We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

6,900
Open access books available

185,000
International authors and editors

200M
Downloads

Our authors are among the

154
Countries delivered to

TOP 1%
most cited scientists

12.2%
Contributors from top 500 universities



WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com



Chapter 8

Will Understanding Methotrexate Modes of Action Teach us About Rheumatoid Arthritis?

Charles F. Spurlock III, Nancy J. Olsen and Thomas M. Aune

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/59901

1. Introduction

The traditional paradigm for autoimmunity dates back over a century to the German bacteriologist, and early pioneer of immunology, Paul Ehrlich, who postulated that if the immune system encounters an autoantigen, damaging outcomes ensue. He described the autoimmune phenomenon as "horror autotoxicus" or the horror of self-toxicity. Today, this basic idea persists even in the 'modern era' utilizing biochemical and molecular-based approaches to immunology. We are taught that recognition of self as foreign by the adaptive immune system is the basis for autoimmunity. Thus, the identity of the autoantigen(s) responsible for these illnesses remains the Holy Grail for scientists committed to uncovering the origins of these diseases.

While many are focused on the identity of this antigen, we have opted for a slightly different approach to this centuries-old problem. We would argue that the identity of the autoantigen is not as important as the cell that sees this antigen. Our approach suggests a failure of the responding immune cell, particularly the T helper cell. In fact, recognition of self is essential for T cell survival and immune homeostasis. The immune system must recognize 'self' in order to protect the host.[1, 2] In lieu of traditional approaches that may involve animal models, our work has focused on the patients and their immune cells to investigate the molecular underpinnings of disease. We have also observed that common therapies to treat autoimmune disease, particularly, rheumatoid arthritis (RA), while efficacious, have ill-defined mechanisms elucidating their function. Therefore, a large portion of our investigation of rheumatoid arthritis examines methotrexate (MTX) responses using *in vitro* models and primary cells from RA patients receiving MTX therapy. MTX, which remains the 'gold standard' for the treatment of RA, has become a tool for us to better understand this disease. The resulting body of work

INTECH
open science | open minds

© 2015 The Author(s). Licensee InTech. This chapter is distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/3.0), which permits unrestricted use, distribution, and eproduction in any medium, provided the original work is properly cited.

reveals novel mechanisms not only for low-dose MTX action but has permitted us to learn a great deal about disease pathogenesis. An important consequence of this MTX-centric approach, has led to us to uncover new understandings for how the human immune system may function, which we will explore in this chapter. In general, many molecular defects are seen in T cells from subjects with RA. MTX alters expression/activity of many of these same targets *in vivo* and in tissue culture models. Studies in tissue culture models have allowed us to unravel some of the phenotypic changes that result from MTX treatment. These are summarized in Table 1.

Gene Target	Status in RA	MTX Target	MTX Function
c-Fos(*FOS*)	UC	+	Increases apoptosis sensitivity
c-Jun (*JUN*)	UC	+	Increases apoptosis sensitivity
CHEK2 DNA-PKcs (*PRKDC*)	UE UE	- +	- Increases lincRNA-p21 transcripts
JNK2 (*MAPK9)*	UE	+	Increases apoptosis sensitivity and p53 protein expression
lincRNA-p21 p21 (*CDKN1A)*	UE UE	+ +	Reduces NF-κB activity Activates cell cycle checkpoints
p53 (*TP53)*	UE	+	Activates cell cycle checkpoints and reduces NF-κB activity
NF-κB activity RanGAP1 (*RANGAP1*)	OE UE	+ -	Reduces active NF-κB -

Table 1. Molecular defects in rheumatoid arthritis and methotrexate targets. UC = unchanged, UE = under expressed, OE = over expressed.

2. Body

2.1. Rheumatoid arthritis

Rheumatoid arthritis is a chronic, inflammatory condition of the small and large joints characterized by inflammation of the synovium, or lining of the joint.[3] While the precise etiology of this disease remains unknown, the growing appreciation for the molecular basis of this disease has provided several clues. Particular emphasis has been placed on the cell types found in the joint spaces of patients with active disease.[4, 5] Lymphocytes are the most

http://dx.doi.org/10.5772/59901

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 infiltrating cell types in the synovium.[6] Of the T lymphocytes found in the RA synovium, it has been reported that the majority are CD4+CD45RO+ memory cells.[7] B cells constitute about 5% of the sublining synovial cells.[7] 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.[7] However, in active RA 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.[7, 8] Many studies have suggested that Type A cells in RA display an activated phenotype and via 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." [7, 9, 10] 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.[7] 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.[7, 9] c-Rel, RelA, and RelB function as the major transactivation subunits. Unless activated, these subunits reside in the cytoplasm along with their inhibitor, IκB.[7] Phosphorylation of IκB causes IκB to be degraded by the proteasome, thus releasing NF-κB dimers to migrate to the nucleus where they localize to promoter regions of target genes.[7] Electromobility shift assays show constitutively high levels of p50 and p65 proteins 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.[9] 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.[11]

In addition to increased levels of NF-κB, both synoviocytes and T cells in RA exhibit defects in expression and function of the guardian protein p53 leading to inability of these cells to undergo apoptosis and to resulting loss of genomic integrity.[8, 12] p53 is a critical regulator of cell cycle progression and reduced p53 levels or inactivating p53 mutations 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 etanercept, which selectively blocks the inflammatory cytokine, TNF-α.[13] These newer biologic therapies have added to the ability

of 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.[14, 15] How, then, can we design therapies to target these observed defects? In the mid-twentieth century, we witnessed the birth of molecular medicine and the era of intelligent drug design. While most of the drugs developed during this period sought to treat cancer, very few of these early medications remain first in class therapies today and have since been replaced by more targeted therapies. Yet, one drug, MTX, first developed more than a half century ago, remains the standard of care for the treatment of RA.

2.2. Overview and history of MTX

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.[16-18] 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).[18-20] 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.[18]

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.[18, 21] Thus, blockade of DHFR was implicated as a therapeutic target of chemotherapeutic doses of aminopterin. Isolation of this enzyme allowed for the creation of more potent inhibitors of DHFR. Specifically, another folate analog, MTX, was identified in a study of leukemia-bearing mice and when compared to aminopterin increased survival in these mice.[18] From these initial data in mice, two reports found that MTX at very high doses cured women diagnosed with choriocarcinoma, a malignant trophoblastic cancer of the placenta.[19] This was the first solid tumor to be cured by a drug in humans and stimulated interest in investigating the effects of MTX in additional forms of cancer.[18, 22] Of particular interest was the reduced side effect profile observed in the MTX-treated cohort. Compared to radiation or alkylating agents that can lead to infertility or additional malignancies, MTX monotherapy did not produce these deleterious effects.[18] Today, MTX is currently used in the treatment of large cell or high grade lymphomas, head and neck cancer, breast cancer, bladder cancer, and osteogenic sarcoma.[18] It is often used in combination with other therapies including 6-mercaptopurine (6MP). Studies have shown that the combination of MTX, 6MP, vincristine, and prednisone improve patient outcomes in the treatment of ALL.[18] In particular, a treatment regiment first prescribing MTX and following with 6MP in sequence improves cure rates.[18]

http://dx.doi.org/10.5772/59901

Given the immunosuppressive potential of aminopterin and MTX 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.[18, 23] 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 (1-2 mg/day).[18] Due to these discomforts, MTX, which closely resembles aminopterin, was substituted.[18] Patients were able to tolerate MTX reasonably well at low doses. The role of aminopterin, and later MTX, 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.[18] It is interesting to note that this early report describing the therapeutic potential of MTX or other folate analogs was largely ignored for a quarter century. It would not be until the late 1980s that MTX is approved for the treatment of rheumatoid arthritis.[24]

Given the therapeutic potential for MTX 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 MTX is actively transported into cells through reduced folate transporter 1 (RFT-1).[25] MTX, 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).[26] These long-lived MTX polyglutamates remain in the liver of patients for a long period as well as in the bone marrow myeloid precursors.[26] Polyglutamation of MTX occurs within 12-24 hours after treatment and polyglutamates constitute the active form of the drug.[26, 27] Thus, MTX is commonly referred to as a pro-drug, a compound that undergoes a biochemical modification to become its active form. 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 MTX therapy is to increase the cellular cytotoxicity profile. Alterations to this pathway in the form of mutated RFT-1 or DHFR can lead to MTX resistance in cancer patients.[19, 26] Interestingly, cancer subjects resistant to MTX often exhibit increased levels of DHFR protein. 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.[26] This is currently an area of active exploration and future studies are required to determine the exact mechanisms.

While MTX is still used in the modern treatment of cancer, it is in the treatment of rheumatoid arthritis that physicians have observed MTX's greatest, long-term effectiveness. Often heralded as the drug that revolutionized the field of rheumatology, low-dose, once-weekly MTX 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

MTX in 1988 for the treatment of rheumatoid arthritis, it was assumed that the mechanism of action by which MTX exerts its anti-inflammatory effects in rheumatoid arthritis would closely resemble the mechanism of action found in the treatment of cancer. However, despite considerable experience with MTX in the treatment of RA, we are still uncovering clues as to the exact mechanism or mechanisms MTX employs to produce its anti-inflammatory effects.

2.3. MTX and adenosine

Given the pro-inflammatory, anti-apoptotic phenotype exhibited by both synoviocytes and T cells in RA, and the ability of MTX to mitigate indices of inflammation it is logical to question if MTX may exert its anti-inflammatory properties through modulation of these pathways. For the past 30 years, the precise mechanisms employed by MTX to exert its anti-inflammatory effects in RA have been the focus of thorough investigation.[18, 25, 28-41] In the treatment of cancer, MTX 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 MTX does not reverse its anti-inflammatory effects in randomized, blinded trials.[16, 18, 40, 42] Thus, other mechanisms have been proposed. A prevailing theory is that MTX exerts its mechanism of action through a number of different mechanisms including release of adenosine that function in parallel to blockade of nucleotide synthesis.[16] 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).[17, 27, 36, 40, 42, 43] Polyamine reduction has been posited as one anti-inflammatory mechanism since polyamines can be converted to lymphotoxins.[42] However, use of 3-deazaadensoine, a transmethylation inhibitor, does not demonstrate a significant clinical benefit in RA patients.[42] Yet, low-doses of MTX also inhibit chemotaxis in monocytes through a process reversed by S-adenosylmethionine supporting the contribution of this pathway in RA.[44] The retention of MTX polyglutamates in cells exceeds its half-life in plasma, suggesting that the MTX 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 MTX therapy.[16, 42] Increased AICAR levels are strong inhibitors of adenosine monophosphate (AMP) and adenosine deaminases, 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.[42, 45] MTX has also been shown to enhance vasodilation leading to increased blood flow through inhibition of adenosine deamination in whole blood in humans.[46] The direct quantification of MTX-mediated adenosine release in humans receiving MTX has been unsuccessful largely because the half life of adenosine in blood and tissue is very brief making these measurements technically challenging.[42, 47] In animal models, however, the anti-inflammatory effects of MTX are mediated by adenosine using the carrageenan-induced air pouch model of inflammation and reversals with A2A adenosine receptor antagonists and supplementation of adenosine deaminase.[42, 48] Thus, one mechanism by which MTX achieves its anti-inflammatory effects is by stimulating increased synthesis and release of adenosine, which in turn, activates adenosine receptors to block various pro-inflammatory paths.

http://dx.doi.org/10.5772/59901

2.4. Novel mechanisms for MTX action

Other studies have also shown that MTX inhibits T cell activation, induces apoptosis, and alters expression of T cell cytokines and adhesion molecules.[28, 32, 49, 50] 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.[31] 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 MTX or additional biochemical consequences of DHFR inhibition by MTX may produce these anti-inflammatory properties observed in subjects with RA receiving low-dose MTX as therapy.

While initially developed as a chemotherapeutic, MTX (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.[16, 18] RA patients treated with MTX experience reduced pain, and improved joint score and function typically within three months of initiation 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.

Our initial studies in RA examined differences in expression patterns 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.[51-53] Our expectation was that we would identify genes that encode proteins typically involved in pro-inflammatory processes. Instead, we found that patients with RA significantly underexpressed a panel of genes that are typically considered prototypical 'cancer genes' in peripheral blood mononuclear cells that encode proteins required for cell cycle arrest, maintenance of genomic integrity, and induction of apoptosis. Many cancers have inactivating mutations in these genes. Specifically, these studies established that defects in expression of *CHEK2*, *TP53*, *CDKN1A*, and *CDKN1B* that encode checkpoint kinase 2, p53, cyclin kinase inhibitor 1A or p21, and cyclin kinase inhibitor 1B or p27, respectively, conferred an inability for RA lymphocytes to undergo apoptosis in response to gamma irradiation.[12] The major obstacle moving forward was how to link these observations to inflammation and RA disease pathogenesis.

Since mechanisms by which low-dose MTX achieve therapeutic benefit in RA are incompletely understood and how the above deficiencies in cell cycle regulation and apoptosis may contribute to RA pathogenesis, we chose to initiate studies to compare RA subjects on MTX to RA subjects not receiving MTX therapy. Initially, we found that RA subjects receiving MTX therapy exhibited increased expression of genes encoding Fos and Jun that form the AP-1 transcription factor. In addition, expression of a number of genes induced by the AP-1

transcription factor is elevated in RA subjects receiving MTX therapy. Further, we were also able to reproduce these findings in tissue culture models via dose-dependent induction of *JUN* and *FOS* expression in T cells treated with sub-micromolar concentrations of MTX.[54]

One signaling pathway that activates the AP-1 transcription factor is via activation of Jun-N-terminal kinase (JNK), a MAP kinase, which phosphorylates Jun resulting in increased transcriptional activity of AP-1. MTX also activates JNK in our tissue culture models and MTX-dependent activation of JNK is responsible for the observed increases in *JUN* and *FOS* transcript levels and increased AP-1 activity. Through JNK activation, MTX increases the sensitivity of T cells to undergo apoptosis by production of reactive oxygen species and alteration of the transcriptional profile in favor of genes whose protein products promote apoptosis including Jun mRNA. Thus the resistance to apoptosis in RA T cells we described previously is reversed by MTX treatment. This process is also mediated by MTX-dependent inhibition of dihydrofolate reductase (DHFR). Besides reduction of folates, DHFR also catalyzes the reduction of dihydrobiopterin (BH_2) to tetrahydrobiopterin (BH_4) and BH_4 is a necessary cofactor of all nitric oxide synthases. MTX also blocks the DHFR catalyzed reduction of BH_2 to BH_4. Loss of BH_4 causes a process called nitric oxide synthase 'uncoupling' that results in production of reactive oxygen species such as hydrogen peroxide by nitric oxide synthases rather than production of nitric oxide. It is this nitric oxide synthase 'uncoupling' that leads to JNK activation and altered sensitivity to apoptosis.

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 also 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. BH_4 depletion is known to cause a shift in cytokine profiles from a pro-inflammatory profile to an anti-inflammatory profile and our work shows that BH_4 depletion also increases sensitivity of lymphocytes to apoptosis, a process that may improve clearance of self-reactive inflammatory lymphocytes by apoptosis.

We further probed the mechanism by which MTX increases sensitivity of cells to apoptosis by asking if MTX restores the cell cycle checkpoint deficiencies we described previously. Since MTX increases activity of JNK in our tissue culture models, we asked if levels of JNK are decreased in subjects with RA not receiving MTX. We found highly significant deficiencies of *MAPK9* (JNK2) expression in rheumatoid arthritis.[55] This represents the major JNK protein expressed by lymphocytes. Analysis of other MAPK family members including most known ERK and p38 isoforms did not reveal any significant differences in healthy controls versus RA cohorts receiving or not receiving MTX. Following our gene expression studies, we analyzed protein expression in RA lymphocytes and found that these subjects exhibit reduced JNK protein expression. Analysis of additional autoimmune diseases also indicated that this *MAPK9* deficiency observed in RA was not unique to RA, but also extends to multiple sclerosis (MS). It is interesting to note that both RA and MS exhibit similar molecular defects in PBMC specifically through reduced checkpoint kinase 2 (*CHEK2*), p53 and ataxia telangiectasia (AT) mutated (ATM) expression.[56] The contributions of JNK to these defects remains to be explored. We found that MTX increased levels of both p53 and the downstream target p21 in

http://dx.doi.org/10.5772/59901

MTX-treated cells via JNK, which was further confirmed *in vivo* by analyzing transcript levels of *TP53* and *CDKN1A* 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.[57] 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 activate NF-κB or alternative pro-inflammatory, pro-survival pathways leading to the 'sterile inflammation' observed in RA pathogenesis.

2.5. The cell cycle checkpoint deficiency-NF-κB activation connection in RA

Our studies outlined above clearly establish that MTX is a strong transcriptional activator, both in tissue culture models as well as in RA patients as part of their therapy. This is achieved in large part via activation of JNK. One of the best-studied proteins induced by MTX is p53, which itself is a strong transcriptional activator and the gene expression program induced by p53 allows p53 to carry out many of its cellular functions such as cell cycle arrest and induction of apoptosis. Further, transcript levels of genes encoding p53 and its transcriptional targets are largely depressed in RA patients. However, whether losses of these gene transcripts and corresponding proteins can contribute to the pro-inflammatory state characteristic of RA or how they might contribute to this pro-inflammatory state is less clear.

The NF-κB transcription factor is probably one of the best-characterized pro-inflammatory transcription factors. Many genes that encode pro-inflammatory cytokines, chemokines, and lymphocyte adhesion molecules possess NF-κB binding sites in their promoters and require activation of NF-κB for their increased expression in response to extracellular inflammatory stimuli. For these reasons, our next series of experiments analyzed the influence of MTX upon transcriptional activity 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 NF-κB activity in the PBMC of RA patients receiving and not receiving MTX. In T lymphocytes, we found that MTX is a strong inhibitor of activation of NF-κB in response to various extracellular stimuli. In T cell tissue culture models, MTX inhibits activation of NF-κB via BH_4 depletion and JNK activation. Further, the inhibition of NF-κB activity in T cells by MTX is dependent upon MTX-mediated induction of p53. In patients with RA, NF-κB activity is chronically elevated in T helper cells and this elevation is reversed by MTX therapy. Taken together, we believe these studies provide a direct link between elevated activity of the pro-inflammatory, pro-cell survival transcription factor, NF-κB in RA and depressed levels of the pro-apoptotic, pro-cell cycle control transcription factor, p53, and show how induction of p53 by MTX results in subsequent loss of NF-κB activity in RA T helper cells.

Synovial fibroblast-like cells also activate NF-κB in response to extracellular stimuli and elevated levels of NF-κB activity have been demonstrated in RA synovial tissues. Therefore, we asked if MTX also inhibits activation of NF-κB in response to extracellular stimuli and if this inhibition is achieved via BH_4 depletion and JNK activation. Low concentrations of MTX effectively inhibit NF-κB activation in synovial fibroblasts in tissue culture. However, MTX

does not act by depleting BH_4 and activating JNK as it does in T cells. In fact, genes characteristically induced by MTX in T cells, e.g. *TP53*, *CDKN1A*, *JUN*, are not induced by MTX in synovial fibroblasts. This appears to be because nitric oxide synthase enzymes are expressed at much lower levels in synovial fibroblasts than in T cells and thus generation of reactive oxygen species via nitric oxide synthase 'uncoupling' is inefficient. Rather, inhibition of NF-κB activation in synoviocytes appears to be mediated by adenosine release and activation of adenosine receptors.[58] This follows earlier work implicating the potential role of adenosine synthesis and activation of adenosine receptors in the anti-inflammatory effects of MTX.[27, 29, 45-47, 59-62] Thus, we conclude that MTX modulates NF-κB through distinct mechanisms and these effects are specific to different cell types (Figure 1).

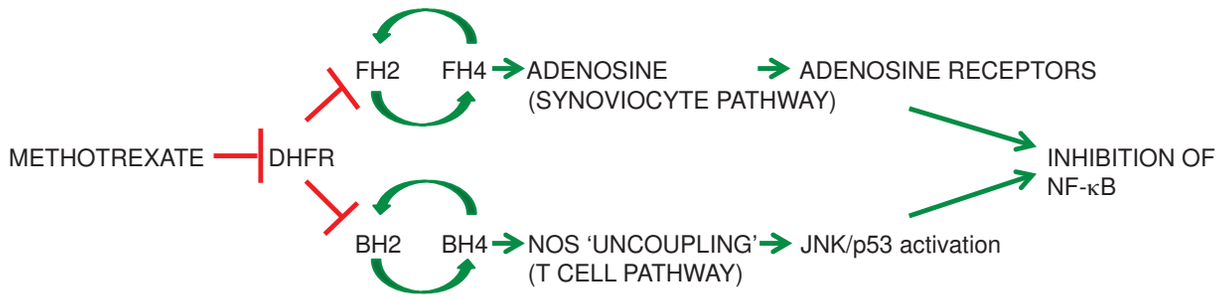


Figure 1. Schematic illustrating alternate pathways of MTX-mediated inhibition of NF-κB activity in T cells and synoviocytes.

We have explored the connection between NF-κB and p53 further, as these transcription factors are two central regulators of the adaptive immune response. NF-κB modulates the response to exogenous stimuli, whereas p53 modulates intrinsic stress responses through initiation of "cell cycle arrest, apoptosis, or senescence, eliminating clones of cells with DNA damage and its resulting mutations".[63] In general terms, NF-κB and p53 are functionally antagonistic. NF-κB is considered a pro-survival, pro-inflammatory transcription factor while p53 is an anti-survival, anti-inflammatory transcription factor. The precise mechanisms explaining the connection between p53 and NF-κB in the context of immune cells remains largely unexplored and is likely to be stimulus-, cell-, and/or disease-specific. The basic understanding in a healthy cell is that DNA damage, hypoxia, or oncogene activation elicit p53 responses that activate cell cycle arrest, senescence or apoptosis, targeting genes that are pro-apoptotic such as *PUMA*, or induce cell cycle arrest proteins such as p21.[63, 64] In the context of DNA damage, ATM is activated which leads to inhibition of MDM-2, an E3 ubiquitin ligase, and subsequent activation of p53.[65-67] While classically thought to be a modulator of cell survival or apoptosis, activation of p53 also creates metabolic consequences via reduced levels of aerobic glycolysis.[63, 68] One example of this regulation is p53-dependent activation of *TIGAR* (*T*P53-*i*nduced *g*lycolysis and *a*poptosis *r*egulator) that decreases fructose-2,6-bisphosphate levels leading to lower rates of cellular glycolysis.[63, 69] Like p53, NF-κB is typically activated via post-translational modifications via degradation of IκB or MDM-2/MDM-4 in response to exogenous signals such as infectious agents, viruses, toll-like receptor agonists, antigen receptors or through inflammatory cytokines, such as TNF-α or interleukin-1β.[63] Thus, NF-κB activation

http://dx.doi.org/10.5772/59901

leads to transcription of mRNAs that encode inflammatory proteins such as: cytokines, IL-6, GM-CSF; cheomokines, IL-8, RANTES, MCP-1; enzymes, COX-2, PLA2; and adhesion molecules VCAM-1 and ICAM-1.(9-11, 70] Interestingly, production of pro-inflammatory cytokines such as IL-1β and TNF-α creates an amplification loop that can lead to constitutive activation of the NF-κB signaling pathway. Therefore, strict regulation of this pathway needs to be employed to avoid persistent NF-κB activity, which could create the basis for chronic inflammatory disease, autoimmunity, and even certain cancers. Metabolically, activation of NF-κB enhances glycolysis and increases glucose transporters (GLUT3) leading to higher amounts of glucose uptake.[63]

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.[63] It has also recently become appreciated that the metabolic fates of RA T cells are reprogrammed. RA T cells are energy deficient as evidenced by reduced glucose consumption, lactate production, and intracellular stores of ATP.[71, 72] Yang et al identified defects in 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3), a critical regulator of glycolysis, as the mediator of the observed defects as constitutive overexpression of PFKFB3 repaired the glycolytic insufficiency. Most interestingly, the study also demonstrated that deficiencies of PFKFB3 reduce ROS levels in cells. Our studies with methotrexate suggest that increased ROS generation is a therapeutic benefit of MTX therapy as apoptotic death of proliferating T cells is essential for T cell homeostasis. Thus, the contribution of metabolic 'rewiring' and the therapeutic potential of targeting these biomarkers represent attractive targets for clinical intervention.

To induce cell cycle arrest or apoptosis, the transcriptional program activated by p53 is mediated, in part, by induction of the long non-coding RNAs (lncRNAs), lincRNA-p21 and PANDA.[73, 74] lncRNAs are relatively newly discovered species of RNA. lncRNAs are transcribed from genes that look like protein-coding genes. Approximately 10,000 lncRNA genes have been discovered in the human genome so they may be as abundant as protein-coding genes.[75] These genes contain exons and introns and lncRNAs are spliced to mature lncRNAs just like mRNAs. The difference between lncRNAs and mRNAs is that lncRNAs are littered with translational stop codons throughout their sequence and thus cannot be translated into proteins and therefore exist as RNA species. As a class, lncRNAs have multiple functions. Major functions are the stimulation or inhibition of transcription of protein-coding genes. These target protein-coding genes are oftentimes, but not always, located in close proximity in the genome to the gene encoding the effector lncRNA. These lncRNAs generally act by recruiting the epigenetic machinery to target gene loci to establish activating or repressive histone marks. lncRNAs also interfere with translation of proteins. Additional mechanisms of action of lncRNAs are to regulate function, stability and activity of proteins. Thus, lncRNAs exhibit a broad spectrum of activities that play key roles in many cellular processes.[76-80]

Further, individual lncRNAs can have multiple modes of action. An example is lincRNA-p21. One function of lncRNA-p21 is to repress transcription of certain genes in response to p53 activation.[73] A second function is to modulate translation of certain mRNAs.[81] A third function is to modulate the stability of the transcription factor HIF-1α, thus regulating its activity.[82] A fourth function is to stimulate transcription of *CDKN1A*, the gene that encodes p21 required for cell cycle arrest.[83] Thus, besides being abundant species of RNAs, individual lncRNAs can possess multiple functions, which increases their cellular phenotypic imprint.

Because both transcript and protein levels of p53 and p21 are depressed in RA and are MTX target genes, we were interested to learn if the lncRNAs, lincRNA-p21 or PANDA, are differentially regulated in RA and/or may be MTX target genes. We have found that lincRNA-p21 transcript levels are depressed in RA T cells and lincRNA-p21 is a MTX target gene in T cells.[84] However, TP53 and lincRNA-p21 levels do not correlate with each other in T cells from subjects with RA or healthy controls suggesting that levels of p53 do not determine levels of lincRNA-p21 in T cells as they do in other cell types. Further, although lincRNA-p21 is strongly induced by MTX in T cells in our tissue culture models and lincRNA-p21 levels are restored to normal in RA patients receiving MTX therapy, induction of lincRNA-p21 does not appear to be dependent upon p53 activation under these conditions in these cell types. In T cells, induction of lincRNA-p21 by MTX is also not mediated by BH_4 depletion, nitric oxide synthase 'uncoupling' and JNK activation or by adenosine release and adenosine receptor activation.[84]

Increased DNA damage is also observed in RA T cells. The two major sentinels of DNA damage responses are the enzymes ATM and DNA-PKcs and these enzymes are also deficient in RA T cells.[57] Thus, these enzyme deficiencies may explain the accumulation of DNA damage observed in RA T cells. In T cells, stimulation with low concentrations of MTX results in activation of DNA-PKcs (phosphorylation) but not activation of ATM. Induction of lincRNA-p21 by MTX requires DNA-PKcs activation. In RA T cells, MTX therapy also restores *PRKDC* (the gene that encodes DNA-PKcs) transcript levels to normal. Mechanistically, we do not understand how MTX activates DNA-PKcs and not ATM in T cells. We also do not understand how activation of DNA-PKcs leads to induction of lincRNA-p21. One obvious mechanism would be via induction of p53 but this appears not to be the case. It may be that p21 contributes to this process but that remains to be investigated. Further studies will be necessary to more fully understand this mechanism.

We also asked if activation of DNA-PKcs and induction of lincRNA-p21 by MTX contributes to MTX-dependent activation of NF-κB in response to extracellular stimuli, such as TNF-α. This is clearly the case. Inhibition of DNA-PKcs, but not ATM, reverses MTX-dependent inhibition of TNF-α mediated NF-κB activation. Further, use of siRNAs to deplete either p53 mRNA or lincRNA-p21 reverses the ability of MTX to inhibit TNF-α mediated NF-κB activation. Thus, we conclude from these studies that multiple pathways are activated in T cells by methotrexate to achieve its anti-inflammatory effects. A graphic summary of the pathways we have discovered as a result of these studies is summarized in Figure 2.

http://dx.doi.org/10.5772/59901

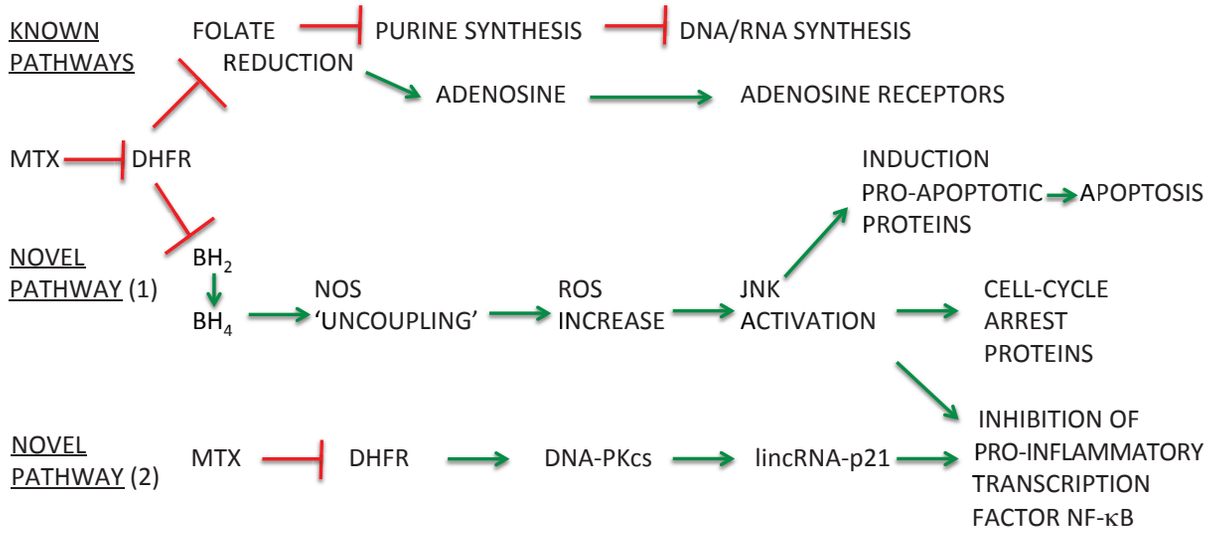


Figure 2. Summary of previously known and new mechanisms of methotrexate action

2.6. Other considerations

It has also been shown that telomeres of CD4+ cells are shortened in subjects with rheumatoid arthritis. On average, the telomeres of lymphocytes and even progenitor cells, such as CD34+ hematopoietic stem cells, are 1.5kb shorter compared to control resulting in accelerated immune system aging. [85, 86] Given that the immune system divides on average once a year and the average telomeric base pair (bp) loss is approximately 50bp, the immune system of RA subjects is approximately 25-30 years older than that of an unaffected individual. This phenotype is present in early disease and in untreated patients. So a question to ask is how are these traits conferred? Genetic studies have informed our knowledge of this disease by revealing the association of human leukocyte antigen (HLA) serotypes with autoimmune disease susceptibility. In the case of rheumatoid arthritis, susceptibility is associated with the HLA-DR4 allele. The relative risk for RA is four times greater in carriers compared to unaffected individuals with current female to male ratios suggesting an approximate 3:1 distribution.[87-89] Specifically, HLA-DRB1*04 remains the most important genetic risk factor for rheumatoid arthritis. If you examine healthy donors, and track the telomeric length of HLA-DRB1*04 +/- individuals as they age, donors with a positive HLA-DRB1*04 haplotypes exhibit premature aging in their CD4+ T cells with average telomeric length approximately 1.0-1.5kb shorter than HLA-DRB1*04 negative individuals.[64-66] It was further observed that this phenomenon of early immune aging was also found in the neutrophils of HLA-DRB1*04 positive individuals with concomitant accumulation of pre-senescent CD4+ T cells in healthy HLA-DRB1*04+ individuals measured by accumulation of CD28 null T cells in the total CD4+ lymphocyte population.[90-92]

In general, telomere loss is a measure of what is termed cellular senescence. Cellular senescence can arise by a number of mechanisms that include DNA damage, deficiencies of DNA damage response and repair pathways, as well as elevated NF-κB activity. It has also been argued that

cellular senescence is a pathogenic mechanism in RA. Further, in experimental models, loss of p21, p27 or p53 can produce cellular senescence as well as pro-inflammatory or autoimmune phenotypes. Similarly, increased NF-κB activation can produce pro-inflammatory or autoimmune phenotypes. These same defects are seen in RA. Thus, one can imagine a continuous pathogenic loop of deficiencies in proteins involved in DNA damage responses and cell cycle control and increased NF-κB activity culminating in RA pathogenesis (Figure 3). MTX-dependent restoration of these deficiencies in DNA damage response and cell cycle control proteins that culminate in inhibition of chronic NF-κB activation reinforces this point. What is unclear is if there are dominant 'drivers' in this pathway or will any of the aforementioned defects that arise produce this pathogenic loop? This model raises the question of whether these defects arise via genetic or environmental mechanisms and understanding this question may improve our understandings of the origins of this disease. There may be other methods to interfere with this pathogenic loop. If developed, these methodologies may aid in the treatment of RA.

3. Conclusions

One hundred years ago, the only drug in the physician armamentarium to manage RA was aspirin.[93] 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.[24] 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 MTX, that physicians saw significant improvement in long-term outcomes, especially when MTX was combined with other therapies. When we initiated our studies to examine the anti-inflammatory properties of MTX, we thought that there would be a single biomarker we could target to achieve the same outcome with less toxicity, as subjects taking MTX 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.[94] 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 envision 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

http://dx.doi.org/10.5772/59901

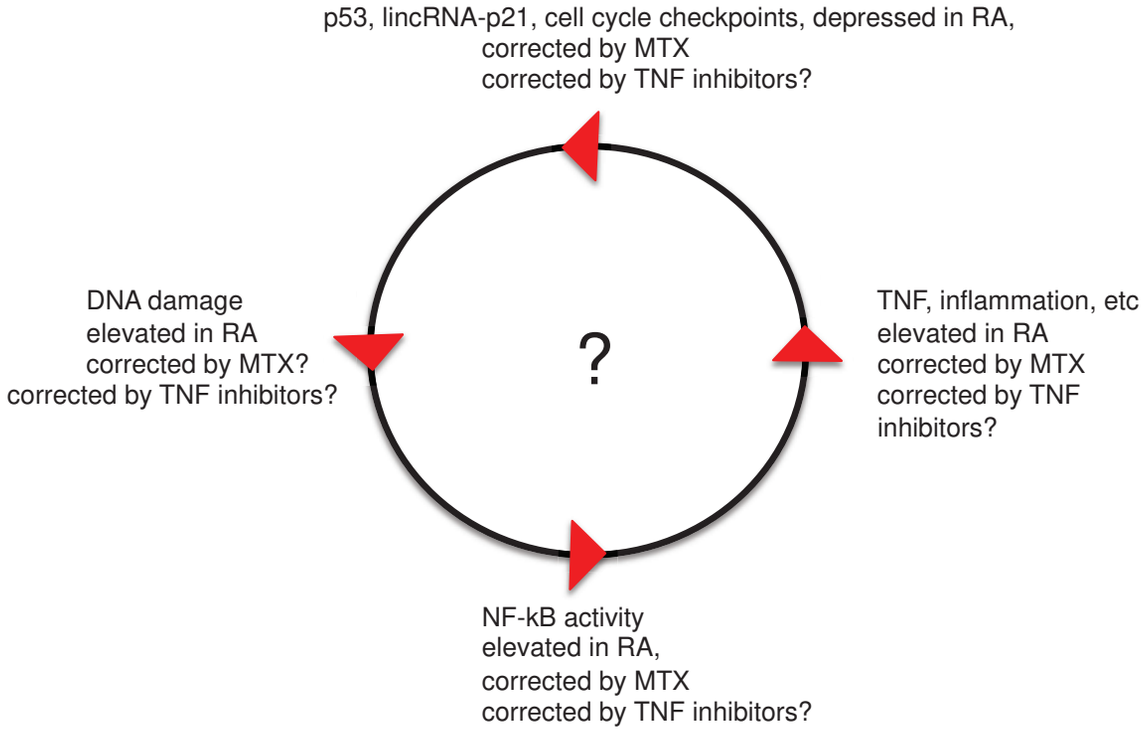


Figure 3. Hypothetical mechanistic loop that connects known molecular defects in RA to pathogenesis.

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 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.[95, 96] 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.[95] We would propose a new model whereby the source of 'danger' is actually internal or intracellular and not external, which we plan to explore.

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.[97-104] Smoking is well established as a significant environmental risk factor for RA. Normally, activation of cell cycle checkpoints and the DNA damage response 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, DNA-PKcs, ATM, and cell cycle checkpoints, JNK2, p53, p21, p27, CHEK2, RANGAP1 are defective, resulting in failure to repair DNA and loss of genomic integrity.[53, 105-108] 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. Future studies are planned to examine the contribution of these defects to the pathogenesis of RA.

4. Nomenclature

Gene symbol-Protein names

ATM – Atm, Ataxia Telangiectasia Mutated

Functions: senses DNA damage and initiates DNA repair pathways and pathways to induce cell cycle arrest or apoptosis, also involved in telomere maintenance

PRKDC – DNA-PKcs, DNA-dependent protein kinase catalytic subunit

Functions: senses DNA damage and initiates similar pathways as Atm, also involved in non-homologous recombination, necessary for successful formation of T and B cell receptors

CHEK2 – Chek2, Chk2, checkpoint kinase 2

Functions: activated by Atm in response to DNA damage, phosphorylates and activates p53

TP53 – p53, tumor protein p53,

Functions: transcription factor, participates in an array of stress responses inducing cell cycle arrest, apoptosis, senescence, DNA repair, or changes in metabolism.

CDKN1A – Cdkn1a, p21, cyclin-dependent kinase inhibitor 1a

Functions: Inhibits activity of cyclin-CDK2 or –CDK4 complexes to inhibit cell cycle progression at G1, p53 target gene

CDKN1B – Cdkn1b, p27, kip1, cyclin-dependent kinase inhibitor 1b

Functions: Inhibits activity of cyclin E-CDK2 or cyclin D-CDK4 complexes to inhibit cell cycle progression at G1, p53 target gene

MAPK9 – Mapk9, Jnk2, c-Jun N-terminal kinase

Functions: Phosphorylates a number of transcription factors including c-Jun to activate the AP-1 transcription factor and regulate stress responses and apoptosis, also pro-inflammatory as many genes that encode cytokines and chemokines have AP-1 binding sites in their promoter

JUN – c-Jun, Jun proto-oncogene

Functions: along with Fos, forms the AP-1 transcription factor

FOS – Fos, c-Fos

Functions: along with Jun, forms the AP-1 transcription factor

RANGAP1 – RanGAP1, Ran GTPase activation protein 1

Functions: GTPase activator for the nuclear Ras-related protein, Ran, and converts it from the active state to the GDP-bound inactive state

RelA – NF-κB p65 subunit, nuclear factor-kappa B

Functions: transcription factor involved in many cellular processes commonly categorized as a pro-survival, pro-inflammatory transcription factor

Telomeresregions of repetitive DNA sequences at the ends of each chromosome, telomere ends shorten after each cell division, cellular senescence occurs when the telomeres become too short and this inhibits further cell division

Acknowledgements

Supported by grants from the National Institutes of Health (R21 AR063846, R01 AI044924, R42 AI53948), the National Center for Advancing Translation Sciences (UL1TR000445), the American College of Rheumatology Within Our Reach grant program (ACR124405) and the National Science Foundation Graduate Research Fellowship Program (DGE0909667).

Author details

Charles F. Spurlock III[1], Nancy J. Olsen[2] and Thomas M. Aune[1,3*]

*Address all correspondence to: tom.aune@vanderbilt.edu

1 Department of Medicine, Vanderbilt University School of Medicine, Nashville, TN, USA

2 Division of Rheumatology, Department of Medicine, Penn State M.S. Hershey Medical Center, Hershey, Pennsylvania, USA

3 Department of Pathology, Microbiology and Immunology, Vanderbilt University School of Medicine, Nashville, Tennessee, USA

References

[1] Stefanova I, Dorfman JR, Tsukamoto M, Germain RN. On the role of self-recognition in T cell responses to foreign antigen. Immunol Rev. 2003;191:97-106.

[2] Stefanova I, Dorfman JR, Germain RN. Self-recognition promotes the foreign antigen sensitivity of naive T lymphocytes. Nature. 2002;420(6914):429-34.

[3] Olsen NJ, Spurlock CF, Aune TM. Methotrexate induces production of IL-1 and IL-6 in the monocytic cell line U937. Arthritis Res Ther. 2014;16(1).

[4] St. Clair EW, Pisetsky DS, Haynes BF. Rheumatoid arthritis. Philadelphia: Lippincott Williams & Wilkins; 2004. xiv, 555 p. p.

[5] Goronzy JJ, Weyand CM. Developments in the scientific understanding of rheumatoid arthritis. Arthritis Res Ther. 2009;11(5):249.

[6] Lundy SK, Sarkar S, Tesmer LA, Fox DA. Cells of the synovium in rheumatoid arthritis. T lymphocytes. Arthritis Res Ther. 2007;9(1):202.

[7] Bartok B, Firestein GS. Fibroblast-like synoviocytes: key effector cells in rheumatoid arthritis. Immunological Reviews. 2010;233(1):233-55.

[8] Firestein GS, Echeverri F, Yeo M, Zvaifler NJ, Green DR. Somatic mutations in the p53 tumor suppressor gene in rheumatoid arthritis synovium. Proc Natl Acad Sci U S A. 1997;94(20):10895-900.

[9] Firestein GS. NF-kappaB: Holy Grail for rheumatoid arthritis? Arthritis and Rheumatism. 2004;50(8):2381-6.

[10] Tak PP, Firestein GS. NF-kappaB: a key role in inflammatory diseases. J Clin Invest. 2001;107(1):7-11.

[11] Simmonds RE, Foxwell BM. Signalling, inflammation and arthritis: NF-kappaB and its relevance to arthritis and inflammation. Rheumatology (Oxford). 2008;47(5): 584-90.

[12] Maas K, Westfall M, Pietenpol J, Olsen NJ, Aune T. Reduced p53 in Peripheral Blood Mononuclear Cells From Patients With Rheumatoid Arthritis Is Associated With Loss of Radiation-Induced Apoptosis. Arthritis and Rheumatism. 2005;52(4):1047-57.

[13] Dichamp I, Bourgeois A, Dirand C, Herbein G, Wendling D. Increased nuclear factor-kappaB activation in peripheral blood monocytes of patients with rheumatoid arthritis is mediated primarily by tumor necrosis factor-alpha. J Rheumatol. 2007;34(10): 1976-83.

[14] Gabriel SE. Why do people with rheumatoid arthritis still die prematurely? Ann Rheum Dis. 2008;67:30-4.

[15] Gabriel SE, Crowson CS, Kremers HM, Doran MF, Turesson C, O'Fallon WM, et al. Survival in rheumatoid arthritis: a population-based analysis of trends over 40 years. Arthritis and Rheumatism. 2003;48(1):54-8.

[16] Cronstein BN. Low-Dose Methotrexate: A Mainstay in the Treatment of Rheumatoid Arthritis. Pharmacological Reviews. 2005;57(2):163-72.

[17] Cronstein BN. Molecular therapeutics. Methotrexate and its mechanism of action. Arthritis and Rheumatism. 1996;39(12):1951-60.

[18] Cronstein BN, Bertino JR. Methotrexate: Springer; 2000.

[19] Chabner BA, Roberts TG, Jr. Timeline: Chemotherapy and the war on cancer. Nat Rev Cancer. 2005;5(1):65-72.

[20] Farber S, Diamond L, Mercer RD, Sylvester RF, Wolff JA. Temporary remissions in acute leukemia in children produced by folic antagonist, 4-aminopteroylglutamic acid (aminopterin).. N Engl J Med. 1948;238:787–93.

[21] Huennekens FM. In search of dihydrofolate reductase. Protein Sci. 1996;5(6):1201-8.

[22] Bertino JR. Karnofsky memorial lecture. Ode to methotrexate. J Clin Oncol. 1993;11(1):5-14.

[23] Gubner R, August S, Ginsberg V. Therapeutic suppression of tissue reactivity: II. Effect of aminopterin in rheumatoid arthritis and psoriasis. Am J Med Sci. 1951;221:176–82.

[24] Pincus T, Yazici Y, Sokka T, Aletaha D, Smolen JS. Methotrexate as the "anchor drug" for the treatment of early rheumatoid arthritis. Clin Exp Rheumatol. 2003;21(5 Suppl 31):S179-85.

[25] Jolivet J, Cowan KH, Curt GA, Clendeninn NJ, Chabner BA. The pharmacology and clinical use of methotrexate. N Engl J Med. 1983;309:1094-104.

[26] Kremer JM. Toward a better understanding of methotrexate. Arthritis and Rheumatism. 2004;50(5):1370-82.

[27] Chan ES, Cronstein BN. Molecular action of methotrexate in inflammatory diseases. Arthritis research. 2002;4(4):266-73.

[28] Paillot R, Genestier L, Fournel S, Ferraro C, Miossec P, Revillard JP. Activation-dependent lymphocyte apoptosis induced by methotrexate. Transplant Proc. 1998;30(5): 2348-50.

[29] Montesinos MC, Desai A, Delano D, Chen JF, Fink JS, Jacobson MA, et al. Adenosine A2A or A3 receptors are required for inhibition of inflammation by methotrexate and its analog MX-68. Arthritis Rheum 2003;48:240-7.

[30] Johnston A, Gudjonsson JE, Sigmundsdotti H, Ludviksson BJ, Valdimarrsson H. The anti-inflammatory action of methotrexate is not mediated by lymphocyte apoptosis, but by the suppression of activation and adhesion molecules. Clinical Immunology. 2005(114):154-63.

[31] Phillips DC, Woollard KJ, Griffiths HR. The anti-inflammatory actions of methotrexate are critically dependent upon the production of reactive oxygen species. Br J Pharmacol. 2003;138:501-11.

[32] Constantin A, Loubet-Lescoulié P, Lambert N, Yassine-Diab B, Abbal M M, B., de Préval C, et al. Antiinflammatory and immunoregulatory action of methotrexate in the treatment of rheumatoid arthritis: evidence of increased interleukin-4 and inter-

leukin-10 gene expression demonstrated in vitro by competitive reverse transcriptase-polymerase chain reaction. Arthritis Rheum 1998;41:48-57.

[33] Olsen NJ, Murray LM. Antiproliferative effects of methotrexate on peripheral blood mononuclear cells. Arthritis Rheum. 1989;32:378-85.

[34] Williams HJ, Willkens RF, Samuelson COJ, Alarcón GS, Guttadauria M, Yarboro C, et al. Comparison of low-dose oral pulse methotrexate and placebo in the treatment of rheumatoid arthritis. A controlled clinical trial. Arthritis Rheum. 1985;28:721-30.

[35] Braun J, Kastner P, Flaxenberg P, Wahrisch J, Hanke P, Demary W, et al. Comparison of the clinical efficacy and safety of subcutaneous versus oral administration of methotrexate in patients with active rheumatoid arthritis: results of a six-month, multicenter, randomized, double-blind, controlled, phase IV trial. Arthritis Rheum. 2008;58:73-81.

[36] Cronstein BN. The mechanism of action of methotrexate. Rheum Dis Clin North Am. 1997;23(4):739-55.

[37] Dervieux T, Furst D, Lein DO, Capps R, Smith K, Caldwell J, et al. Pharmacogenetic and metabolite measurements are associated with clinical status in patients with rheumatoid arthritis treated with methotrexate: results of a multicentred cross sectional observational study. Ann Rheum Dis 2005;64:1180-5.

[38] Dalrymple JM, Stamp LK, O'Donnell JL, Chapman PT, Zhang M, Barclay ML. Pharmacokinetics of oral methotrexate in patients with rheumatoid arthritis. Arthritis Rheum 2008;58:3299-308.

[39] van Dieren JM, Kuipers EJ, Samsom JN, Nieuwenhuis EE, van der Woude CJ. Revisiting the immunomodulators tacrolimus, methotrexate, and mycophenolate mofetil: their mechanisms of action and role in the treatment of IBD. Inflamm Bowel Dis. 2006;12:311-27.

[40] Tian H, Cronstein BN. Understanding the mechanisms of action of methotrexate: implications for the treatment of rheumatoid arthritis. Bull NYU Hosp Jt Dis. 2007;65:168-73.

[41] Braun J, Rau R. An update on methotrexate. Curr Opin Rheumatol. 2009;21:216-23.

[42] Chan SLC, Cronstein BN. Methotrexate-how does it really work? Nature Reviews/ Rheumatology. 2010;6:175-8.

[43] Cronstein BN. Molecular mechanism of methotrexate action in inflammation. Inflammation. 1992;16(5):411-23.

[44] Nesher G, Moore TL, Dorner RW. In vitro effects of methotrexate on peripheral blood monocytes: modulation by folinic acid and S-adenosylmethionine. Ann Rheum Dis. 1991;50(9):637-41.

[45] Morabito L, Montesinos MC, Schreibman DM, Balter L, Thompson LF, Resta R, et al. Methotrexate and sulfasalazine promote adenosine release by a mechanism that re-

quires ecto-5'-nucleotidase-mediated conversion of adenine nucleotides. J Clin Invest. 1998;101(2):295-300.

[46] Riksen NP, Barrera P, van den Broek PH, van Riel PL, Smits P, Rongen GA. Methotrexate modulates the kinetics of adenosine in humans in vivo. Ann Rheum Dis. 2006;65(4):465-70.

[47] Dolezalová P, Krijt J, Chládek J, Nemcová D, Hoza J. Adenosine and methotrexate polyglutamate concentrations in patients with juvenile arthritis. Rheumatology (Oxford). 2005;44:74-9.

[48] Cronstein BN, Naime D, Ostad E. The antiinflammatory mechanism of methotrexate. Increased adenosine release at inflamed sites diminishes leukocyte accumulation in an in vivo model of inflammation. J Clin Invest. 1993;92(6):2675-82.

[49] Wessels JAM, Huizinga TWJ, Guchelaar H-J. Recent insights in the pharmacological actions of methotrexate in the treatment of rheumatoid arthritis. Rheumatology (Oxford). 2008;47:249-55.

[50] Genestier L, Paillot R, Fournel S, Ferraro C, Miossec P, Revillard JP. Immunosuppressive properties of methotrexate: apoptosis and clonal deletion of activated peripheral T cells. J Clin Invest 1998;102:322-8.

[51] Maas K, Chan S, Parker J, Slater A, Moore J, Olsen NJ, et al. Cutting edge: molecular portrait of human autoimmune disease. J Immunol. 2002;169:5-9.

[52] Olsen N, Sokka T, Seehorn CL, Kraft B, Maas K, Moore J, et al. A gene expression signature for recent onset rheumatoid arthritis in peripheral blood mononuclear cells. Ann Rheum Dis. 2004;63:1387-92.

[53] Liu Z, Maas K, Aune TM. Identification of gene expression signatures in autoimmune disease without the influence of familial resemblance. Hum Mol Genet. 2006;15(3):501-9.

[54] Spurlock CF, 3rd, Aune ZT, Tossberg JT, Collins PL, Aune JP, Huston JW, 3rd, et al. Increased sensitivity to apoptosis induced by methotrexate is mediated by JNK. Arthritis and Rheumatism. 2011;63(9):2606-16.

[55] Spurlock CF, 3rd, Tossberg JT, Fuchs HA, Olsen NJ, Aune TM. Methotrexate increases expression of cell cycle checkpoint genes via JNK activation. Arthritis and Rheumatism. 2012;64(6):1780-9.

[56] Deng X, Ljunggren-Rose A, Maas K, Sriram S. Defective ATM-p53-mediated apoptotic pathway in multiple sclerosis. Annals of neurology. 2005;58(4):577-84.

[57] Shao L, Fukii H, Ines C, Oishi H, Goronzy JJ, Weyand CM. Deficiency of the DNA repair enzyme ATM in rheumatoid arthritis. J Exp Med. 2009;206(6):1435-49.

[58] Spurlock CF, 3rd, Gass HMt, Bryant CJ, Wells BC, Olsen NJ, Aune TM. Methotrexate-mediated inhibition of nuclear factor kappaB activation by distinct pathways in T cells and fibroblast-like synoviocytes. Rheumatology. 2015; 54:178-187

[59] Cristalli G, Lambertucci C, Marucci G, Volpini R, Dal Ben D. A2A adenosine receptor and its modulators: overview on a druggable GPCR and on structure-activity relationship analysis and binding requirements of agonists and antagonists. Current pharmaceutical design. 2008;14:1525–52.

[60] Jacobson KA, Gao ZG. Adenosine receptors as therapeutic targets. Nature reviews Drug discovery. 2006;5:247–64.

[61] Montesinos MC, Yap JS, Desai A, Posadas I, McCrary CT, Cronstein BN. Reversal of the antiinflammatory effects of methotrexate by the nonselective adenosine receptor antagonists theophylline and caffeine: evidence that the antiinflammatory effects of methotrexate are mediated via multiple adenosine receptors in rat adjuvant arthritis. Arthritis and Rheumatism. 2000;43(3):656-63.

[62] Majumdar S, Aggarwal BB. Methotrexate suppresses NF-kappaB activation through inhibition of IkappaBalpha phosphorylation and degradation. J Immunol. 2001;167:2911-20.

[63] Ak P, Levine AJ. p53 and NF-κB: different strategies for responding to stress lead to a functional antagonism. FASEB. 2010;24:3643-52.

[64] Zhang XP, Liu F, Wang W. Two-phase dynamics of p53 in the DNA damage response. Proc Natl Acad Sci U S A. 2011;108(22):8990-5.

[65] Lee J-H, Paull TT. ATM Activation by DNA Double-Strand Breaks Through the Mre11-Rad50-Nbs1 Complex. Science. 2005;308(5721):551-4.

[66] Liu S, Opiyo SO, Manthey K, Glanzer JG, Ashley AK, Amerin C, et al. Distinct roles for DNA-PK, ATM and ATR in RPA phosphorylation and checkpoint activation in response to replication stress. Nucleic Acids Res. 2012;40(21):10780-94.

[67] Maya R, Balass M, Kim ST, Shkedy D, Leal JF, Shifman O, et al. ATM-dependent phosphorylation of Mdm2 on serine 395: role in p53 activation by DNA damage. Genes Dev. 2001;15(9):1067-77.

[68] Matoba S, Kang JG, Patino WD, Wragg A, Boehm M, Gavrilova O, et al. p53 regulates mitochondrial respiration. Science. 2006;312(5780):1650-3.

[69] Bensaad K, Tsuruta A, Selak MA, Vidal MN, Nakano K, Bartrons R, et al. TIGAR, a p53-inducible regulator of glycolysis and apoptosis. Cell. 2006;126(1):107-20.

[70] McInnes IB, Schett G. Cytokines in the pathogenesis of rheumatoid arthritis. Nature Reviews Immunology. 2007;7(6):429-42.

[71] Yang Z, Goronzy JJ, Weyand CM. The glycolytic enzyme PFKFB3/phosphofructokinase regulates autophagy. Autophagy. 2014;10(2):382-3.

[72] Yang Z, Fujii H, Mohan SV, Goronzy JJ, Weyand CM. Phosphofructokinase deficiency impairs ATP generation, autophagy, and redox balance in rheumatoid arthritis T cells. J Exp Med. 2013;210(10):2119-34.

[73] Huarte M, Guttman M, Feldser D, Garber M, Koziol MJ, Kenzelmann-Broz D, et al. A large intergenic noncoding RNA induced by p53 mediates global gene repression in the p53 response. Cell. 2010;142(3):409-19.

[74] Hung T, Wang Y, Lin MF, Koegel AK, Kotake Y, Grant GD, et al. Extensive and coordinated transcription of noncoding RNAs within cell-cycle promoters. Nat Genet. 2011;43(7):621-9.

[75] Rinn JL, Chang HY. Genome regulation by long noncoding RNAs. Annu Rev Biochem. 2012;81:145-66.

[76] Wang KC, Yang YW, Liu B, Sanyal A, Corces-Zimmerman R, Chen Y, et al. A long noncoding RNA maintains active chromatin to coordinate homeotic gene expression. Nature. 2011.

[77] Gupta RA, Shah N, Wang KC, Kim J, Horlings HM, Wong DJ, et al. Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis. Nature. 2010;464(7291):1071-6.

[78] Tsai MC, Manor O, Wan Y, Mosammaparast N, Wang JK, Lan F, et al. Long noncoding RNA as modular scaffold of histone modification complexes. Science. 2010;329(5992):689-93.

[79] Guttman M, Amit I, Garber M, French C, Lin MF, Feldser D, et al. Chromatin signature reveals over a thousand highly conserved large non-coding RNAs in mammals. Nature. 2009;458(7235):223-7.

[80] Khalil AM, Guttman M, Huarte M, Garber M, Raj A, Rivea Morales D, et al. Many human large intergenic noncoding RNAs associate with chromatin-modifying complexes and affect gene expression. Proc Natl Acad Sci U S A. 2009;106(28):11667-72.

[81] Yoon JH, Abdelmohsen K, Srikantan S, Yang X, Martindale JL, De S, et al. LincRNA-p21 suppresses target mRNA translation. Molecular Cell. 2012;47(4):648-55.

[82] Yang F, Zhang H, Mei Y, Wu M. Reciprocal regulation of HIF-1alpha and lincRNA-p21 modulates the Warburg effect. Molecular Cell. 2014;53(1):88-100.

[83] Dimitrova N, Zamudio JR, Jong RM, Soukup D, Resnick R, Sarma K, et al. LincRNA-p21 activates p21 in cis to promote Polycomb target gene expression and to enforce the G1/S checkpoint. Mol Cell. 2014;54(5):777-90.

[84] Spurlock CF, 3rd, Tossberg JT, Matlock BK, Olsen NJ, Aune TM. Methotrexate Inhibits NF-kappaB Activity Via Long Intergenic (Noncoding) RNA-p21 Induction. Arthritis & Rheumatology. 2014;66(11):2947-57.

[85] Koetz K, Bryl E, Spickschen K, O'Fallon WM, Goronzy JJ, Weyand CM. T cell homeostasis in patients with rheumatoid arthritis. Proc Natl Acad Sci U S A. 2000;97(16): 9203-8.

[86] Colmegna I, Diaz-Borjon A, Fujii H, Schaefer L, Goronzy JJ, Weyand CM. Defective proliferative capacity and accelerated telomeric loss of hematopoietic progenitor cells in rheumatoid arthritis. Arthritis Rheum. 2008;58(4):990-1000.

[87] Ritchie MD, Denny JC, Crawford DC, Ramirez AH, Weiner JB, Pulley JM, et al. Robust replication of genotype-phenotype associations across multiple diseases in an electronic medical record. Am J Hum Genet. 2010;86(4):560-72.

[88] Weyand CM, Goronzy JJ. Association of MHC and rheumatoid arthritis. HLA polymorphisms in phenotypic variants of rheumatoid arthritis. Arthritis research. 2000;2(3):212-6.

[89] Weyand CM, Goronzy JJ. HLA polymorphisms and T cells in rheumatoid arthritis. International reviews of immunology. 1999;18(1-2):37-59.

[90] Hohensinner PJ, Goronzy JJ, Weyand CM. Targets of immune regeneration in rheumatoid arthritis. Mayo Clin Proc. 2014;89(4):563-75.

[91] Weyand CM, Yang Z, Goronzy JJ. T-cell aging in rheumatoid arthritis. Curr Opin Rheumatol. 2014;26(1):93-100.

[92] Schonland SO, Lopez C, Widmann T, Zimmer J, Bryl E, Goronzy JJ, et al. Premature telomeric loss in rheumatoid arthritis is genetically determined and involves both myeloid and lymphoid cell lineages. Proc Natl Acad Sci U S A. 2003;100(23):13471-6.

[93] Ragan C. Rheumatoid arthritis; the natural history of the disease and its management. Bull N Y Acad Med. 1951;27(2):63-74.

[94] Swinney DC, Anthony J. How were new medicines discovered? Nat Rev Drug Discov. 2011;10(7):507-19.

[95] Matzinger P. Friendly and dangerous signals: is the tissue in control? Nat Immunol. 2007;8(1):11-3.

[96] Gallucci S, Matzinger P. Danger signals: SOS to the immune system. Curr Opin Immunol. 2001;13(1):114-9.

[97] Kaarniranta K, Salminen A. Control of p53 and NF-KB signaling by WIP1 and MIF: Role in cellular senescence and organismal aging. Cellular Signaling. 2010;23:747-52.

[98] Perry JJP, Tainer JA. All Stressed Out Without ATM Kinase. Sci Signal. 2011;4(167).

[99] Hadian K, Krappmann D. Signals from the Nucleus: Activation of NF-kappa B by Cytosolic ATM in the DNA Damage Response. Sci Signal. 2011;4(156).

[100] Bredemeyer AL, Helmink BA, Innes CL, Calderon B, McGinnis LM, Mahowald GK, et al. DNA double-strand breaks activate a multi-functional genetic program in developing lymphocytes. Nature. 2008;456(7223):819-23.

[101] Matsuoka S, Ballif BA, Smogorzewska A, McDonald ER, Hurov KE, Luo J, et al. ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. Science. 2007;316(5828):1160-6.

[102] Wu ZH, Shi Y, Tibbetts RS, Miyamoto S. Molecular linkage between the kinase ATM and NF-kappaB signaling in response to genotoxic stimuli. Science. 2006;311(5764): 1141-6.

[103] Parker A, Izmailova ES, Narang J, Badola S, Le T, Roubenoff R, et al. Peripheral blood expression of nuclear factor-kappab-regulated genes is associated with rheumatoid arthritis disease activity and responds differentially to anti-tumor necrosis factor-alpha versus methotrexate. J Rheumatol. 2007;34:1817-22.

[104] Oleinik NV, Krupenko NI, Krupenko SA. Cooperation between JNK1 and JNK2 in activation of p53 apoptotic pathway. Oncogene. 2007;26:7222-30.

[105] Gutierrez GJ, Tsuji T, Cross JV, Davis RJ, Templeton DJ, Jiang W, et al. JNK-mediated Phosphorylation of Cdc25C Regulates Cell Cycle Entry and G(2)/M DNA Damage Checkpoint. Journal of Biological Chemistry. 2010;285(19):14217-28.

[106] Quimby BB, Dasso M. The small GTPase Ran: interpreting the signs. Curr Opin Cell Biol. 2003;15(3):338-44.

[107] Weyand CM, Fujii H, Shao L, Goronzy JJ. Rejuvenating the immune system in rheumatoid arthritis. Nat Rev Rheumatol. 2009;5(10):583-8.

[108] Fujii H, Shao L, Colmegna I, Goronzy JJ, Weyand CM. Telomerase insufficiency in rheumatoid arthritis. Proc Natl Acad Sci U S A. 2009;106(11):4360-5.

IntechOpen

IntechOpen

IntechOpen