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Methotrexate Suppresses NF-κB Activation Through Inhibition of IκBα Phosphorylation and Degradation

Sekhar Majumdar and Bharat B. Aggarwal

Methotrexate (MTX), a folate antagonist, is a commonly used anti-inflammatory, antiproliferative, and immunosuppressive drug whose mode of action is not fully established. Due to the central role of NF-κB in these responses, we postulated that MTX must mediate its effects through suppression of NF-κB activation. We investigated the effects of MTX on NF-κB activation induced by TNF in Jurkat cells. The treatment of these cells with MTX suppressed TNF-induced NF-κB activation with optimum effects occurring at 10 μM MTX for 60 min. These effects were not restricted to Jurkat cells because other cell types were also inhibited. Besides TNF, MTX also suppressed the NF-κB activation induced by various other inflammatory stimuli. The suppression of TNF-induced NF-κB activation by MTX correlated with inhibition of IκBα degradation, suppression of IκBα phosphorylation, abrogation of IκBα kinase activation, and inhibition of NF-κB-dependent reporter gene expression. Because ecto 5’ nucleotidase inhibitor (α,β-methylene adenosine-5’-diphosphate) blocked the effect of MTX, adenosine mimicked the effect of MTX, and adenosine A2b receptor antagonist (3,7-dimethyl-1-propargylxanthine) reversed the inhibitory effect of MTX, we suggest that MTX suppresses NF-κB activation by releasing adenosine. A partial reversal of MTX-induced NF-κB suppression by thymidine and folic acid indicates the role of the thymidylate synthase pathway also. Overall, our results clearly demonstrate that MTX suppresses NF-κB activation through the release of adenosine, which may contribute to the role of MTX in anti-inflammatory, immunomodulatory, and antiproliferative effects.


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Abbreviations used in this paper: MTX, methotrexate; RA, rheumatoid arthritis; DHFR, dihydrofolate reductase; AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; IKK, IκBα kinase; SEAP, secretory alkaline phosphatase; APCCP, α,β-methylene adenosine-5’-diphosphate; DMPX, 3,7-dimethyl-1-propargylxanthine.
Materials and Methods

Materials

Bacteria-derived human rTNF with a sp. act. of $5 \times 10^7$ U/mg was kindly provided by Genentech (South San Francisco, CA). Penicillin, streptomycin, RPMI 1640 medium, and FBS were obtained from Life Technologies (Grand Island, NY). Tris, glycin, NaCl, SDS, PMA, BSA, and MTX were obtained from Sigma-Aldrich (St. Louis, MO). The polyclonal Abs used were as follows: anti-p65, against the epitope corresponding to amino acids mapping within the amino-terminal domain of human NF-κB p65; anti-p50, against a 15-aa peptide mapping at the nuclear localization region of NF-κB p50; anti-IκBα, against amino acids 297–317 mapping at the carboxyl terminus of IκBα/MAD-3; anti-c-Rel and anti-cyclin D1 against amino acids 1–295, which represents full-length cyclin D1 of human origin. All these Abs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-IκBα (Ser15) Ab was purchased from New England Biolabs (Beverly, MA). Anti-IκB kinase (IKK) or anti-IKKα Abs were kindly provided by Imgenex (San Diego, CA).

Cell lines

The cell lines T-Jurkat (T cells), HeLa (human epithelial cells), and U937 (human histiocytic lymphoma) were obtained from American Type Culture Collection (Manassas, VA). HeLa cells were maintained in MEM, and the other cell lines were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. For most studies, Jurkat cells were used because these cells express both types of TNFR, and TNF-induced responses in this cell type have been well characterized in our laboratory. Normal human diploid fibroblasts were obtained from Dr. O. Periera-Smith (Baylor College of Medicine, Houston, TX), and human vascular endothelial cells were obtained from Dr. C. W. Smith (Baylor College of Medicine).

NF-κB activation assays

To determine NF-κB activation, EMSAs were conducted essentially as described (27). Briefly, nuclear extracts prepared from TNF-treated cells (2 × 10^6/ml) were incubated with 32P-end-labeled 45-mer dsNF-κB oligonucleotide (4 μg protein with 16 fmol DNA) from the HIV long terminal repeat, 5'-TTGTATCAAGGGACCTTTCCGTGGGACTTTCCAGGGAG GCGTGG-3' (underlining indicates NF-κB binding sites) for 15 min at 37°C, and the DNA-protein complex formed was separated from free oligonucleotide on 6.6% native polyacrylamide gels. A double-stranded mutated oligonucleotide, 5'-TTGTATCAACTCAGTTCCGTGGGACTTTCCAGGGAG GCGTGG-3', was used to examine the specificity of binding of NF-κB to the DNA. The specificity of binding was also examined by competition with the unlabeled oligonucleotide. For supershift assays, nuclear extracts prepared from TNF-treated cells were incubated with the Abs against either p50 or p65 of NF-κB for 30 min at room temperature before the complex was analyzed by EMSA. Abs against c-Rel B and cyclin D1 and preimmune serum were included as negative controls. The dried gels were visualized, and radioactive bands were quantitated by a PhosphorImage (Molecular Dynamics, Sunnyvale, CA) using ImageQuant software.

Degradation of IκBα

To determine the levels of IκBα, postnuclear (cytoplasmic) extracts were prepared (28) from TNF-treated cells and resolved on 10% SDS-polyacrylamide gels. After electrophoresis, the proteins were electrotransferred to nitrocellulose filters, probed with rabbit polyclonal Abs against IκBα, and detected by ECL (Amersham, Arlington Heights, IL). The bands obtained were quantitated using Personal Densitometer Scan v1.30 using Imagequant software version 3.3 (Molecular Dynamics).

IκBα phosphorylation

To determine the effect of MTX on IκBα phosphorylation, cytoplasmic extracts were prepared from cells (2 × 10^6/cells/ml) treated with 10 μM MTX for 2 h and then treated with 0.1 nM TNF for different times. The extracts were then resolved on 10% SDS-PAGE and analyzed by Western blot using Abs against either IκBα or phosphorylated IκBα. After electrophoresis, the proteins were detected by chemiluminescence (Amersham).

MTX BLOCKS TNF-MEDIATED NF-κB ACTIVATION

The IKK assay was performed by a method described previously (29). Briefly, IKK complex from cytoplasm was precipitated with Ab to IKKα (Imgenex, San Diego, CA), followed by treatment with 20 μl protein A/G-Sepharose (Pierce Endogen, Rockford, IL). After 2 h, the beads were washed with lysis buffer and then assayed in kinase assay mixture containing 50 mM HEPES (pH 7.4), 20 mM MgCl₂, 2 mM DTT, 20 μCi

FIGURE 1. Effect of MTX on the TNF-dependent NF-κB activation. A, Jurkat cells (2 × 10⁶/ml) were preincubated at 37°C for 2 h with different concentrations (0–20 μM) of MTX followed by 30 min of incubation with 0.1 nM TNF. After these treatments, nuclear extracts were prepared and then assayed for NF-κB as described in Materials and Methods. B, Cells were preincubated at 37°C with 10 μM MTX for the indicated times and then tested for NF-κB activation after treatment either with or without 0.1 nM TNF at 37°C for 30 min. After these treatments, nuclear extracts were prepared and then assayed for NF-κB activation.
[α-32P]ATP, 10 μM unlabeled ATP, and 2 μg substrate GST-κBα (1–54). After incubation at 30°C for 30 min, reaction was terminated by boiling with 5 μl 5% SDS sample buffer for 5 min. Finally, the protein was resolved on 10% polyacrylamide gel under reducing conditions, the gel was dried, and the radioactive bands were visualized by PhosphorImager. To determine the total amounts of IKKα and IKKβ in each sample, 60 μg of the cytoplasmic protein was resolved on 7.5% acrylamide gels and then electrophoresed to a nitrocellulose membrane; the membrane was blocked with 5% nonfat milk protein for 1 h and then incubated with either anti-IKKα or anti-IKKβ (1/500 dilution) for 1 h. The membrane was then washed and treated with HRP-conjugated secondary anti-mouse IgG Ab and finally detected by chemiluminescence (Amersham).

**NF-κB-dependent reporter gene transcription**

The effect of MTX on TNF-induced NF-κB-dependent reporter gene transcription was measured as previously described (30). Briefly, HeLa cells (0.1 × 10^6 cells/well) were plated in six-well plates, pretreated with 10 μM MTX for 2 h, and then transiently transfected by the calcium phosphate method with 1 ml containing 0.5 μg NF-κB promoter DNA linked to the heat-stable secretory alkaline phosphatase (SEAP) gene. The total final amount of DNA was maintained at 3 μg by the addition of the control plasmid pCMVFLAG1 DNA. To examine TNF-induced reporter gene expression, we transfected cells with the SEAP expression plasmid for 10 h before treating them with TNF (1 nM). Twenty-four hours later, the SEAP activity was determined according to the protocol essentially as described by the manufacturer (CLONTECH Laboratories, Palo Alto, CA) using 96-well fluorescent plate reader (Fluoroscan II; Labsystems, Chicago, IL) with excitation set at 360 nm and emission at 460 nm.

**AP-1 activation assay**

To determine the TNF-induced AP-1 activation, 6 μg nuclear extract prepared as described above was incubated with 16 fmol 32P-end-labeled AP-1 consensus oligonucleotide, 5’-CGCTTGA TGAGTCGACCGGAA-3’ (Promega, Madison, WI; bold indicates AP-1 binding site) for 30 min, and the DNA-protein complex formed was separated from free oligonucleotide on 6% native polyacrylamide gels. The specificity of binding was examined by competition with unlabeled oligonucleotide. Visualization and quantitation of radioactive bands were done as described above.

**Results**

In this report, we examined the effect of MTX, a highly watersoluble compound, on NF-κB activation induced by TNF and various other inflammatory stimuli. The concentration of MTX and the duration of exposure used had no effect on cell viability or on the expression of TNFRs (data not shown).

**MTX inhibits TNF-dependent NF-κB activation**

Jurkat cells were preincubated for 2 h with different concentrations of MTX and then treated with TNF (0.1 nM) for 30 min at 37°C, and then nuclear extracts were prepared and assayed for NF-κB activation by EMSA. As shown in Fig. 1A, MTX inhibited TNF-mediated NF-κB activation in a dose-dependent manner, with maximum inhibition occurring at 10 μM. MTX by itself did not activate NF-κB. We next tested the length of incubation required for MTX to block TNF-induced NF-κB activation. The cells were incubated with MTX for 120, 60, 30, and 15 min before the addition of TNF, at the same time as the addition of TNF, or 5, 15, and 30 min after the addition of TNF. The cells were treated with TNF for 30 min. Only when the cells were pretreated for 60 or 120 min with MTX (10 μM) was maximum inhibition of NF-κB activation observed (Fig. 1B). Cotreatment or post-treatment with MTX did not inhibit NF-κB activation significantly (Fig. 1B).

**FIGURE 2.** A, Supershift and specificity of NF-κB activation. Nuclear extracts were prepared from untreated or TNF (0.1 nM)-treated Jurkat cells (2 × 10^6/ml), incubated for 15 min with different Abs and unlabeled NF-κB, and then assayed for NF-κB, as described in Materials and Methods. B, In vitro effect of MTX on DNA binding of NF-κB protein. Nuclear extracts were prepared from 0.1 nM TNF-treated Jurkat cells; 4 μg/sample nuclear extract protein was treated with indicated concentrations of MTX for 2 h at room temperature and then assayed for DNA binding by EMSA. C, Effect of MTX on TNF-induced AP-1 activation. Jurkat cells (2 × 10^6/ml) were pretreated with different concentrations of MTX (5, 10, and 20 μM) for 2 h followed by 30 min of incubation with 0.1 nM TNF. Nuclear extracts were prepared and examined for AP-1 activation as described in Materials and Methods.
Activated NF-κB that is inhibited by MTX consists of p50 and p65 subunits

Because NF-κB is a family of proteins, various combinations of Rel/NF-κB protein can constitute an active NF-κB heterodimer that binds to a specific sequence in DNA (26). To show that the retarded band visualized by EMSA in TNF-treated cells was indeed NF-κB, we incubated nuclear extracts from TNF-activated cells with Ab to either the p50 (NF-κB1) or the p65 (RelA) subunit of NF-κB. Both shifted the band to a higher molecular mass (Fig. 2A), thus suggesting that the TNF-activated complex consisted of p50 and p65 subunits. Neither preimmune serum nor such irrelevant Abs as anti-cyclin D1 had any effect. Excess unlabeled NF-κB (100-fold) caused complete disappearance of the band, but mutant oligonucleotide did not, indicating the specificity of NF-κB.

MTX does not interfere with the DNA-binding ability of NF-κB proteins

It has been shown that N-tosyl-phenylalanine-chloromethyl ketone, which is a serine protease inhibitor, herbimycin A, which is a protein tyrosine kinase inhibitor, and caffeic acid phenethyl ester down-regulate NF-κB activation by chemical modification of the NF-κB subunits, thus preventing its binding to DNA (31–33). To determine whether MTX also modifies NF-κB proteins, we incubated nuclear extracts prepared from TNF-activated cells with different concentrations of MTX in vitro, and EMSA was performed (Fig. 2B). MTX concentrations as high as 20 μM did not modify the ability of NF-κB to bind to the DNA. Therefore, MTX inhibits NF-κB activation through a mechanism different from that of N-tosyl-phenylalanine-chloromethyl ketone, herbimycin A, and caffeic acid phenethyl ester.

MTX exhibits minimal effect on TNF-induced AP-1 activation

Most agents that activate NF-κB also activate another transcription factor, AP-1. To determine whether MTX also blocks TNF-induced AP-1 activation, Jurkat cells were preincubated for 2 h with different concentrations of MTX, treated with TNF (0.1 nM) for 30 min at 37°C, and then nuclear extracts were prepared and assayed for AP-1 activation by EMSA. As shown in Fig. 2C, TNF-activated AP-1 and the supershift analysis with specific Abs against c-fos and c-jun indicated that TNF-induced AP-1 consisted of c-fos and c-jun (data not shown). A concentration of MTX (5 μM), which inhibited most of NF-κB activation, had minimal effect on TNF-mediated AP-1 activation. Higher concentration of MTX (20 μM) inhibited AP-1 by 25%. Thus, these results indicate that MTX is more potent in suppressing NF-κB activation than AP-1.

Inhibition of NF-κB activation by MTX is not cell-type specific

That distinct signal transduction pathways could mediate NF-κB induction in epithelial and lymphoid cells has been demonstrated (34). All of the effects of MTX described until now were observed in human Jurkat T cells. Therefore, we also studied whether MTX could also block TNF-induced NF-κB activation in epithelial (HeLa) cells and myeloid (U937) cells. Five micromolar MTX inhibited most of TNF-induced NF-κB activation in all cell types. A complete inhibition was observed with 10 μM MTX, thus suggesting that this effect of MTX is not cell-type specific (Fig. 3, A and B).

MTX inhibits TNF-induced NF-κB activation in normal cells

Whether MTX also affects NF-κB in normal cells was examined. As shown in Fig. 4, TNF activated NF-κB in human foreskin fibroblasts and in HUVECs, and pretreatment with MTX inhibited the activation. Thus, our results suggest that the suppressive effect of MTX is not restricted to tumor cells.

MTX blocks phorbol ester-, okadaic acid-, and ceramide-mediated activation of NF-κB

Besides TNF, NF-κB is also activated by a wide variety of other agents including phorbol ester, okadaic acid, LPS, and ceramide. However, the signal transduction pathway induced by these agents may differ (35–38). Therefore, we examined the effect of MTX on the activation of NF-κB by various agents. As shown in Fig. 5, MTX blocked the activation of NF-κB by PMA, okadaic acid, and ceramide, only partially blocked the effect of H2O2, and had no effect on serum-activated LPS-induced NF-κB activation. These results suggest that MTX may act at a step where all these agents (except LPS) converge in the signal transduction pathway leading to NF-κB activation. In retrospect, the lack of effect of LPS is not surprising because Jurkat cells are known not to express LPS receptors.

MTX inhibits TNF-dependent phosphorylation and degradation of IκBα

The translocation of NF-κB to the nucleus is preceded by the phosphorylation, ubiquitination, and proteolytic degradation of IκBα (23). To determine whether inhibition of TNF-induced NF-κB activation was due to inhibition of IκBα degradation, we pretreated cells with 10 μM MTX for 2 h, exposed them to 0.1 nM TNF for
different times, and then examined them for NF-κB in the nucleus by EMSA and for IκBα in the cytoplasm by Western blot. As shown in Fig. 6A, TNF activated NF-κB in the control cells in a time-dependent manner but had no effect in MTX-pretreated cells. TNF induced IκBα degradation in control cells to a maximum at 15 min, but in MTX-pretreated cells, TNF-induced IκBα degradation was suppressed (Fig. 6B). In TNF-treated cells, a complete resynthesis of IκBα occurred at 60 min, when NF-κB is still active. The resynthesis of IκBα is dependent on NF-κB activation. Why NF-κB inactivation did not occur with full resynthesis of IκBα is not clear. However, this suggests that the inactivation of NF-κB by newly synthesized IκBα is a slow process. To determine whether inhibition of TNF-induced IκBα degradation by MTX was due to suppression of IκBα phosphorylation, we examined the hyperphosphorylated form of IκBα by Western blot, using Ab that detects only the serine-phosphorylated form of IκBα. Fig. 6C clearly shows that TNF induced the phosphorylation of IκBα as early as 5 min, and MTX suppressed IκBα phosphorylation.

**MTX inhibits TNF-induced IκB activation**

Because TNF-induced phosphorylation of IκBα is mediated through IKKβ, these results suggest that MTX must inhibit IKKβ activation. Indeed, as shown in Fig. 6D (upper panel), in immune-complex kinase assays, TNF activated IKKβ in a time-dependent manner, and MTX treatment completely suppressed the activation. Under these conditions, MTX had no effect on IKKα (middle panel) and IKKβ protein levels (lower panel).

**MTX represses TNF-induced NF-κB-dependent reporter gene expression**

Although we have shown by EMSA that MTX blocks NF-κB activation and phosphorylation and degradation of IκBα, DNA binding alone does not always correlate with NF-κB-dependent gene transcription, suggesting the role of additional regulatory steps (39). To determine the effect of MTX on TNF-induced NF-κB-dependent reporter gene expression, we transiently transfected MTX-pretreated or untreated cells with the NF-κB-regulated SEAP reporter construct and then stimulated the cells with TNF. An almost 4-fold increase in SEAP activity over the vector control was noted upon stimulation with TNF (Fig. 7). TNF-induced SEAP activity was almost completely abolished by dominant-negative IκBα, indicating the specificity. When the cells were pretreated with MTX, TNF-induced NF-κB-dependent SEAP expression was inhibited in a dose-dependent manner. These results demonstrate that MTX also represses NF-κB-dependent reporter gene expression induced by TNF.

**MTX suppresses TNF-induced NF-κB activation through adenosine release**

Recent studies indicate that treatment of cells with MTX causes release of adenosine, which could contribute to its anti-inflammatory and immunoregulatory effects (15). Whether MTX inhibits NF-κB activation through adenosine release was investigated using two independent approaches. The first approach involved suppression of NF-κB activation by treatment of cells with adenosine, and the second involved the use of inhibitors to block
production of adenosine. To determine whether adenosine blocks TNF-mediated NF-κB activation, human skin fibroblasts (normal) and human Jurkat T cells were pretreated with different amounts of adenosine and then examined for TNF-induced NF-κB activation. Our results indicate that preincubation of both the cells with adenosine for 2 h inhibited TNF-mediated NF-κB activation in a dose-dependent manner with optimum effect at ~10 μM concentration (Fig. 8).

The adenosine is produced in the cells from adenosine monophosphate, a reaction catalyzed by the enzyme ecto-5'-nucleotidase. This enzyme is inhibited in a competitive manner by 3,7-dimethyl-1-propargylxanthine (DMPX). To establish that MTX suppresses NF-κB activation by enhancing adenosine release, we treated Jurkat cells with MTX in the presence or absence of DMPX for 2 h and then with TNF (0.1 nM) for different times and then analyzed by Western blot using Abs against phosphorylated IκBα. β-actin shows equal protein loading. C, Effect of MTX on TNF-induced phosphorylation of IκBα. Cells (2 × 10⁶/ml) were incubated first with MTX (10 μM) for 2 h and then with TNF (0.1 nM) for different times and then analyzed for IKK by the immune-complex kinase assay as described in Materials and Methods. The presence of IKKα and IKKβ protein were detected by Western blot using Abs specific against IKKα and IKKβ.

To further understand the role of adenosine on MTX-mediated suppression of NF-κB activation, we examined the effect of type A2 adenosine receptor antagonist, 3,7-dimethyl-1-propargylxanthine (DMPX). As shown in Fig. 9B, MTX suppressed the TNF-induced NF-κB activation, and DMPX reversed it in a concentration-dependent manner. These results also suggest that the inhibitory effect of MTX on NF-κB activation is mediated through adenosine.

Folinic acid and thymidine reverse the MTX-mediated NF-κB inhibition

MTX exerts cytotoxic properties by inhibiting DHFR and other folate-dependent enzymes, such as thymidylate synthase, in T cells, which can be reversed by the addition of folic acid or thymidine (6). To determine whether these molecules reverse the suppression of TNF-induced NF-κB activation in Jurkat cells, we pretreated cells with MTX in the presence of folic acid or thymidine and then activated NF-κB by TNF. As shown in Fig. 9B, TNF induced NF-κB activation, and it was suppressed by MTX.
Both folinic acid and thymidine reversed this suppression, although only partially. Under these conditions, uridine had no effect. These results suggest that pyrimidine biosynthesis also plays a critical role in the TNF signaling pathway leading to NF-κB activation.

Discussion

Because several in vitro and in vivo anti-inflammatory, immunomodulatory, and antiproliferative effects assigned to MTX require suppression of NF-κB activation, we tested the hypothesis that MTX may directly block NF-κB activation. We found that MTX was indeed a potent inhibitor of TNF-induced activation of NF-κB, and this inhibition was not cell-type specific. The suppression was observed in both normal and tumor cells. Besides TNF, MTX also blocked NF-κB activation induced by a wide variety of other inflammatory agents. NF-κB-dependent reporter gene transcription was also suppressed by MTX. The suppression of NF-κB by MTX accompanied inhibition of IκBα phosphorylation and degradation. Our results also indicate that MTX mediated its effects through the pyrimidine and purine biosynthesis pathways.

Several lines of evidence indicate that MTX suppresses TNF-induced NF-κB activation in Jurkat cells. These include suppression of binding of NF-κB to the DNA, inhibition of IκBα phosphorylation, inhibition of IκBα degradation, abrogation of activation of IKK, and suppression of NF-κB-dependent reporter gene transcription. Recent reports indicate that different inflammatory agents may activate NF-κB through mechanisms that consist of some overlapping and some nonoverlapping steps (34–38, 40). How MTX blocks NF-κB activation by TNF is not clear. Its suppression of NF-κB activation by a wide variety of agents suggests that MTX must act at a step common to all agents. Most inhibitors of NF-κB activation such as curcumin, silymarin, and oleandrin mediate their effects through suppression of phosphorylation and degradation of IκBα (41–43). Like these agents, our results indicate that MTX also blocked both IκBα phosphorylation and degradation. However, these results differ from that described for caffeic acid phenethyl ester or mesalamine, which block NF-κB activation without any effect on IκBα phosphorylation or degradation (33, 44). Caffeic acid phenethyl ester modifies the NF-κB protein so that it can no longer bind to DNA, whereas MTX had no effect on the binding of NF-κB proteins to DNA. Recent report indicates that TNF-induced IκBα phosphorylation occurs through activation of IKKβ (45), and our results indicate that MTX inhibits the TNF-induced activation of IKKβ, thus leading to the suppression of NF-κB activation.

Our results indicate that, besides inhibiting NF-κB activation by a wide variety of inflammatory stimuli, MTX also blocked NF-κB activation in various cell types (both normal and tumor). Although there is some evidence for distinct signal transduction pathways leading to NF-κB induction in epithelial and lymphoid cells (34), our results indicate that MTX inhibits both cell types. The cell types involved in RA, the major target of MTX, include fibroblasts, macrophages, and T cells (46). Our results indicate that NF-κB activation in all these cells is inhibited. Some reports indicate that MTX has antiangiogenic effects, most likely mediated through its effects on endothelial cells. Our results indicate that MTX also inhibits NF-κB activation in endothelial cells.

![FIGURE 7. Effect of MTX on the TNF-induced NF-κB-dependent SEAP reporter gene expression. HeLa cells, untreated or treated with different concentrations of MTX, were transiently transfected with IκBα-dominant-negative mutant plasmid linked to the SEAP gene. After 24 h in culture with TNF, cell supernatants were collected and assayed for SEAP activity as described in Materials and Methods. Results are expressed as fold activity over the nontreated control. The specificity of the assay was examined by suppression of TNF-induced NF-κB reporter activity by IκBα-dominant-negative mutant plasmid.](http://www.jimmunol.org/doi/abs/10.4049/jimmunol.2917)

![FIGURE 8. Effect of adenosine on TNF-induced NF-κB activation in normal skin fibroblasts (A) and human T cell line (Jurkat cells, B). Both the cell types (2 × 10⁶/ml) were preincubated at 37°C for 2 h with different concentrations of adenosine followed by 30 min of incubation with 0.1 nM TNF. Nuclear extracts were prepared and tested for NF-κB activation as described in Materials and Methods.](http://www.jimmunol.org/doi/abs/10.4049/jimmunol.2917)
MTX also blocked TNF-induced NF-κB-mediated gene transcription. Several inflammatory genes have been shown to be regulated by NF-κB, including cyclooxygenase-2, inducible NO synthase, matrix metalloproteinase-9, cell surface adhesion molecules (e.g., ICAM-1, endothelial cell-lucocyte adhesion molecule-1, and VCAM-1), urokinase-type plasminogen activator, TNF, IL-1, IL-2, IL-6, and GM-CSF (47). Several of these genes are involved in RA and in tumor growth and metastasis (12, 46). Our results imply that MTX may down-regulate the expression of these genes. Indeed, the down-regulation of IL-1, IL-6, TNF, and IL-8 has been reported (12).

The mechanism by which MTX suppresses TNF-induced NF-κB activation was also investigated. Our results demonstrate that adenosine blocks TNF-induced NF-κB activation in different cell types (Fig. 8). Additionally, the inhibitor of 5′-ectonucleotidase, APCP, also completely reversed the effect of MTX on TNF-stimulated NF-κB activation (Fig. 9A). Further, the adenosine receptor antagonist, DMPX, reversed the MTX-mediated suppression of TNF-induced NF-κB activation (Fig. 9B). MTX has been shown to exert a wide range of anti-inflammatory actions that are mediated in part by the release of adenosine from different cell types (15). MTX inhibits the conversion of AICAR to formyl-AICAR, a reaction catalyzed by the enzyme AICAR transformylase (15, 18, 19), and leads to intracellular accumulation of AICAR. Excess AICAR inhibits the conversion of AMP to inosine monophosphate by AMP deaminase. AMP is rapidly converted to adenosine by surface expressed 5′-ectonucleotidase (CD73). Adenosine is a potent endogenous regulator of a variety of physiologic processes through specific receptors on cell surfaces (15) and binds to four different types of G protein-coupled cell surface molecules, termed the A1, A2a, A2b, and A3 adenosine receptors. After binding to the cell surface receptor, adenosine alters immune cell production of soluble mediators such as cytokines, free radicals, and arachidonic acid metabolites (2). For instance, TNF expression has been shown to be suppressed by adenosine by interaction with A3 adenosine receptors (48). Taken together, these results, for the first time, suggest that the regulatory effect of MTX on NF-κB is mediated by adenosine.

Although Jurkat cells used in our study have been shown to express A2 type adenosine receptors (49, 50), whether these cells also express 5′-ectonucleotidase (also called CD73) is controversial. CD73 is a glycoprotein generally expressed on the surface of various cell types (51). The expression of CD73 on lymphocytes depends on their state of differentiation and function. It has also been shown that PMA, IL-1, PGE2, and TNF could up-regulate the expression of CD73 in different cell types (51, 52). Although Jurkat T cells are considered CD73 negative, when activated with anti-CD38 mAb, a cell surface expression of CD73 was observed (53). Whether the source of adenosine in our studies is from activation of CD73 or some other 5′-nucleotidase enzyme is not clear. The reversal of effects of MTX by APCP suggests the involvement of membrane-associated 5′-nucleotidase.

Our results also demonstrate that both folinic acid and thymidine partially reversed the inhibitory effect of MTX on NF-κB, whereas uridine had no effect. How these agents reverse the effect of MTX is not clear, but MTX is known to inhibit the enzyme DHFR and thus prevent the regeneration of tetrahydrofolate from dihydrofolate (2). Folinic acid antagonizes the effect of MTX by increasing intracellular levels of tetrahydrofolate. MTX also inhibits the enzyme thymidylate synthase and depletes cells of thymidine, which may result in antiproliferative and cytotoxic effects. Thus, our results indicate that the pyrimidine biosynthesis pathway also plays a critical role in TNF-mediated NF-κB activation.
RA is characterized by the proliferation of synovium, which leads to joint destruction. TNF is one of the major cytokines that is elevated in the synovial fluid and presumably involved in the disease process by up-regulation of a multitude of inflammatory mediators. The success of anti-TNF Abs and soluble TNFRs in clinical trials has led to a search for signaling pathways that control TNF production as well as its function. NF-κB activation has been reported to play a pivotal role in synovial cell proliferation (54) and has been suggested as a therapeutic target (55). Recently, we have reported that the antirheumatic drug leflunomide also inhibits TNF-mediated NF-κB activation (29, 56). Our present results suggest that suppression of NF-κB activity by MTX is another novel pathway through which MTX could mediate its effect in RA. The suppression of NF-κB by MTX by a variety of inflammatory stimuli in different cell types may explain the anti-inflammatory, immunomodulatory, and antiproliferative effects of MTX.

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