNA-p21, a long intergenic non-coding RNA, activity of NF-κB, and responses to methotrexate in rheumatoid arthritis (RA) by analyzing patient samples and cell culture models.

Methods. Expression levels of long non-coding RNAs and messenger RNAs were determined by quantitative reverse transcription-polymerase chain reaction. Western blotting and flow cytometry were used to quantify levels of intracellular proteins. Intracellular NF-κB was determined using an NF-κB luciferase reporter plasmid.

Results. RA patients expressed reduced basal levels of lincRNA-p21 and increased basal levels of phosphorylated p65 (Rel A), a marker of NF-κB activation. RA subjects not receiving MTX expressed lower levels of lincRNA-p21 and higher levels of phosphorylated p65 compared to RA subjects receiving low-dose MTX. Using a cell culture model, we found that MTX induced lincRNA-p21 through a DNA-PKcs-dependent mechanism. Deficiencies of *PRKDC* mRNA levels in RA subjects were also corrected by

MTX, *in vivo*. Further, MTX lowered NF-κB activity in TNF-α treated cells through a DNA-PKcs-dependent mechanism via induction of lincRNA-p21. Finally, we found that depressed levels of *TP53* and lincRNA-p21 increased NF-κB activity in cell lines. Decreased levels of lincRNA-p21 did not alter *NFKB1* or *RELA* transcripts. Rather, lincRNA-p21 physically bound to *RELA* mRNA.

Conclusion. Our findings support a model whereby depressed levels of lincRNA-p21 in RA contribute to increased NF-κB activity. MTX decreases basal levels of NF-κB activity by increasing lincRNA-p21 by a DNA-PKcs dependent mechanism.

Introduction

Rheumatoid arthritis (RA) is the most common autoimmune disease affecting more than one million adults in the United States.[127] While the etiology of RA remains unknown, the disease is characterized by small and large joint erosion and disability that manifests across decades. Newer biologic therapies, such as those that inhibit activity of TNF-α, have enhanced the physician's ability to improve outcomes and decrease disability.[21] However, despite these advances, excess mortality observed in patients with RA continues and recent data suggest that the mortality gap between RA patients and the rest of the population continues to widen.[20]

Although the origins of RA remain unknown, T cells in RA exhibit loss of genomic integrity via reduced expression of proteins responsible for cell cycle arrest and DNA damage repair.[84,85] These include: reduced expression of sentinel DNA damage response proteins, ATM (ataxia telangiectasia mutated) and DNA-PKcs (DNA-protein kinase catalytic subunit), the tumor suppressor protein, p53, which is an ATM target protein, the cyclin-dependent kinase inhibitors p21 and p27, and stress kinases, such as JNKs (c-Jun-N-terminal kinases) that also respond to DNA damage.[77,84,146]

Genes that encode these proteins are also methotrexate (MTX) response genes.[132,163] MTX is a potent inhibitor of dihydrofolate reductase (DHFR), which is classically thought to reduce purine and pyrimidine biosynthesis via depletion of tetrahydrofolate, thymidylic acid, and thymidylate synthase resulting in cell cycle arrest.[34] Adenosine synthesis and activation of adenosine receptors may also contribute to anti-inflammatory effects of MTX.[3,23,36,41] Inhibition of DHFR by MTX also blocks reduction of dihydrobiopterin (BH_2) to tetrahydrobiopterin (BH_4) leading to nitric oxide synthase (NOS) 'uncoupling' and production of reactive oxygen species (ROS). Increased ROS activates JNK increasing expression of p53 and p21 via JNK-dependent mechanisms and enhances cellular apoptotic responses.[132,163]

Effects of p53 are mediated in part by induction of the long non-coding RNAs (lncRNA), lincRNA-p21 and PANDA.[178,179] LncRNAs represent a new

class of RNAs. To date, 1,000's of lncRNA genes have been identified in a mammalian genome.[180,181] Genes encoding lncRNAs are similar to protein coding genes and contain exons and introns. Exons are spliced to produce mature lncRNAs. However, unlike protein coding genes, lncRNAs do not code for proteins due to presence of multiple translational stop codons.[181] One function of lncRNAs is to activate or repress transcription of protein-coding genes and thus lncRNAs play key roles in determining a cell's transcriptional program.[182] For example, lincRNA-p21 and PANDA play critical roles in mediating cellular responses to p53 activation.[178,179]

Besides its role in apoptosis, p53 possesses anti-inflammatory properties demonstrated in tissue culture and animal models.[183] In contrast, NF-κB is a transcription factor generally considered to exhibit pro-survival and pro-inflammatory properties.[184] Thus, biologically, p53 and NF-κB may be considered to be antagonistic.[173,185] However, it is unknown if p53 directly inhibits NF-κB activity and vice versa and how this inhibition may occur mechanistically.[173,186] Here, we sought to explore the interrelationship between p53, its target lncRNAs, and NF-κB activity in the context of RA and MTX therapy. We find that lincRNA-p21 is under-expressed in RA and MTX restores lincRNA-p21 levels to normal, *in vivo*. Similarly, NF-κB activity is elevated in RA, *in vivo*, and MTX restores NF-κB activity to normal, *in vivo*. Analysis in cell models demonstrates that MTX induction of lincRNA-p21 is via DNA-PKcs dependent mechanisms and that lincRNA-p21 inhibits NF-κB

activity, in part, by sequestering *RELA* mRNA. Collectively, our results demonstrate that lincRNA-p21 is a key negative regulator of NF-κB activity. We suggest that reduced expression of lincRNA-p21 in RA may contribute to the inflammation associated with this disease and that a portion of the anti-inflammatory properties of MTX may result from induction of lincRNA-p21.

Materials and Methods

Reagents and Cell Culture. MTX was from Sigma-Aldrich (St. Louis, MO). Human TNF-α was from BD Biosciences (Bedford, MA). KU-55933 and NU-7441 inhibitors were from Tocris Biosciences. The following primary antibodies were used: mouse monoclonal anti-actin (sc-8432; Santa Cruz Biotechnology), polyclonal rabbit-anti-JNK (sc-471; Santa Cruz Biotechnology), polyclonal rabbit anti-p53 (NB200-171; Novus Biologicals), polyclonal rabbit anti-phospho-p65 (ab10684; Abcam), monoclonal anti-phospho-ATM (NB110-55475; Novus Biologicals), polyclonal rabbit anti-phospho-DNA-PKcs (ab18192; Abcam). FITC-conjugated goat anti-rabbit Ig (554020; BD) was used as secondary antibody. The NF-κB-luciferase reporter (NF-κB-luc) plasmid containing five κB elements was a gift from Dean Ballard (Vanderbilt).

Cell culture. Jurkat T cells were from the American Type Culture Collection (ATCC). The THP-1 human monocytic cell line was a gift from

Jacek Hawiger (Vanderbilt). Cells were cultured in RPMI 1640 medium (1μg/mL folic acid) as described previously.[132] Concentrations of 0.1μM or 1.0μM MTX were used and culture periods ranged from 24 hours (activated primary T cells) to 48 hours (Jurkat T cells) of continuous exposure to MTX.[132,163] Pharmacokinetic analysis indicates that ingestion of a 20-mg tablet of MTX produces plasma MTX concentrations of ~0.5μM after 1 hour and ~0.1μM after 10 hours.[137,163]

Transient transfections and luciferase measurements. Jurkat T cells were transfected with the NF-κB-luc reporter plasmid and Silencer Select siRNAs (Ambion, Life Technologies) for the specified targets using the Cell Line Nucleofector Kit V according to the supplied protocol (Amaxa, Koeln, Germany). Plasmid amounts were equalized across transfections. THP-1 cells were transfected with Silencer Select siRNAs using Lipofectamine RNAiMAX (Life Technologies) according to the manufacturer's protocol. Luminescence was measured on a TD-20/20 Luminometer (Turner Designs) using Steady-Glo (E2510; Promega) according to the supplied protocol.

RNA isolation, cDNA synthesis, and real-time PCR. Total RNA was isolated using Tri-Reagent (Molecular Research Center, Cincinnati, OH) for cell culture assays or by PAXgene tube (Qiagen, Germantown, MD) for patient studies, purified with the RNeasy MiniElute Cleanup Kit (Qiagen), and quantified using a Nano Drop 1000 spectrophotometer. Complementary

DNA (cDNA) was reverse transcribed from total RNA with the SuperScript III First-Strand Synthesis Kit (Life Technologies) using oligo(dT) and purified using the Qiagen QiaQuick PCR purification kit. Real-time qPCR (ABI-7300 Real Time PCR System; Applied Biosystems) was performed in duplicate using custom or inventoried TaqMan gene expression assays in volumes of 25μL with 50ng cDNA and TaqMan Gene Expression Master Mix. *GAPDH* was used for normalization.

Flow Cytometry. Cells were suspended in PBS with 10% FBS and 0.1% azide and fixed with BD Cytofix Buffer, permeabilized using BD Phospho Perm/Wash Buffer (BD Biosciences), and labeled with primary antibodies for 24 hours at 4°C followed by incubation with FITC-labeled secondary antibodies for 1 hour at 4°C. Cells were analyzed using a 3-laser BD LSRII flow cytometer at the Vanderbilt Medical Center Flow Cytometry Core facility. Supplemental analysis was performed using FlowJo (Treestar, Ashland, OR).

Biotin lincRNA-p21 pull down assay and bioinformatic analysis of lincRNA-p21 bindings sites with mRNAs. The vector expressing partial human lincRNA-p21 (pcDNA3-lincRNA-p21) was a gift from Dr. Myriam Gorospe (National Institute on Aging, NIH). Biotinylated transcripts were synthesized using the MaxiScript T7 kit (+ strand) or the MaxiScript SP6 kit (-- strand) (Ambion) as described previously.[187] Briefly, biotinylated transcripts were incubated with THP-1 total RNA, heated to

55°C, and allowed to cool for 1hr. Complexes were isolated using streptavidin-coupled Dynabeads (Invitrogen, Life Technologies, Grand Island, NY). The RNA present in the pull-down was measured using RT-qPCR. The NCBI BLAST tool (blast.ncbi.nlm.nih.gov) was used to determine regions of complementarity between lincRNA-p21 and *JUNB* mRNA and *RELA* mRNA [187].

Study Populations. Patient cohorts used for qRT-PCR measurements are summarized in Supplementary Table 5-1. RA, SLE, and SS patients met the American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) classification criteria. From these cohorts, western blotting analysis was performed on 9 control subjects (age: 48 ± 8, 7 females, 2 males, 8 Caucasian, 1 African American) with no current chronic or acute infection and no family history of autoimmune disease, and 8 RA patients (age: 41 ± 12, 7 females, 1 male, 6 Caucasian, 2 African American). Demographic characteristics among control and disease cohorts did not differ significantly. Studies were approved by the Vanderbilt University Human Research Protection Program and Institutional Review Board. Written informed consent was obtained at the time of blood draw.

Western Blotting. Immunoblotting was performed as described previously.[132] Briefly, PBMC were isolated from whole blood using BD Vacutainer Cell Preparation Tubes. Whole cell lysates were resolved by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes

overnight at 4°C. Membranes were washed and blocked with Odyssey Blocking Buffer (Li-COR Biosciences, Lincoln, NE) for 1 hr at room temperature. Membranes were incubated with primary antibodies overnight at 4°C. Membranes were washed and incubated with fluorescently labeled IRDye 700/800 antibodies diluted in Odyssey blocking buffer in the dark. Blots were washed and resuspended in TBS prior to scanning and band quantification using the Li-COR Odyssey Infrared Imaging System (Li-COR Biosciences, Lincoln, NE).

Microarrays. Microarray analyses were performed previously (Gene Expression Omnibus: GSE21761 and GSE3447).[147,179]

Statistical analysis. Statistically significant differences between tissue culture experiment groups were determined by Student's t-test. *P* values less than 0.05 were considered significant. Unless otherwise indicated, results are representative of at least three independent experiments. Comparisons of human subject groups were calculated in two-way comparisons using Bonferroni's method to correct for multiple testing, GraphPad Prism Software (La Jolla, CA).

Results

TP53 and lincRNA-p21 expression in rheumatoid arthritis

To initiate our studies, we conducted gene expression studies across three autoimmune diseases including rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and Sjogren's syndrome (SS). We found that subjects with RA exhibited reduced expression of *TP53* and lincRNA-p21 relative to control subjects (CTRL) (Figure 5-1A). These deficiencies were not observed in either the SLE or SS cohorts. We concluded from these studies that lincRNA-p21 and *TP53* levels are significantly reduced in subjects with RA, but this deficiency is not a common property of all inflammatory autoimmune diseases. Further, we measured expression of PANDA, an additional lncRNA induced by p53 activation. There was no significant difference in PANDA expression between RA and CTRL cohorts.

The original publication describing discovery and function of lincRNA-p21 included microarray analysis to identify genes positively or negatively regulated by lincRNA-p21 or *TP53* based upon changes in expression levels following siRNA-mediated knockdown of either lincRNA-p21, *TP53* or the combination.[179] Therefore, we reasoned that genes repressed by lincRNA-p21 or *TP53* knockdown should be under-expressed in RA and genes induced by lincRNA-p21 or *TP53* knockdown should be over-expressed in RA if lincRNA-p21 or *TP53* levels in RA contribute to RA differentially expressed gene

(DEG) profiles and this is exactly what we observed. In RA, approximately 15% of the genes assayed were either over or under expressed (Figure 5-1B). Of these over- or under-expressed gene sets, greater than 25% of the DEGs correspond with the DEG profiles obtained from microarray analysis of cells treated with lincRNA-p21 or *TP53* siRNAs. These results are consistent with the hypothesis that under-expression of lincRNA-p21 and *TP53* played a large role in establishing the unique RA mononuclear cell 'transcriptome'. Given these findings, we asked if transcript levels of *TP53* correlated with transcript levels of lincRNA-p21. In a cohort of control subjects (CTRL; N=20) we found no significant correlation between *TP53* and lincRNA-p21 transcript levels (Figure 5-1C). We conclude from these data that basal transcript levels of lincRNA-p21 were not necessarily dependent upon basal levels of *TP53* and that p53-independent mechanisms may contribute to basal levels of lincRNA-p21 in mononuclear cells. Therefore, we employed pathway analyses to interrogate overlapping RA, lincRNA-p21, and *TP53* gene sets.[188] Known NF-κB response genes, such as IL-8, were highly represented in this analysis.
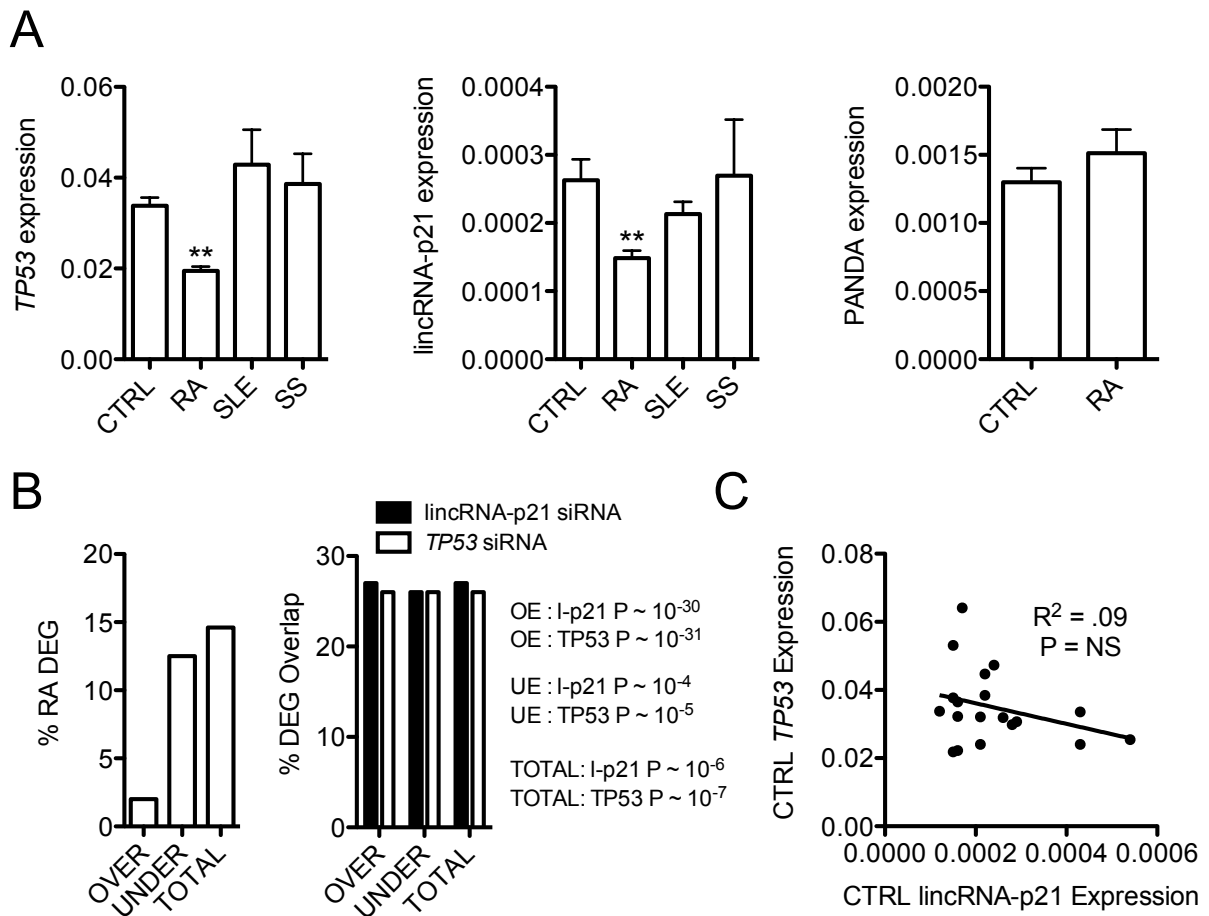
Figure 5-1. Reduced expression of lincRNA-p21 in rheumatoid arthritis. A, *TP53*, lincRNA-p21, and PANDA transcript levels were measured by qRT-PCR and normalized to *GAPDH*. Whole blood samples from CTRL (n = 45), RA (n = 18), SLE (n = 24), and SS (n = 12) subjects were collected into PaxGene tubes. **B,** Comparison of RA differentially expressed genes (DEGs) and genes significantly over-expressed (OE) or under-expressed (UE) following lincRNA-p21 or *TP53* RNA interference. Chi-squared test was used to calculate P values. **C,** Correlation between lincRNA-p21 and *TP53* transcripts in CTRL subjects (n = 20).

MTX therapy restores lincRNA-p21 expression by DNA-PKcs activation.

We next ascertained if commonly prescribed therapies for RA altered lincRNA-p21 expression. MTX is often referred to as the anchor therapy for RA. Therefore, we asked if subjects receiving (RA+MTX) or not receiving (RA-MTX) once-weekly doses of MTX exhibited different lincRNA-p21 transcript levels. We found that the RA+MTX cohort exhibited higher levels of lincRNA-p21 transcripts than the RA-MTX cohort (Figure 5-2A). Further, no significant difference existed between the CTRL and RA+MTX cohort. We conclude from this cross-sectional study that MTX restored lincRNA-p21 expression, *in vivo,* in RA.

We next asked if MTX directly induced lincRNA-p21 expression in cells. We found that treatment of either the transformed Jurkat T cell line or activated primary T cells with 0.1 μM MTX resulted in a 10-15 fold increase in lincRNA-p21 transcript levels (Figure 5-2B). The degree of induction of lincRNA-p21 by MTX in both cell types was greater than the degree of induction of either JNK or p53 by MTX (Figure 5-2C). Previous work demonstrated that MTX-mediated activation of JNK in cells induced increased expression of proteins contributing to cell cycle arrest and apoptosis.[132] In certain cell types, MTX also induces increased synthesis of adenosine and activation of adenosine receptors.
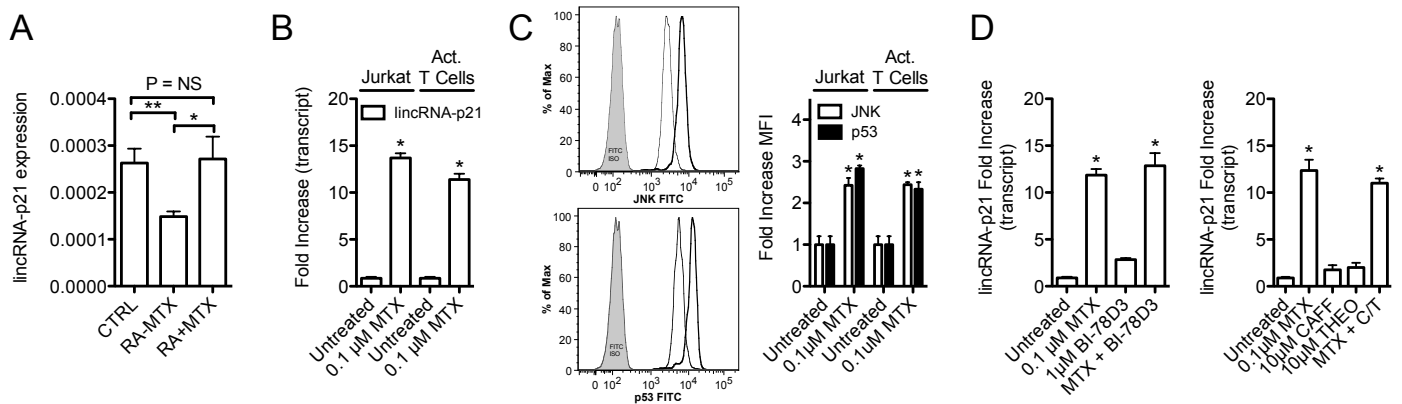
Figure 5-2. Methotrexate increases expression of lincRNA-p21. A, LincRNA-p21 expression levels in CTRL (n = 45), RA-MTX (n = 18), and RA+MTX (n = 18) subjects were determined by quantitative RT-PCR. Results are expressed as mean lincRNA-p21 relative to *GAPDH* transcript levels. **B,** LincRNA-p21 transcript levels in Jurkat cells or activated T cells +/- MTX. Results are expressed as in **A**. **C,** Jurkat cells or activated T cells were cultured with 0.1μM MTX and intracellular protein measurements determined by flow cytometry. A representative flow diagram for JNK and p53 shows background fluorescence (left, shaded gray) and results obtained with untreated (middle) or MTX-treated (right) cells. Fold increase in mean fluorescence intensity (MFI) of four independent experiments is shown in the right graph. **D,** LincRNA-p21 expression in Jurkat cells cultured with MTX and the JNK inhibitor, BI-78D3, (left panel) or adenosine receptor antagonists, caffeine (CAFF) and theophylline (THEO) (right panel). Results are expressed as in **A**. * = p < 0.05, ** = p < 0.005. Values are the mean ± S.D. NS = not significant

Thus, we determined if either inhibition of JNK activation using the small molecular weight JNK antagonist BI-78D3 or adenosine receptor activation using the general adenosine receptor antagonists, caffeine and theophylline, was sufficient to prevent MTX-mediated elevation of lincRNA-p21 transcript levels. We found that inhibition of JNK activity by BI-78D3 did not abrogate MTX-mediated induction of lincRNA-p21 (Figure 5-2D, left panel). We also found that adenosine receptor antagonists, caffeine and theophylline, did not block induction of lincRNA-p21 by MTX (Figure 5-2D, right panel). Taken together, these results suggest that other pathways are activated by MTX to induce lincRNA-p21 in these tissue culture models.

The original report describing the function of lincRNA-p21 demonstrated that p53 activates human lincRNA-p21 in response to DNA damage.[179] Deficiencies in the DNA damage response have also been reported in mononuclear cells from subjects with RA including reduced expression of p53 and two sentinels of the DNA damage response, ATM and DNA-PKcs.[84,146] Therefore, we asked if MTX-induced phosphorylation of ATM or DNA-PKcs. To do so, we treated Jurkat cells with MTX and performed intracellular flow cytometry with specific antibodies to detect phosphorylated-ATM and phosphorylated-DNA-PKcs. We found that MTX treatment of Jurkat cells only modestly increased phosphorylation of ATM (Figure 5-3A).
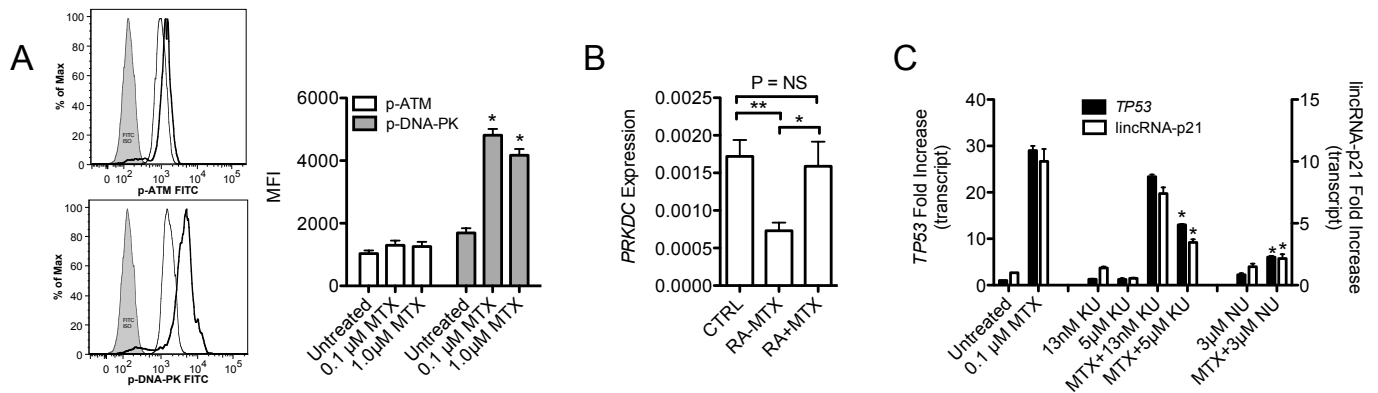
Figure 5-3. Methotrexate induces lincRNA-p21 via DNA-PKcs activation. A, Jurkat cells were treated with the indicated concentrations of MTX for 48 hours and levels of the indicated phosphorylated proteins were measured by flow cytometry. Representative flow diagrams for phosphorylated-ATM and phosphorylated-DNA-PKcs show background fluorescence (left, shaded gray), untreated cells (middle) and MTX-treated (right) Jurkat cells. * = p < 0.05 versus untreated cells. **B,** *PRKDC* transcript levels relative to *GAPDH* were measured in CTRL (n = 45), RA-MTX (n = 18), and RA+MTX (n = 18) subjects. **C,** MTX-treated Jurkat cells were co-cultured for 48 hours with varying concentrations of KU-55933 (KU) or NU-7441 (NU) and transcript levels of *TP53* and lincRNA-p21 determined by qRT-PCR. * = p < 0.05 versus MTX-treated cells. See Figure 2 for other definitions.

In contrast, MTX markedly increased phosphorylation of DNA-PKcs in Jurkat cells. We also compared DNA-PKcs (*PRKDC*) transcript levels in the healthy CTRL cohort to the RA+MTX and RA-MTX cohorts. We found that the RA-MTX cohort exhibited reduced expression levels of *PRKDC* compared to the CTRL cohort (Figure 5-3B). The RA+MTX cohort expressed *PRKDC* at levels equivalent to the CTRL cohort and significantly higher than the RA-MTX cohort. We conclude from these studies that pharmacologic doses of MTX increased *PRKDC* transcript levels in RA subjects to near control values. Next, we asked using an *in vitro* assay if induction of *TP53* and lincRNA-p21 by MTX in Jurkat cells could be reversed with the addition of an ATM or DNA-PKcs specific inhibitor. To accomplish this, we supplemented cultures with KU-55933, which at low concentrations (10-20nM) inhibits phosphorylation of ATM but at high concentrations inhibits phosphorylation of both ATM and DNA-PKcs (5-10μM).[189-191] Supplementation of MTX-treated cultures with the KU-55933 inhibitor at low concentrations to selectively inhibit ATM did not significantly alter induction of lincRNA-p21 or *TP53* by MTX (Figure 5-3C). In contrast, treatment with MTX and high concentrations of KU-55933 that inhibit both ATM and DNA-PKcs significantly reduced induction of lincRNA-p21 and *TP53* transcripts by MTX suggesting that induction of *TP53* and lincRNA-p21 by MTX resulted from activation of DNA-PKcs rather than activation of ATM. To directly test this hypothesis, we employed the specific inhibitor of DNA-PKcs phosphorylation,

NU-7441[192,193], and found that treatment of Jurkat cells with NU-7441 directly inhibited MTX-mediated induction of lincRNA-p21 and *TP53* transcripts to nearly the same level. Taken together, these results provide evidence that MTX increases expression of lincRNA-p21, at least in part, through activation of DNA-PKcs.

Methotrexate inhibits NF-κB activity via lincRNA-p21 activation.

Since deficiencies of p53 exacerbate inflammatory disease in murine models, rheumatoid arthritis is a disease characterized by p53 deficiency, and NF-κB is a critical pro-inflammatory and pro-survival transcription factor, we postulated that *TP53* and/or lincRNA-p21 may directly interfere with NF-κB activity in tissue culture models. To explore this hypothesis, we measured activity of NF-κB using a luciferase reporter construct. We initiated our studies in cell lines co-transfected with the NF-κB luciferase reporter and Silencer Select siRNAs for *TP53* and lincRNA-p21. Cultures were untreated or treated with MTX for 48 hours. TNF-α was added during the last 24 hours of culture to stimulate NF-κB activity. Cultures treated MTX and TNF-α (transfected with a scrambled siRNA control (Neg. Ctrl)) exhibited reduced activation of NF-κB compared to cultures treated with TNF-α (Figure 5-4A). Addition of *TP53* or lincRNA-p21 siRNAs significantly abrogated MTX-mediated inhibition of NF-κB activity in Jurkat cells.
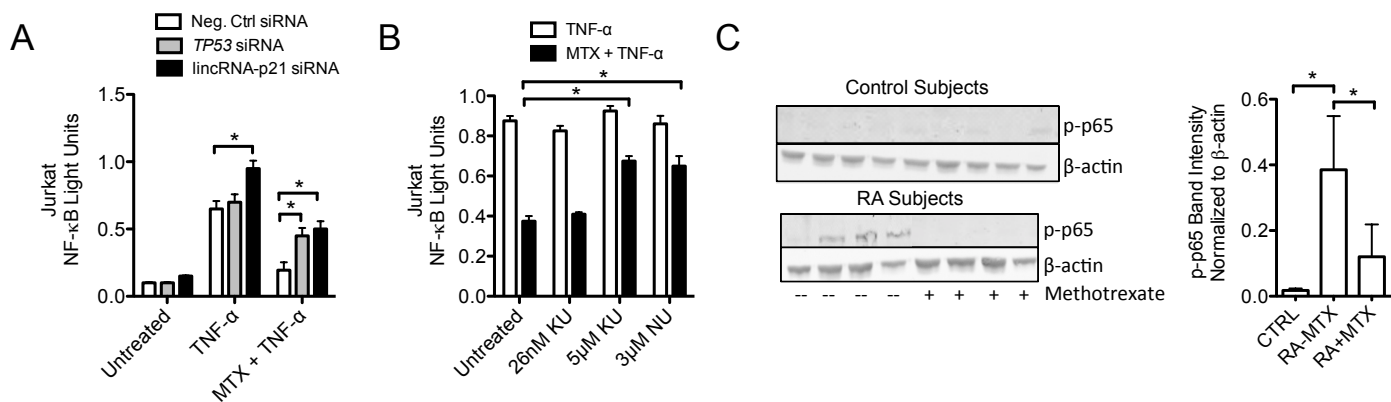
Figure 5-4. Methotrexate reduces NF-κB activity via DNA-PKcs activation and lincRNA-p21 induction. A, Jurkat cells were transfected with an NF-κB luciferase reporter construct in the presence of *TP53* (gray) and lincRNA-p21 (black) siRNAs or a scrambled siRNA control (Neg. Ctrl, white). Cells were treated with methotrexate (MTX) for 48 hours. TNF-α (5ng) was added to cell cultures after 24 hours. Results are expressed in light units. **B,** As in A, Jurkat cells were transfected with an NF-κB luciferase reporter construct and treated with MTX in the presence or absence of KU-55933 (KU) or NU-7441 (NU). Results are expressed as in **A**. **C,** Western blotting for phosphorylated p65 (P-p-65). Whole cell lysates were prepared in CTRL (n = 9), RA+MTX (n = 4) and RA-MTX subjects (n = 4). Right panel shows quantitation of band intensity relative to b-actin. **A-C**, * = p < 0.05

Since we found that MTX induced lincRNA-p21 through activation of DNA-PKcs, we asked if inhibition of DNA-PKcs using KU-55933 (KU) or NU-7441 (NU) also blocked inhibition of NF-κB activity by MTX. Low concentrations of KU-55933 that only inhibit ATM phosphorylation failed to prevent MTX-mediated inhibition of NF-κB activity. In contrast, high concentrations of KU-55933 that inhibit phosphorylation of both ATM and DNA-PKcs or addition of the DNA-PKcs-specific inhibitor, NU-7441, effectively blocked inhibition of NF-κB activity by MTX in our cell culture model (Figure 5-4B). One of the most abundant NF-κB family members is the p50-p65 complex. Phosphorylation of p65 (Rel A), in response to exogenous stimuli, such as TNF-α, increases NF-κB transcriptional activity.[194] Therefore, we elected to use this biomarker to measure basal levels of NF-κB activity in control and RA subjects by western blotting for phosphorylated-p65 (P-p65). Whole cell lysates were prepared from PBMC harvested from CTRL and RA subjects. Surprisingly, we were able to detect elevated basal levels of P-p65 in RA PBMC but not in CTRL (Figure 5-4C). Increased levels of P-p65 were not detected, however, in RA subjects receiving once-weekly doses of MTX. Our conclusion from these studies is that chronic NF-κB activation exists in RA and is corrected by MTX therapy, *in vivo*.

The above results demonstrated that induction of lincRNA-p21 and/or *TP53* transcripts by MTX contributed to MTX-mediated inhibition of

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activation of NF-κB by TNF-α. However, they did not demonstrate if basal levels of lincRNA-p21 and/or *TP53* transcripts contributed to basal or stimulus-dependent (TNF-α) NF-κB activity. To explore this, we used siRNAs to specifically reduce basal levels of lincRNA-p21 or *TP53* transcripts in two different cell lines, THP-1 cells, which is of the monocyte lineage, and Jurkat cells. In THP-1 cells, siRNA-mediated specific reduction of lincRNA-p21 or *TP53* transcripts resulted in increased basal and TNF-α induced NF-κB activity (Figure 5-5A). In Jurkat cells, siRNA-mediated reduction of lincRNA-p21 levels, but not *TP53* levels, resulted in increased basal and TNF-α induced NF-κB activity. This difference is due, in part, to decreased efficiency of siRNA-mediated knockdown of *TP53* transcripts in Jurkat cells. These studies demonstrated that lincRNA-p21 is a direct negative regulator of both basal and stimulus-induced NF-κB activity.

Previous studies have demonstrated that lincRNA-p21 inhibits expression of genes encoding pro-survival proteins to induce apoptosis in response to DNA damage.[178,179,187] Further, lincRNA-p21 binds to mRNAs, such as *JUNB* and *CTNNB1* and inhibits their translation.[179,187] Thus, we reasoned that lincRNA-p21 may inhibit NF-κB activity by altering transcript levels of genes that encode proteins critically involved in NF-κB signaling pathways or that lincRNA-p21 may actually bind to these mRNAs and thus lower rates of translation. We performed two studies to help discriminate between these two possibilities.

Figure 5-5. Association of *RELA* and lincRNA-p21 transcripts. A, THP-1 (left) or Jurkat (right) cells were transfected with an NF-κB luciferase reporter construct in the presence of specific siRNAs targeting lincRNA-p21 (black), *TP53* (gray), or a scrambled siRNA control (Neg. Ctrl, white). Luminescence was quantified 48 hours post-transfection. TNF-α (5ng) was administered to cultures at 24 hours post-transfection. * = p < 0.05 **B,** THP-1 cells were transfected with a lincRNA-p21 siRNA and qRT-PCR measurements of the indicated transcripts measured 48 hours post-transfection. * = p < 0.05 versus cells transfected with a negative control siRNA. **C,** Sites of high complementarity between *RELA* and *JUNB* mRNA and lincRNA-p21 and **D,** relative enrichment as determined by qRT-PCR of *RELA* and *JUNB* mRNAs purified with a lincRNA-p21 biotinylated probe. * = p <0.05 versus 18S and *NFKB1* transcript levels.

We employed lincRNA-p21 specific siRNAs to knockdown levels of lincRNA-p21. We found that loss of lincRNA-p21 did not produce a corresponding gain in transcript levels of *NFKB1* or *RELA* suggesting that lincRNA-p21 did not interfere with cellular NF-κB activity by lowering *NFKB1* or *RELA* transcript levels (Figure 5-5B). To test the alternate hypothesis, that lincRNA-p21 may associate with *RELA* and/or *NFKB1* mRNAs and inhibit their translation, we used a bioinformatics approach to identify regions of complementarity between lincRNA-p21 and *JUNB* (as control) and *RELA*, the gene whose protein product encodes for p65. We identified 8 regions of high complementarity between lincRNA-p21 and *JUNB*, mirroring previously published findings[187] and further identified 6 sites of high complementarity between lincRNA-p21 and *RELA* (Figure 5-5C). The interactions between endogenous levels of lincRNA-p21 and *JUNB* and *RELA* were quantified using an antisense, biotinylated lincRNA-p21 RNA as described previously.[187] Similar to the known ability of lincRNA-p21 to associate with *JUNB,* we found that lincRNA-p21 had a significantly higher interaction with *RELA* transcripts than *NFKB1* or 18S transcripts (Figure 5-5D). Our work suggests that one way lincRNA-p21 may function is through binding to mRNAs that encode proteins critical for NF-κB transcriptional activity.

Discussion

Increased expression of P-p65, a necessary component of NF-κB transcriptional activity, and decreased expression of lincRNA-p21 are present in RA subjects not receiving MTX therapy. Treatment with MTX corrects both defects, *in vivo*. Our results support a model whereby MTX induces lincRNA-p21 expression via a DNA-PKcs dependent pathway and that lincRNA-p21 directly inhibits both basal and TNF-α stimulated NF-κB activity. Inhibition of NF-κB activity results, at least in part, by the ability of lincRNA-p21 to sequester *RELA* mRNA. Thus, reduced expression of lincRNA-p21 and p53 contributes to increased activity of the pro-inflammatory and pro-survival transcription factor NF-κB. We propose that reduced expression of these pro-apoptotic and anti-inflammatory RNAs and proteins in RA contributes to the chronic inflammation observed in RA.

MTX is generally considered an anchor therapy for treatment of RA. Its anti-inflammatory effects are incompletely understood. Two known pathways activated by MTX are stimulation of adenosine release and activation of adenosine receptors, which has anti-inflammatory properties, and activation of JNK enzymes to induce pro-apoptotic proteins leading to heightened sensitivity to apoptosis.[132] Activation of DNA-PKcs by MTX appears to be independent of these two pathways. Phosphorylation (activation) of DNA-PKcs and ATM in response to DNA damage is reasonably

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well understood. However, phosphorylation of ATM is not observed in response to MTX suggesting that induction of DNA damage by MTX is not responsible for phosphorylation of DNA-PKcs in our culture systems. DNA-PKcs activation also plays a critical role in cellular responses to replicative stress.[73] Thus, it is possible that MTX may induce replicative stress via inhibition of DHFR or other mechanisms to activate DNA-PKcs independent of ATM. Using selective inhibitors of DNA-PKcs and ATM, we clearly show that induction of lincRNA-p21 and *TP53* by MTX is dependent upon DNA-PKcs and not ATM and that MTX mediated inhibition of NF-κB activity in our culture systems requires DNA-PKcs. Our interpretation is that MTX induces phosphorylation of DNA-PKcs leading to induction of *TP53* and lincRNA-p21. In turn, elevated activity of p53 and lincRNA-p21 inhibits basal and stimulus-induced NF-κB activity.

LincRNA-p21 is upstream of *CDKN1A* (Supplemental Figure 5-1) in the genome and belongs to a class of lncRNAs induced by p53 in response to DNA damage.[182] In blood cells, baseline levels of *TP53* and lincRNA-p21 are not well correlated. In the absence of DNA damage, genes responsive to *TP53* 'knockdown' or lincRNA-p21 'knockdown' are not identical (16). Both classes of genes are highly over-represented in the class of genes found to be over or under-expressed in RA. Thus, we believe our results and results of others support the notion that *TP53* and lincRNA-p21 transcript levels are regulated by both common pathways in response to, for example, extensive

DNA damage as well as by independent pathways. Further, gene expression programs regulated by p53 and lincRNA-p21 are not identical. Further experimentation will be required to identify underlying mechanisms leading to reduced expression of *TP53* and lincRNA-p21 in RA and the full spectrum of consequences in RA produced by reduced expression of *TP53* and lincRNA-p21.

Two mechanisms are known by which lincRNA-p21 affects cellular function. First, in the nucleus, lincRNA-p21 mediates transcriptional repression of target genes through association with heterogeneous nuclear ribonucleoprotein-K (hnRNP-K).[179] Second, in the cytoplasm, lincRNA-p21 associates with target mRNAs, such as *JUNB* and *CTNNB1*, to prevent their translation.[187] In our analysis, reduced lincRNA-p21 levels do not alter transcript levels of NF-κB genes, *NFKB1* or *RELA*. Rather, lincRNA-p21 binds *RELA* mRNA thus possibly interfering with mRNA translation, a result consistent with the second mechanism. However, we cannot rule out the possibility that lincRNA-p21 may inhibit transcription of other mRNAs that encode proteins required for NF-κB activation or represses translation of these same mRNAs. Nevertheless, our results indicate that lincRNA-p21 possesses regions of mRNA sequence homology with *RELA* mRNA and associates with *RELA* mRNA under physiologic conditions.

Existence of defects in the transcriptional response leading to cell cycle arrest and apoptosis in RA are well established. These same defects are

corrected, at least in part, by one of the major therapies for RA, MTX.[163] What has been unclear is how these defects may contribute to underlying chronic inflammation that is a hallmark of RA. We propose that one mechanism is that p53 and lincRNA-p21 are negative regulators of NF-κB activity. As such, reduced levels of p53 and lincRNA-p21 produce enhanced basal and stimulus-dependent NF-κB activity and restoration of levels of p53 and lincRNA-p21 by MTX also lowers NF-κB activity, a major driver of the pro-inflammatory state of RA. Future studies will be required to determine if understanding interplay between these two pathways can be exploited for therapeutic benefit in RA.

CHAPTER VI

GENERAL DISCUSSION AND CONCLUSION

While initially developed as a chemotherapeutic, methotrexate (MTX) has been the mainstay for RA treatment for nearly four decades. 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.[1,3] RA patients treated with MTX experience reduced pain, and improved joint score and function typically within three months of treatment. The tight control and suppression of inflammation in early stages of disease has been advocated as the basis of documented disease modifying effects. Yet, the mechanisms accounting for the anti-inflammatory effects of MTX remain incompletely understood. Questions also remain as to the specific targets necessary to develop new therapeutics beyond MTX for the treatment of RA.

The initial studies of rheumatoid arthritis in our laboratory examined differential expression of genes in healthy control subjects and patients diagnosed with autoimmune disease. The goal of these experiments was to identify a subset of genes that could distinguish between healthy individuals and patients with autoimmune disease. We found that patients with rheumatoid arthritis significantly underexpressed a panel of genes that are typically considered prototypical cancer genes in peripheral blood

mononuclear cells. Specifically, these studies established that defects in expression of *CHEK2, TP53, CDKN1A,* and *CDKN1B* conferred an inability for RA lymphocytes to undergo apoptosis in response to gamma irradiation.[18] The major obstacle moving forward was how to link these observations to RA disease pathogenesis. Further, we found that a subset of RA subjects exhibited increased expression of *JUN* target mRNA. A unique characteristic of these RA subjects was that they were all receiving low-dose methotrexate therapy. From this observation, we were then able to reproduce in tissue culture MTX-dependent *JUN* activation in T cells treated with sub-micromolar doses of MTX. These experimental findings have been the area of intensive study during my time in the laboratory and have led to the elucidation of a novel series of biochemical pathways for low-dose MTX therapy.

In the first chapter we found that Jun-N-terminal kinase (JNK) a MAP kinase, is activated in response to methotrexate therapy. Through JNK, methotrexate increases the sensitivity of T cells to apoptosis through production of reactive oxygen species and alteration of the transcriptional profile in favor of genes whose protein products promote apoptosis including activation of *JUN* mRNA. This process is mediated by MTX inhibition of dihydrofolate reductase (DHFR), which blocks the reduction of dihydrobiopterin (BH_2) to tetrahydrobiopterin (BH_4). Since our *in vivo* studies of RA patients on low-dose MTX therapy revealed elevated levels of

the prototypical JNK-target gene, *JUN*, in response to MTX, our data support the notion that the JNK pathway is activated by MTX, *in vivo*, and may contribute to the efficacy of MTX in inflammatory disease. Specifically, we now hypothesize that the therapeutic efficacy of MTX may arise at least in part from its ability to deplete BH_4 causing both a shift in cytokine profiles from a pro-inflammatory profile to an anti-inflammatory profile and initiation of the clearance of self-reactive inflammatory lymphocytes by apoptosis.

In chapter two, we further probed the mechanism by which MTX increases sensitivity of cells to apoptosis and asked if methotrexate restores the cell cycle checkpoint deficiencies we have observed previously. Since MTX increased levels of JNK in tissue culture, we asked if levels of JNK were decreased in subjects with RA not receiving MTX. We found highly significant deficiencies of *MAPK9* (JNK2) expression in rheumatoid arthritis. Analysis of other MAPK family members including most known ERK and p38 isoforms did not reveal any significant difference in healthy controls versus RA cohorts receiving or not receiving methotrexate. Following our gene expression studies, we analyzed protein expression in RA lymphocytes and found that these subjects exhibit reduced JNK expression. Analysis of additional autoimmune diseases also indicated that this *MAPK9* deficiency observed in RA was not unique, but also extends to multiple sclerosis (MS). It is interesting to note that RA and MS exhibit similar molecular defects in

PBMC specifically through reduced checkpoint kinase 2 (*CHEK2*), p53 and ataxia telangiectasia (AT) mutated (ATM) expression.[83] The contribution of JNK to these defects remains to be explored. We found that MTX increased levels of both p53 and the downstream target p21 in MTX-treated cells via JNK, which was further confirmed *in vivo* analyzing transcript levels of *TP53* and *CDKN1A* mRNAs in subjects receiving and not receiving MTX therapy. These MTX-mediated effects are critically dependent upon MTX depletion of BH_4, generation of ROS, and activation of JNK. Through loss of ATM, RA T cells accumulate a significant amount of DNA damage.[84] One hypothesis is that DNA damage repair deficiencies coupled with depressed levels of p53 and JNK blunt the central pathways of apoptosis resulting in cell survival but loss of genomic integrity. Cell survival comes at a cost and the cost is persistent DNA damage, which may induce NF-κB or alternative pro-inflammatory, pro-survival pathways leading to the 'sterile inflammation' observed in RA pathogenesis.

Our next series of experiments analyzed the influence of methotrexate upon transcriptional levels of NF-κB, a central regulator of the inflammatory response, in two cells types: T cells and primary fibroblast-like synoviocytes (FLS) from RA subjects. We also examined the PBMC of RA patients receiving and not receiving MTX and found that increased levels of phosphorylated p65 were present in the cohort of RA subjects not receiving MTX, but this signal was absent in RA patients currently on methotrexate.

In tissue culture experiments, administration of MTX decreased TNF-α-dependent activation of NF-κB in both synoviocytes and T cells, but the observed mechanism was divergent across the two cell types. In T cells, the BH$_4$ pathway we have described possessed anti-inflammatory properties via induction of JNK and p53 as depletion of both of these proteins with dominant negative mutants abrogated the inhibitory effects of MTX on T cells *in vitro*. In contrast, reduced activity of NF-κB in FLS was reversed not by BH$_4$ or JNK. Rather, adenosine receptor antagonists caffeine and/or theophylline almost completely reversed the effects of MTX in these cells, which follows closely with much of the published work concerning the mechanism of action of MTX over the past two decades.[13,23,38-40,106,108,195] Thus, we conclude that MTX modulates NF-κB through distinct mechanisms in these cell types.

We further explored the connection between NF-κB and p53, as these are two central regulators of the adaptive immune response. NF-κB modulates the response to exogenous stimuli, whereas p53 modulates the intrinsic stress responses through initiation of "cell cycle arrest, apoptosis, or senescence, eliminating clones of cells with DNA damage and its results mutations".[173] Examination of the PBMC and synovium of RA subjects demonstrates that NF-κB is significantly overexpressed. Also present are reduced levels of p53. p53 drives induction of genes that both prevent DNA damage and repair damaged DNA. Together with NF-κB, these master

regulators of internal and external stimuli must achieve a careful balance. Each transcription factor responds to a different form of cellular stress, adopting two very different strategies that have evolved into mutually exclusive processes under normal physiologic conditions.[173] A downstream target of p53 activity is the activation of the long, non-coding RNA, lincRNA-p21. Similar to our p53 findings, we observed decreased levels of lincRNA-p21. This deficiency of lincRNA-p21 expression was corrected in subjects receiving MTX. Introduction of specific siRNA duplexes targeting lincRNA-p21 prevented MTX-mediated reduction of NF-κB in T cells. To ascertain mechanism, we examined if induction of lincRNA-p21 by MTX could be abrogated with supplementation of either BH_4 or JNK inhibitors. We find that activation of the BH_4 pathway does not modulate lincRNA-p21 activation. Rather, phosphorylation of DNA-PKcs was necessary to induce expression of lincRNA-p21. Surprisingly, activation of p53 by two distinct pathways results in inhibition of NF-κB activity. We conclude from these observations that multiple pathways exist in T cells to restore the p53 response in RA subjects receiving low doses of MTX. A graphic summary of the pathways we have discovered as a result of these studies is summarized in Figure (6-1).
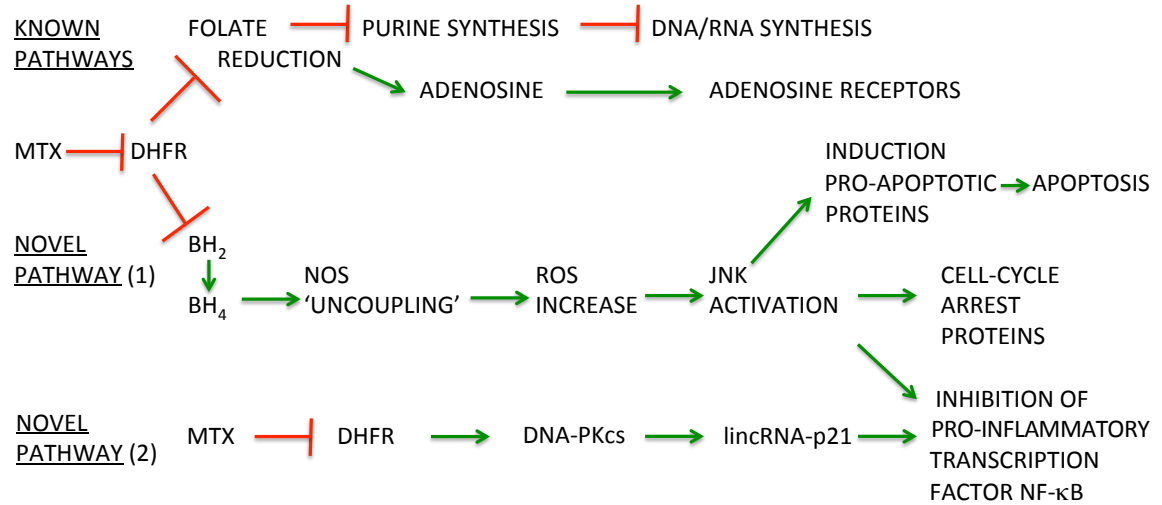
Figure 6-1. **Summary of known and novel mechanisms of MTX action.**

One hundred years ago, the only drug in the physician armamentarium to manage RA was aspirin.[196] Soon after, gold salts were commonly prescribed from approximately 1930-1980. Penicillamine, anti-malarial drugs, and sulfasalazine were subsequently introduced from in the 1970s and 1980s.[10] However, despite introduction of these pharmacologics, the disease course of most RA patients progressed and was not adequately controlled. It wasn't until the introduction of disease-modifying anti-rheumatic drugs, such as methotrexate, that physicians saw significant improvement in long-term outcomes, especially when methotrexate was combined with other therapies. When we initiated our studies to examine the anti-inflammatory properties of methotrexate, we thought that there would be a single biomarker we could target to achieve the same outcome with less toxicity, as subjects taking methotrexate have reported hair loss, nausea, and fatigue. However, as we examine the molecular basis for this drug in RA, we

find that not only does it stimulate the adenosine pathway, which results in reduced NF-κB activation in FLS, but it also activates the BH_4 pathway and induces lincRNA-p21 in T cells. Both of these pathways lower NF-κB transcriptional activity and function *in vivo* in RA.

Targeted therapies that have been approved over the past decade have resulted in many first-in-class drugs. However, the majority of these first in class drugs were the result of phenotypic assay screening, and not through targeted approaches.[197] Interestingly, most targeted approaches result in follow-on drugs that are prescribed in combination with other 'anchor' therapies in human disease. An interesting area of future investigation would be to design small molecules that selectively target BH_2 reduction to BH_4 and alternatively induce lincRNA-p21 expression. Given the diverse effects we see both *in vitro* and *in vivo* in RA patients receiving MTX, it would be difficult to point to a single therapy that could supplant the multi-faceted pathway MTX employs in the management of RA. While these new therapeutics could be of utility, an important future direction of this work is setting the stage for a better understanding the origins of the cellular defects we have observed. We would argue that treatment of the most proximal events in disease pathogenesis lead to more effective therapeutic strategies and improved clinical outcomes. Most newly developed therapies attempt to disrupt the downstream NF-κB activation-pro-inflammatory cytokine loop, such as Enbrel (etanercept) through blockade of TNF-α. We argue that interfering

with the upstream pathways of deficiency cell cycle arrest and DNA repair will produce improved therapeutic outcomes for subjects with RA. To this end, we have considered prevailing theories of immunity and autoimmunity. The general view is that initiation of adaptive immune responses to pathogens is divided into two parts, the recognition of 'danger' via the innate immune system and the recognition of foreign antigen by the adaptive immune system.[198,199] It is widely accepted that a breach in tolerance in the adaptive immune response leads to recognition of self-antigen contributing to autoimmunity. In this model, the source of 'danger' to initiate the immune response to self has never been completely identified.[198] We would propose a new model whereby the source of 'danger' is actually internal or intracellular and not external, which we will explore in a new series of experiments. This model is summarized in Figure 6-2.

ENVIRONMENTAL STIMULI
Oxidative Stress, Cigarette Smoking, UV Radiation, Ionizing Radiation

DNA Damage Accumulation

GENOME MAINTENANCE

Without RA Risk Factors

Properly Functioning
Cell Cycle Checkpoints

DNA Damage Repair

Genome Integrity

With RA Risk Factors: HLA-DRB1

Defects in Apoptosis, Cell Cycle Checkpoint
Machinery, and DNA Repair

NF-κB Activation

Pro-Inflammatory Cytokine Activation

Inflammation and Chronic Synovitis

LOSS OF GENOMIC INTEGRITY

Figure 6-2. Schematic illustrating proposed inter-relationships between environmental exposures and genetic liabilities to produce failure of DNA damage repair, chronic NF-κB activation and inflammation leading to RA.

In our model, DNA damage accumulates every day in individuals as a result of environmental exposures, UV or ionizing radiation, oxidative stress, chemical exposures, cell replication, inflammatory stress in response to infection, normal metabolic activities produce oxidants, or smoking.[69,71,82,153,200-203] Smoking is well established as a significant environmental risk factor for RA. Normally, activation of cell cycle checkpoints and the DNA repair machinery repair DNA damage. However, in RA, via intrinsic mechanisms regulated by the presence of HLA-DRB1*04 alleles or other pathways, these repair mechanisms, ATR, DNA-PKcs, and ATM, and cell cycle checkpoints, JNK2, p53, p21, p27, CHEK2, RANGAP1 are defective, resulting in failure to repair DNA and loss of genomic integrity.[85,175,204-206] Failure to repair DNA and/or cell cycle checkpoint defects

results in chronic NF-κB activation and induction of pro-inflammatory cytokines, producing a continuous cycle of events causing chronic inflammation, which underlies the pathogenesis of RA. If the origins of these defects could be corrected, this may even result in a cure. Future studies are planned to examine the contribution of these defects to the etiopathogenesis of RA.

LIST OF PUBLICATIONS

Spurlock III, C.F., Aune, Z.T., Tossberg, J.T., Collins, P.L., Aune, J.P., Huston, J.W., Crooke, P.S., Olsen, N.J. and Aune, T.M. (2011), Increased sensitivity to apoptosis induced by methotrexate is mediated by JNK. *Arthritis & Rheumatism*, 63:2606-2616. *Reprinted with permission in its entire form under License No. 3346061002521*

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Zhang, F., Tossberg, J.T., Spurlock III, C.F., Yao, S., Aune, T.M. (2014), Expression of IL33 and its epigenetic regulation in multiple sclerosis. *Annals of Clinical and Translational Neurology*. In press.

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APPENDIX A

Supplemental Table 2-1. Regulation of genes encoding proteins involved in apoptosis by MTX is JNK-mediated.

Gene Symbol	MTX,-8M, 48hr MEAN	STDEV	TTEST	MTX,-7M, 48hr MEAN	STDEV	TTEST	MTX, -6M, 48 hr MEAN	STDEV	TTEST
18S	0.9	0.3	NS*	1.5	1.4	NS	1.0	0.7	NS
ACTB	0.5	0.1	NS	1.3	0.7	NS	1.6	0.6	NS
APAF1	0.9	0.3	NS	1.4	0.6	NS	1.8	0.5	NS
BAD	0.7	0.5	NS	1.9	0.3	0.02	2.9	1.7	NS
BAK1	0.9	0.0	NS	2.0	0.4	0.04	2.8	1.3	NS
BAX	0.8	0.5	NS	2.2	1.0	NS	4.0	2.8	NS
BBC3	0.5	0.2	NS	5.9	1.8	0.04	6.0	1.9	0.04
BCAP31	1.6	0.1	NS	1.6	0.5	NS	1.5	0.4	NS
BCL10	1.2	0.5	NS	2.5	1.4	NS	2.7	1.5	NS
BCL2	1.5	0.7	NS	4.6	1.9	NS	4.2	1.4	NS
BCL2A1	2.1	1.3	NS	2.5	1.3	NS	2.9	1.1	NS
BCL2L1	0.4	0.1	NS	1.2	0.3	NS	1.5	0.8	NS
BCL2L11	1.0	0.3	NS	2.9	1.3	NS	4.9	4.1	NS
BCL2L13	0.3	0.0	NS	1.4	1.4	NS	2.0	1.4	NS
BCL2L2	ND**	ND	ND	ND	ND	ND	ND	ND	ND
BCL3	1.2	0.3	NS	10.5	1.9	0.01	14.4	3.9	0.03
BID	2.2	0.4	NS	1.1	0.1	NS	1.2	0.3	NS
BIK	1.4	0.2	NS	3.5	1.0	0.04	2.8	2.1	NS
BIRC1	1.4	0.1	NS	2.1	1.5	NS	1.1	1.8	NS
BIRC2	1.1	0.1	NS	1.1	0.7	NS	1.3	1.0	NS
BIRC3	1.6	0.0	NS	1.9	1.2	NS	2.2	1.2	NS
BIRC4	0.8	0.4	NS	2.5	1.5	NS	6.5	7.5	NS
BIRC5	1.4	0.4	NS	1.9	0.4	0.04	2.1	0.2	0.00
BIRC6	0.5	0.2	NS	1.3	1.0	NS	1.7	1.0	NS
BIRC8	1.6	1.0	NS	3.8	0.4	0.00	10.5	3.6	0.04
BNIP3	1.3	0.3	NS	0.5	0.3	NS	0.5	0.2	0.03
BNIP3L	1.4	0.4	NS	1.0	0.2	NS	1.0	0.1	NS
BOK	0.8	0.2	NS	4.4	1.4	0.01	5.6	1.2	0.01
CARD4	0.2	0.0	NS	1.2	0.9	NS	2.3	1.7	NS
CARD9	1.4	0.4	NS	8.9	2.9	0.04	9.6	2.2	0.02
CASP1	2.2	0.1	NS	6.2	1.4	0.01	7.2	2.3	0.01
CASP10	0.5	0.0	NS	3.2	1.6	NS	3.7	2.1	NS
CASP2	0.6	0.0	NS	2.0	0.7	NS	2.9	1.2	NS
CASP3	1.0	0.1	NS	2.2	1.2	NS	2.3	0.8	NS
CASP4	1.6	0.2	NS	1.8	0.8	NS	1.9	0.7	NS
CASP6	1.8	0.1	NS	2.8	2.2	NS	3.2	1.9	NS
CASP7	1.7	0.7	NS	1.8	1.2	NS	2.6	1.4	NS
CASP8	0.6	0.4	NS	1.9	0.8	NS	2.9	1.5	NS

Gene Symbol	MEAN	STDEV	TTEST	MEAN	STDEV	TTEST	MEAN	STDEV	TTEST
CASP8AP2	1.4	0.0	NS	2.2	0.9	NS	2.9	2.2	NS
CFLAR	1.0	0.3	NS	1.8	0.6	NS	1.9	0.9	NS
CHUK	1.8	0.7	NS	1.8	0.9	NS	1.7	0.8	NS
CRADD	1.6	0.2	NS	1.1	0.4	NS	1.2	0.6	NS
DAPK1	0.6	0.4	NS	3.2	1.6	NS	4.5	3.3	NS
DEDD	1.0	0.2	NS	1.3	0.5	NS	1.4	0.5	NS
DEDD2	0.8	0.1	NS	1.4	0.1	0.01	2.2	0.5	0.04
DIABLO	1.3	0.3	NS	1.4	0.3	NS	1.6	0.5	NS

Gene Symbol	MTX [-8M 48hr]			MTX [-7M 48hr]			MTX [-6M 48 hr]		
	MEAN	STDEV	TTEST	MEAN	STDEV	TTEST	MEAN	STDEV	TTEST
ESRRBL1	2.3	0.3	NS	2.5	1.0	NS	2.6	1.6	NS
FADD	1.0	0.0	NS	1.5	0.7	NS	1.7	0.8	NS
FAS	1.7	0.8	NS	2.8	0.8	NS	3.7	0.4	0.01
GAPDH	1.0	0.0	NS	1.0	0.0	NS	1.0	0.0	NS
HIP1	0.7	0.3	NS	2.3	1.7	NS	2.0	0.9	NS
HRK	1.1	0.4	NS	6.9	2.1	0.04	10.3	2.7	0.03
HTRA2	1.4	0.5	NS	1.5	0.6	NS	1.8	1.0	NS
IKBKB	0.6	0.2	NS	0.8	0.3	NS	1.2	0.5	NS
IKBKE	1.4	0.2	NS	3.4	1.9	NS	3.5	2.3	NS
IKBKG	0.7	0.1	NS	1.9	0.4	0.05	2.5	0.6	0.03
LRDD	0.1	0.0	NS	2.0	1.2	NS	3.0	1.2	NS
LTA	0.9	0.3	NS	2.8	1.2	NS	2.7	1.5	NS
LTB	1.5	1.5	NS	6.1	1.4	NS	13.8	4.9	0.02
MCL1	1.0	0.4	NS	2.8	2.3	NS	4.1	2.9	NS
NALP1	2.5	3.3	NS	1.7	0.9	NS	2.4	1.6	NS
NFKB1	2.3	0.7	NS	2.8	1.3	NS	2.6	1.7	NS
NFKB2	0.3	0.1	NS	1.9	0.3	0.01	2.6	0.3	0.00
NFKBIA	1.7	0.0	NS	2.9	1.1	NS	2.4	1.4	NS
NFKBIB	1.2	0.2	NS	2.4	1.1	NS	2.8	1.8	NS
NFKBIE	0.8	0.1	NS	2.1	0.6	NS	2.8	1.4	NS
PEA15	0.5	0.0	NS	2.5	1.0	NS	2.9	0.9	0.06
PMAIP1	1.6	0.7	NS	2.1	1.4	NS	2.3	1.5	NS
REL	0.9	0.1	NS	2.3	1.0	NS	3.4	1.7	NS
RELA	0.5	0.3	NS	1.3	0.2	NS	1.5	0.3	NS
RELB	1.2	0.3	NS	2.6	1.2	NS	8.3	3.0	NS
RIPK1	0.8	0.2	NS	2.4	1.3	NS	3.2	1.2	NS
TA-NFKBH	0.9	0.3	NS	2.7	1.8	NS	3.1	1.8	NS
TBK1	2.0	0.7	NS	2.1	0.8	NS	2.2	1.3	NS
TNF	1.5	0.7	NS	7.5	3.5	NS	7.4	2.4	0.04
TNFRSF10A	0.9	0.2	NS	9.0	2.7	0.03	8.4	2.1	0.02
TNFRSF10B	0.5	0.3	NS	1.8	0.4	0.05	2.3	0.5	0.03
TNFRSF1A	0.4	0.2	NS	1.3	0.4	NS	1.3	0.5	NS
TNFRSF1B	1.2	0.4	NS	4.9	0.2	0.00	13.7	3.9	0.03
TNFRSF21	0.5	0.0	NS	1.5	0.5	NS	1.6	0.7	NS
TNFRSF25	2.0	0.7	NS	7.2	1.6	0.02	7.4	2.2	0.04
TNFSF10	1.7	0.3	NS	10.2	3.9	0.05	10.6	2.3	0.02

TRADD	1.7	0.4	NS	2.3	0.7	NS	2.6	1.4	NS

Legend: Experimental details as in Fig. 2-2. Mean and S.D. determined from fold difference between treated and untreated cultures.

*NS: not significant

**ND: transcript not detected,

T test: page 1-2 determines significance between untreated and MTX-treated cultures

T test: page 3-4 determines significance between MTX treated cultures and MTX + JNK-inhibitor treated cultures

Gene Symbol	MTX,-6M, 48 hr		MTX,-6M+JNK-I,-5M, 48 hr		
	MEAN	STDEV	MEAN	STDEV	TTEST
18S	1.4	0.5	0.9	0.4	NS
ACTB	1.3	0.5	1.6	1.2	NS
APAF1	1.6	0.7	1.9	0.8	NS
BAD	2.7	1.5	1.2	0.5	NS
BAK1	3.1	0.8	1.1	0.6	NS
BAX	3.4	2.4	2.2	1.8	NS
BBC3	6.5	1.1	2.6	1.4	NS
BCAP31	1.4	0.5	0.9	0.6	NS
BCL10	2.2	1.2	1.2	0.8	NS
BCL2	4.5	1.3	3.2	1.1	NS
BCL2A1	2.8	1.6	1.3	1.1	NS
BCL2L1	1.4	0.5	1.6	0.6	NS
BCL2L11	4.4	4.0	1.6	1.4	NS
BCL2L13	2.6	1.3	1.8	1.2	NS
BCL2L2	ND	ND	ND	ND	ND
BCL3	15.8	3.3	3.1	1.1	0.01
BID	1.3	0.6	0.7	0.4	NS
BIK	2.2	1.1	1.7	0.8	NS
BIRC1	1.8	0.8	2.8	1.2	NS
BIRC2	1.4	0.9	1.1	0.6	NS
BIRC3	2.3	1.5	2.5	1.2	NS
BIRC4	4.5	3.5	3.1	3.1	NS
BIRC5	2.8	0.2	2.0	1.4	NS
BIRC6	1.8	1.5	1.7	1.1	NS
BIRC8	11.4	3.8	1.6	1.4	0.02
BNIP3	0.8	0.6	0.7	0.5	NS
BNIP3L	1.4	0.8	1.5	0.7	NS
BOK	6.3	0.8	2.9	0.6	0.02
CARD4	2.2	1.2	2.9	0.9	NS
CARD9	10.3	2.1	0.9	0.5	0.01
CASP1	8.4	2.5	1.0	0.9	0.00
CASP10	4.7	2.4	2.1	1.9	NS
CASP2	2.6	2.0	3.8	1.7	NS
CASP3	2.7	1.0	1.4	0.8	NS
CASP4	1.7	0.9	1.3	0.9	NS
CASP6	3.4	1.6	1.9	1.2	NS
CASP7	2.2	1.3	1.2	1.0	NS

CASP8	2.6	1.2	2.1	0.9	NS
CASP8AP2	2.5	1.2	0.8	0.6	NS
CFLAR	1.8	0.7	1.2	0.9	NS
CHUK	1.3	1.0	1.5	1.1	NS
CRADD	1.4	0.7	1.4	0.6	NS
DAPK1	3.5	2.1	3.9	1.5	NS
DEDD	1.2	0.3	1.0	0.4	NS
DEDD2	2.3	0.8	1.7	1.1	NS
DIABLO	1.8	0.8	1.5	0.8	NS

Gene Symbol	MTX MEAN	STDEV	MTX [+JNK-I MEAN	STDEV	TTEST
ESRRBL1	2.7	1.3	2.3	1.3	NS
FADD	1.9	1.2	1.9	1.4	NS
FAS	4.3	0.2	2.6	0.4	NS
GAPDH	1.0	0.0	1.0	0.0	NS
HIP1	2.2	1.2	1.4	0.9	NS
HRK	14.1	2.2	1.5	1.1	0.00
HTRA2	1.4	1.4	1.6	1.1	NS
IKBKB	1.4	0.7	1.8	9.0	NS
IKBKE	3.1	2.0	1.0	1.4	NS
IKBKG	2.9	1.6	1.5	1.2	NS
LRDD	3.7	1.6	4.4	1.9	NS
LTA	2.3	0.5	1.2	1.1	NS
LTB	10.9	3.6	2.4	1.1	0.00
MCL1	4.8	3.0	2.5	2.8	NS
NALP1	2.6	1.5	2.4	1.9	NS
NFKB1	2.2	1.1	0.7	0.6	NS
NFKB2	2.3	0.3	2.4	0.5	NS
NFKBIA	2.3	1.1	1.0	0.8	NS
NFKBIB	2.4	1.5	1.9	1.3	NS
NFKBIE	2.7	1.1	1.4	1.1	NS
PEA15	2.5	0.7	2.3	0.9	NS
PMAIP1	2.7	1.1	0.8	0.9	NS
REL	2.8	1.0	2.0	0.7	NS
RELA	1.6	0.5	1.6	0.8	NS
RELB	9.0	3.6	7.5	2.9	NS
RIPK1	2.2	1.7	1.7	1.4	NS
TA-NFKBH	2.1	1.0	2.2	1.4	NS
TBK1	2.6	1.6	0.8	0.7	NS
TNF	8.0	2.4	3.1	1.6	0.02
TNFRSF10A	8.8	2.0	2.7	1.2	0.01
TNFRSF10B	2.2	0.6	1.5	0.7	NS
TNFRSF1A	1.2	0.7	1.2	0.6	NS
TNFRSF1B	14.3	3.3	2.0	1.1	0.01
TNFRSF21	1.8	1.0	3.3	1.5	NS
TNFRSF25	8.8	2.5	0.9	0.8	0.00
TNFSF10	10.3	1.6	0.8	0.9	0.00
TRADD	2.8	1.3	0.7	0.7	NS

Supplemental Table 2-2. Schematic depicts known effects of DHFR inhibition and new pathway described herein.
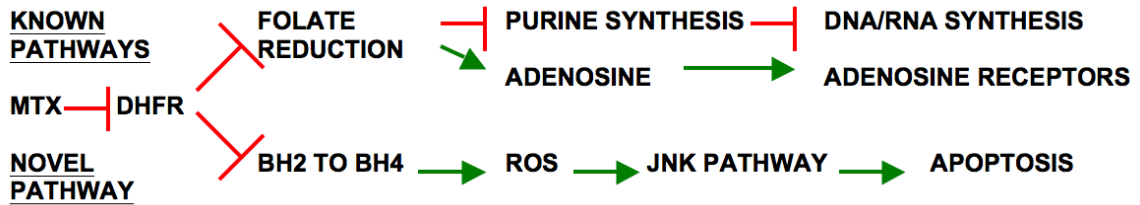
Supplemental Table 3-1. Demographic characteristics of the RA patients and healthy controls and clinical characteristics of the RA patients*

	Controls	RA, no MTX treament	RA, MTX treatment
	(n=43)	(n=18)	(n=24)
Age, mean ± SD yrs	44 ± 11	46 ± 11	48 ± 15
Female	74	78	100
Ethnicity			
Caucasian	74	94	80
African American	15	6	15
Hispanic	6	0	5
Asian	6	0	0
Clinical characteristics			
Disease Duration, mean ± SD years	-	10 ± 9	9 ± 7
Active Disease ‡	-	72	68
Early RA (disease duration < 1 year)	-	22	17
Treatment			
HCQ	-	44	39
Steroids	-	39	61
TNF Inhibitors	-	44	28

* Except where indicated otherwise, values are the percent.
‡ Defined as the presence of at least 3 of the following: morning stiffness > 45 minutes, > 3 swollen joints, > 6 tender joints, and erythrocyte sedimentation > 28 mm/hour. Abbreviations: RA, rheumatoid arthritis; MTX, methotrexate; HCQ, hydroxychloroquine; TNF = tumor necrosis factor

Supplemental Table 3-2. Gene expression studies reveal statistically significant differences in *MAPK9* expression in patients diagnosed with rheumatoid arthritis (RA). Mitogen-activated protein kinases (MAPKs) were assayed in control (CTRL, N=43) and RA subjects (N=18). Expression values are relative to *GAPDH*. *P* values are calculated relative to controls.

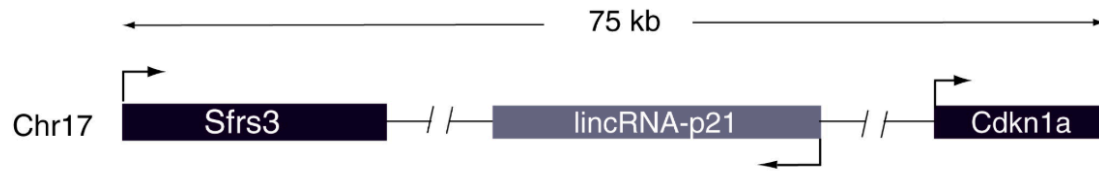| Expression: MAPK gene / GAPDH				
Symbol	Protein	Control	RA	P Value
MAPK8	JNK1	0.00060	0.00060	NS
MAPK9	JNK2	0.00460	0.00210	< 0.0001
MAPK10	JNK3	0.00004	0.00003	NS
MAPK14	p38 α	0.04300	0.03400	NS
MAPK11	p38 β	0.00040	0.00040	NS
MAPK12	p38 γ (ERK3/6)	0.00010	0.00010	NS
MAPK13	p38 δ	0.01200	0.01100	NS
MAPK3	ERK1	0.02500	0.03100	NS
MAPK1	ERK2	0.04600	0.04700	NS
MAPK4	ERK4	ND	ND	
MAPK7	ERK5	0.01200	0.01200	NS
MAPK15	ERK7/8	ND	ND	

Supplemental Table 5-1. Characteristics of the control and autoimmune subject populations

	Controls (n=45)	RA (n=36)	SLE (n=24)	SS (n=12)
Age, mean ± SD yrs	38 ± 11	51 ± 14	42 ± 13	47 ± 13
Female	73	94	94	82
Ethnicity				
Caucasian	58	83	45	58
African American	22	11	33	25
Hispanic	13	6	11	17
Asian	7	-	11	-
Disease Duration, mean ± SD years	-	-	§	8 ± 2
Characteristics of RA Subjects		**(-MTX)** (n = 18)	**(+MTX)** (n = 18)	
Disease Duration, mean ± SD years		10 ± 9	9 ± 7	
Active Disease ‡	-	72	68	
Early RA (disease duration < 1 year)	-	22	17	
Treatment				
HCQ	-	44	39	
Steroids	-	39	61	
TNF Inhibitors	-	44	28	

* Except where indicated otherwise, values are the percent.

‡ Defined as the presence of at least 3 of the following: morning stiffness > 45 minutes, > 3 swollen joints, > 6 tender joints, and erythrocyte sedimentation > 28 mm/hour. § Data are unavailable. Abbreviations: RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; SS, Sjögren's syndrome; MTX, methotrexate; HCQ, hydroxychloroquine

Supplemental Figure 5-1. Schematic representation of the chromosomal location of the lincRNA-p21 gene locus. Arrowheads indicate the orientation of transcription.