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ORIGINAL ARTICLE

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Folinic acid antagonizes methotrexate-induced differentiation of monocyte progenitors

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Abstract *Objective*: The anti-inflammatory action of low-dose methotrexate (MTX) in the treatment of rheumatoid arthritis (RA) appears to be partially impaired by folate supplementation. Here we investigated whether a folate excess impairs monocyte differentiation, a putative anti-inflammatory action of low-dose MTX. *Methods*: Monocyte differentiation of U937 promonocytic cells was assessed by CD11b and CD14 immunostaining and fluorescent absorbent cell sorting (FACS) analysis. Cell proliferation and viability were determined by cell counts and trypan-blue staining, respectively. Nuclear apoptosis was assessed by 7-actinomycin staining. Cells were treated with 10^{-10}–10^{-6} M MTX in the presence or absence of folinic acid. Exposure to 1,25-OH-vitamine D_3 and TGF-β served as a positive control of monocyte differentiation in U937 cells. *Results*: Low-dose MTX-induced monocyte differentiation was marginal when compared with 1,25-OH-D_3 + TGF-β treatment. Low-dose MTX inhibited cell proliferation, induced apoptosis, and reduced cell viability. All the antiproliferative, cytotoxic, and monocyte differentiating effects of MTX were completely reversed by folinic acid. *Conclusions*: Monocyte differentiation is part of the folate-dependent MTX actions.

Keywords Methotrexate · Folinic acid · Monocyte · U937 · Differentiation

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Introduction

Weekly administered low-dose methotrexate (MTX) has developed as a standard treatment for active rheumatoid arthritis (RA) and other inflammatory disorders [1, 2]. Early clinical trials have shown an inhibition of MTX-induced toxicity but almost sustained anti-inflammatory efficacy, despite folate supplementation [3, 4], leading to the widely accepted recommendation of folate supplementation in MTX-treated RA patients [3, 4, 5, 6, 7]. Recently published, large clinical trials and a review of the Cochrane collaboration, however, suggested impaired MTX efficacy by folate supplementation and raised the question again of whether folates might compromise MTX efficacy in RA treatment, at least when given in high concentrations of its active metabolite (folinic acid) [8, 9, 10, 11]. The present discussion [8, 9, 10, 11, 12] shows the urgent need for a deeper understanding of the molecular low-dose MTX effects and in particular of the role of folate antagonism, which might be essential for effective RA treatment.

Despite many efforts in the past, the contribution of different postulated mechanisms of low-dose MTX in RA treatment is still speculative: the clonal deletion of activated T cells was clearly shown to be folate-dependent [13]; but can this mechanism represent the predominant action of low-dose MTX in RA treatment in respect of the clinical efficacy data? Several experiments indicated other anti-inflammatory effects of low-dose MTX, e.g., on the expression of interleukin (IL)-1 and other proinflammatory cytokines [14, 15, 16, 17, 18, 19], but the cellular mechanisms of these effects, e.g., by affecting the cytokine promoter activity, are still unclear. An increased adenosine release through 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase inhibition by low-dose MTX [20] was recently confirmed as an anti-inflammatory action of low-dose MTX in the rat adjuvant arthritis model [21], but the discussion about its clinical relevance for RA treatment is still controversial [21, 22, 23].

Here we aimed to investigate whether impaired folate metabolism is a prerequisite for MTX-induced monocyte differentiation. This cellular phenomenon is associated with reduced IL-1 beta (IL-1β) and IL-8 secretion and enhanced release of the endogenous IL-1 receptor antagonist (IL-1Ra) in RA bone marrow progenitors and in U937 cells [24, 25, 26, 27]. We investigated monocyte differentiation in promonocytic U937 cells by low-dose MTX in the presence or absence of folinic acid, the active form of folates and the enzyme product of dihydrofolate reductase (DHFR). In addition, we searched for a role of adenosine in the context of cell differentiation by applying different receptor-selective and nonselective inhibitors of adenosine signaling pathways. Finally, we reviewed the anti-inflammatory effect of monocyte differentiation by investigating the release of IL-18, a proinflammatory, interferon gamma (IFN-γ), and tumor necrosis factor alpha (TNF-α)-inducing monokine in T cells, with the capacity for IL-1β and IL-8 induction in monocytes [28, 29].

Materials and methods

Cells and reagents

Human promonocytic U937 cells, a myeloid leukemic cell line, are able to overcome the leukemic differentiation block by exposure to dihydroxylated vitamin D_3 (1,25-OH-D_3) in combination with transforming growth factor beta (TGF-β). Methotrexate (Wyeth-Lederle, Münster, Germany) was given in indicated 10^{-6}–10^{-10} M concentrations comprising the concentrations observed in the plasma of RA patients when treated with low-dose MTX [30, 31]. 1,25-OH-D_3 (Roche, Basel, Switzerland) was applied in a concentration of 250 ng/ml. Transforming growth factor beta, used in a 1-ng/ml concentration, was purchased from CellConcept, Umkirch, Germany. Folinic acid (Wyeth-Lederle) and thymidine (Sigma, Deisenhofen, Germany), the enzyme product of the MTX-targeted thymidilate synthase [32], were administered in 100-µM and 10-µM concentrations, respectively. Following the applied concentrations in animal models [21], all specific adenosine antagonists were given in concentrations of 10 µg/ml: the A_1 selective adenosine antagonist 1,3 dipropyl-8-cyclopentylxanthine (DPCPX) and the A_{2B} selective adenosine receptor antagonist enprofylline were provided from Sigma, and the A_{2A} selective inhibitor (4-(2-[-amino-2-(2-furyl)(triazolo(2,3-a)-[1,3,5]triazin-5-ylamino]ethyl)phenol [M241385], and A_3 adenosine receptor selective inhibitor MRS1220 were purchased from Biotrend, Cologne, Germany. The nonspecific adenosine receptor antagonist theophylline, inhibitor of $A_1 + A_2$-receptor subtypes, (Byk Gulden, Konstanz, Germany) was used in two final concentrations of 10 µg/ml and 50 µg/ml. The IL-18 enzyme-linked immunosorbent assay (ELISA) kits were provided from R and D Systems (Wiesbaden, Germany). The IL-18 antibodies for western blotting experiments were a kind gift of Dr. Kalina, Marburg, Germany.

Culture conditions

Five times 10^4 U937 cells/ml were cultured for indicated times and conditions in HAM's F10 medium (Bio Whittaker, Verviers, Bel-

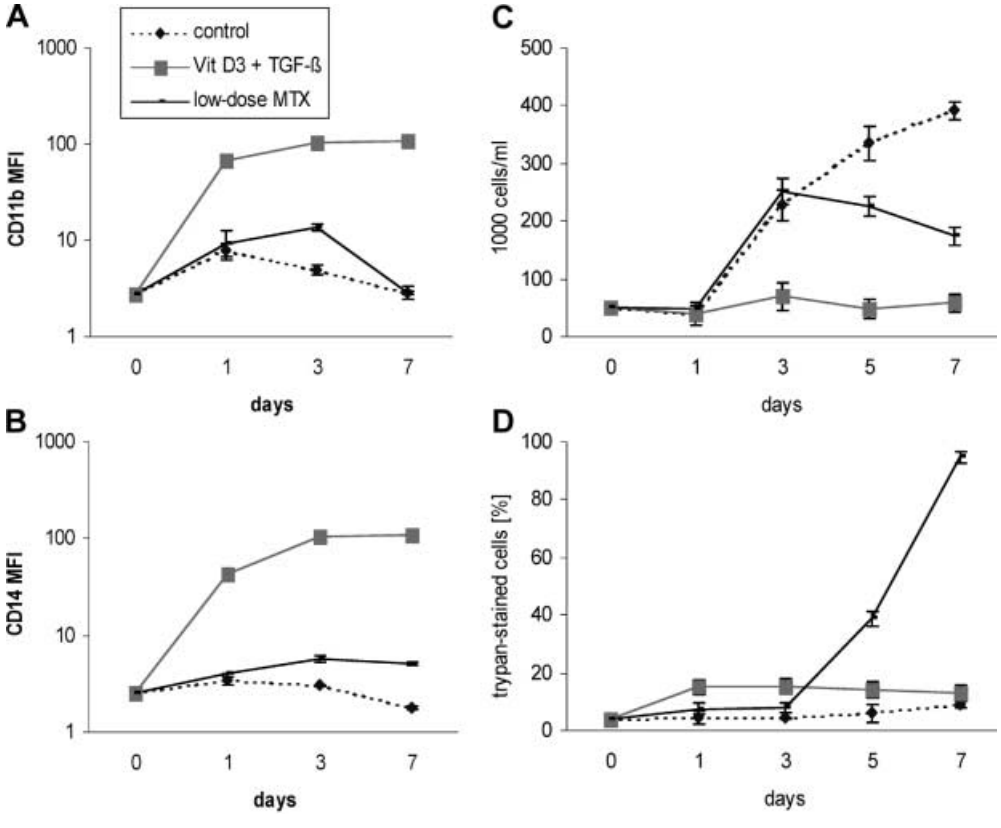

Fig. 1A–D. Time-dependent effect of 10^{-7} M MTX on the expression of differentiation markers CD11b (**A**) and CD14 (**B**), the total cell number (**C**), and cell death (**D**) in a 7-day culture of U937 promonocytic cells. 1,25-OH D_3 + TGF-b stimulation served as a positive control of cell differentiation. Results are presented as mean ± SD. *MFI* mean fluorescence intensity

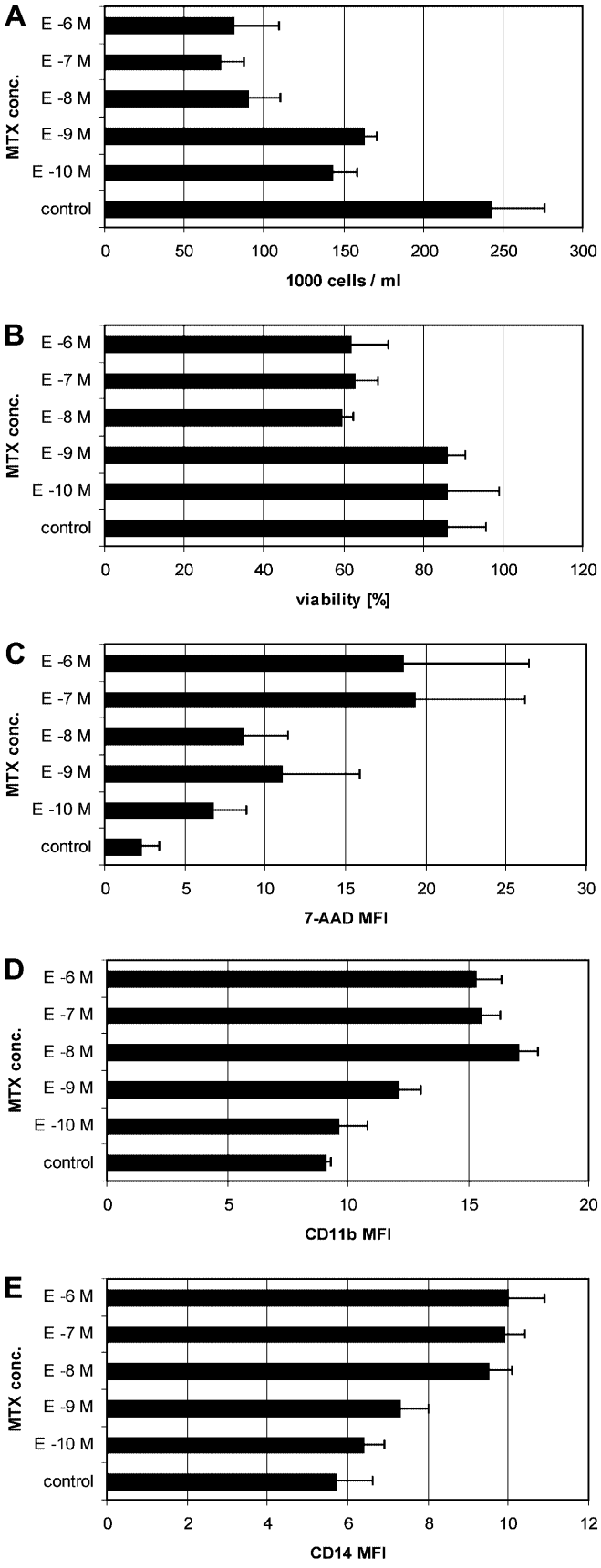

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Fig. 2A–E. Dose-dependence of MTX effects on proliferation (**A**), viability (**B**), nuclear apoptosis as shown by uptake and DNA intercalation of 7-actinomycin D (**C**), and monocyte differentiation, represented by CD11b (**D**) and CD14 expression (**E**) in a 3-day culture of U937 cells. Results in A, B, D, and E are presented as mean ± SD results and in C as mean ± SEM. *MFI* mean fluorescence intensity

gium) and completed with 10% fetal calf serum (FCS) (Boehringer, Ingelheim, Germany), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine in 12-well plates (Nunc, Roskilde, Denmark) in a humidified 5% CO_2 atmosphere at 37°C. All investigated MTX antagonists were administered simultaneously with the MTX, and their endogenous effects were determined in separate control experiments.

Cell proliferation, viability, differentiation, and apoptosis

The cells were removed from the wells, gently washed, and counted in a Neubauer hemocytometer. Cell viability was determined by trypan-blue exclusion. Analyses of the differentiation indicating monocyte surface antigens were performed with phycoerythrin (PE)-conjugated anti-CD11b (integrin αM) and fluorescein-isothiocyanate (FITC)-conjugated anti-CD14 lipopolysaccharide (LPS) receptor mouse monoclonal antibodies from Becton Dickinson, Franklin Lane, N.J., USA. Intercalation of 7-actinomycin-D (7-AAD, Sigma) was used as a marker of nuclear apoptosis [33]. The mean fluorescence intensity (MFI) of these parameters was determined by FACScan (Becton Dickinson).

Interleukin-18 western blotting

Five times 10^6 U937 cells were cultured for 48 h in indicated conditions. After centrifugation and thorough washing with phosphate buffered saline, IL-18 western blotting experiments were performed in the cell pellets. Briefly, total protein lysates were prepared at 4°C in lysis buffer containing 20 mM TRIS (pH 8.0), 137 mM NaCl, glycerol 10%, nonidet P-40, 10 mM ethylenediaminetetra-acetate (EDTA), 100 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), aprotinin, 20 μM Na-orthovanadat, and 4 μM leupeptin. The protein content was determined with a modified Lowry's protein measurement DC Protein Assay Kit (Biorad, Munich, Germany). One hundred micrograms of total cellular proteins per lane were separated by electrophoresis in a 15% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and blotted to nitro-cellulose membranes. The IL-18 was stained with a specific murine monoclonal IL-18 antibody that recognizes both pro-IL-18 and the mature cytokine [34]. The IL-18 ELISAs were performed following manufacturers' instructions in the cell culture supernatants.

Statistics

All experiments were performed in triplicate. Results are presented as the mean ± standard deviation (SD) or standard error of the mean (SEM).

Results

Monocyte differentiation

Low-dose MTX, administered in a clinically relevant 10^{-7} M concentration, promoted monocyte differentia-

tion, as shown by an approximately twofold increase in CD11b and CD14 staining intensity on day 3 when compared with negative controls of untreated cells. Of importance is that 1,25-OH-D_3 + TGF-β-treated promonocytic cells exhibited a 10-fold to 50-fold CD11b or CD14 staining intensity over that of untreated cells (Fig. 1 A, B). High CD11b and CD14 expression in 1,25-OH-D_3 + TGF-β-treated cells was sustained for the whole time of the experiments, but the increase in expressed differentiation markers was more or less transient when cells were exposed to low concentrations of MTX.

Proliferation and viability of monocyte progenitors

Stimulus-free control experiments showed the typical growth curve of rapidly proliferating cells, with a logarithmic phase from days 1 to 3 and retarded cell proliferation with more extended culture duration (Fig. 1C). Corresponding to the differentiation of monocytes, 1,25-OH-D_3 + TGF-β treatment induced an immediate and total growth arrest. In contrast, exposure to low concentrations (10^{-7} M) of MTX retarded cell expansion with a delay of 3 days and caused high rates of trypan-blue-positive cells from days 5 to 7 (Fig. 1D).

Dose-dependence of MTX effects on cell differentiation, proliferation, viability, and apoptosis were assessed on day 3. Methotrexate exposure was escalated in a logarithmic manner from 10^{-10} M to 10^{-6} M concentrations (Fig. 2). We observed a dose-dependent increase in CD11b and CD14 expression and a reduced total number of viable cells upon exposure to MTX, with a cutoff level approximately above 10^{-10}–10^{-9} M MTX concentrations. Inhibited cell expansion was seen to be stepwise over the whole range and in parallel with increasing MTX concentrations and, although with a relatively large variety of obtained dose-related results, a stepwise increase of average 7-AAD staining intensity in parallel indicated nuclear apoptosis.

Antagonists of low-dose MTX were investigated either alone or in combination with 10^{-7} M MTX, which had previously shown the maximal achievable effect of monocyte differentiation by MTX. In these experiments, CD11b and CD14 expression was assessed on day 3, and cell proliferation and viability were determined after 7-day culture duration. Supplementation of folinic acid completely reversed cell proliferation inhibited by MTX (Fig. 3A) and completely prevented MTX-induced cell death (Fig. 3B). In contrast, thymidine reduced cell proliferation and viability, either when given alone (number of total cells 32.7 ± 5.4%, number of viable cells 9.7 ± 2.8% of untreated controls) or in combination with MTX (Fig. 3A, B). Antagonism of MTX and folates, but synergism of MTX and thymidine, were again observed in cell differentiation experiments: MTX-

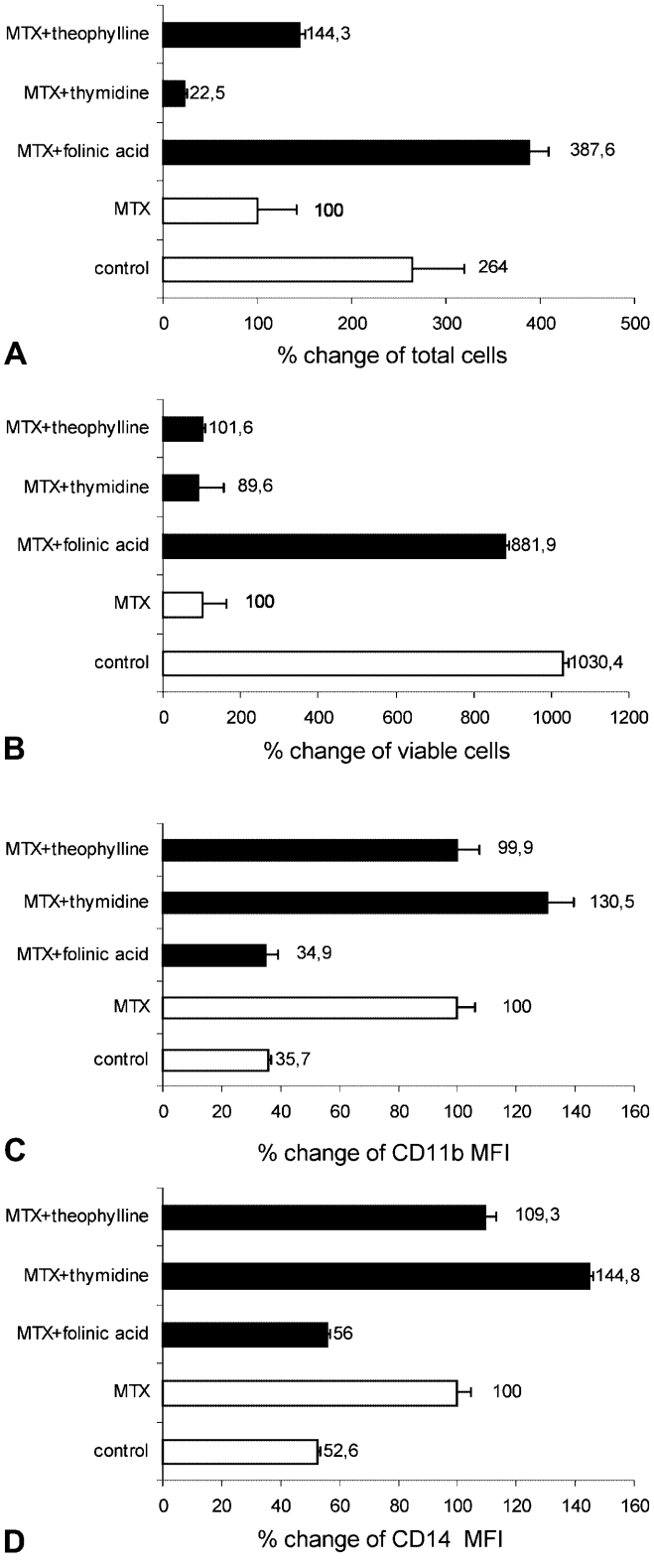

Fig. 3. Effects of folinic acid, thymidine, and theophylline supplementation (*black bars*) on the number of total (**A**) and viable cells (**B**), CD11b (**C**), and CD14 expression (**D**), presented as % change (mean ± SD of three independent experiments) from exposure to 10^{-7} M MTX alone (= 100%, *upper white bars*). *White bars on bottom* represent the untreated control experiments

Table 1. Influences of nonselective and selective adenosine receptor antagonists on the number of total and viable cells, and the expression of differentiation markers in U937 cells exposed to 10^{-7} M MTX. Cell counts were from day 7, and CD11b and CD14 expression from day 3 is expressed as % change of results in comparison to MTX alone (mean ± SD of each of three independent experiments). *MFI* mean fluorescence intensity

Condition	Total cells	Viable cells	CD11b MFI	CD14 MFI
MTX alone	100 ± 6.3	100 ± 1.4	100 ± 6.4	100 ± 2.2
MTX + theophylline (A_1 and A_2 inhibitor)	144.3 ± 6.5	101.6 ± 8.5	99.9 ± 7.4	104.9 ± 4.0
MTX + DPCPX (A_1 inhibitor)	143.0 ± 33.6	234.5 ± 89.7	88.9 ± 11.9	94.3 ± 11.4
MTX + ZM241385 (A_{2a} inhibitor)	203.7 ± 43.9	72.24 ± 4.6	96.0 ± 11.0	100 ± 2.6
MTX + enprofylline (A_{2b} inhibitor)	21.2 ± 3.1	80.4 ± 22.1	88.1 ± 5.1	43.0 ± 2.0
MTX + MRS1220 (A_3 inhibitor)	108.2 ± 14.3	99.1 ± 7.8	128.6 ± 1.6	45.4 ± 0.1

induced CD11b and CD14 expression was completely abolished by folate supplementation, while the addition of thymidine further supported cell differentiation (Fig. 3C, D). Inhibition of MTX-induced adenosine effects by using theophylline in both indicated concentrations caused no significant changes in the number of total and viable cells or the expression of leukocyte differentiation markers (Fig. 3). In contrast, selective inhibition of adenosine receptor subtypes displayed remarkable although divergent cellular effects when given in a combination with low-dose MTX. These results are summarized in Table 1. Finally, we excluded significant influences of theophylline or selective adenosine receptor antagonists on any of the investigated parameters in U937 cells in the absence of MTX (data not shown).

Monocyte differentiation and IL-18 secretion

Exposure to 1,25-OH-D_3 + TGF-β revealed a tenfold higher concentration of secreted IL-18 in the supernatants than in control experiments (Fig. 4), although exposure to these compounds prohibited cell expansion and maintained viability. In agreement with this, 1,25-OH-D_3 + TGF-β treatment depleted IL-18 from cellular stores, as could be shown by western blotting, and very similar observations were made following exposure to the MTX + thymidine combination, but not on MTX alone.

Discussion

The presented experiments addressed the mode of action of low-dose MTX on monocyte differentiation, with a focus on folate-dependent mechanisms. As could be expected from the literature [24, 25, 26, 27], low concentrations of MTX induced the expression of monocyte differentiation markers in U937 cells. Application of higher MTX concentrations ($> 10^{-6}$ M) elicited, if at all, only marginal further increases of CD11b and CD14 expression (data not shown). Low-dose MTX in concentrations used in RA treatment thus revealed the maximal achievable monocyte differentiation-inducing effect of this compound, but this result was marginal when compared with 1,25-OH-D_3 + TGF-β-induced differentiation. In addition, low-dose MTX treatment did not attain an immediate and total proliferation stop, as observed upon 1,25-OH-D_3 + TGF-β treatment and expected from completely differentiated monocytes. These observations clearly show that low-dose MTX failed to induce definitive differentiation into mature monocytes or macrophages.

Measurable cell differentiation only occurred on MTX concentrations with a cytotoxic potential. Supplementation of folinic acid was the most effective way to antagonize completely this MTX cytotoxicity, but it likewise abolished MTX-induced monocyte differentia-

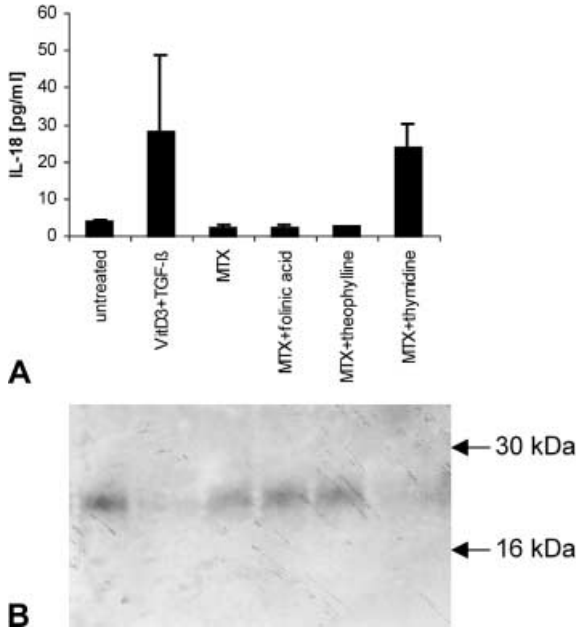

Fig. 4A, B. Monocyte differentiation of U937 cells following exposure to 1,25-OH-D_3 + TGF-β and 10^{-7} M MTX + thymidine is correlated with the release of stored IL-18 protein. **A** The IL-18 concentrations in the supernatants (mean ± SD of three independent experiments). **B** One representative of three similar IL-18 western blotting experiments in the corresponding cell pellets

tion. If monocyte differentiation plays a role for the antirheumatic effects, it definitely contributes to the folate-dependent MTX actions. This circumstance suggests minor relevance for successful RA treatment in respect of nearly maintained MTX efficacy despite folate supplementation [3, 4, 5], but it may be a mechanism relevant for impaired MTX efficacy in folate-supplemented RA patients. The same consideration, however, should be true for the folate-dependent induction of apoptosis in activated T cells [13]. Inhibited proliferation [35] and differentiation induced by low-dose MTX are likewise observed in monocyte lineage progenitor cells and in bone marrow cell cultures, but not in synovial fluid macrophages from RA patients [25], indicating a restriction of cell-differentiating MTX effects on monocytic progenitors with a proliferative potential.

Clinical use of thymidine supplementation has been tested in a limited number of oncology patients [36], and we were interested in thymidine as an alternative antidote for MTX-induced cytotoxicity. Thymidine, when given either alone or in combination with MTX, remarkably supported cell differentiation, and it did not antagonize but instead aggravated the antiproliferative and cytotoxic MTX effects. These results are preliminary and not completely explainable at present. However, thymidine should not be recommended as an alternative antagonist of MTX cytotoxicity with regard to the presented data.

The nonspecific adenosine receptor inhibitor theophylline was, although reversing the anti-inflammatory MTX effect in chronic inflammation of rodents [21], unable to modulate antiproliferative, cytotoxic, or monocyte differentiation-inducing MTX effects in U937 cells. Selective adenosine receptor inhibition in MTX-treated cells again showed a variety of cellular effects dependent on the blocked receptor subtype. Although all four investigated adenosine receptors are inferred to act, when occupied, as anti-inflammatory receptors in different models of acute or chronic inflammation, selective blockage did not reverse the anti-inflammatory MTX effects on the course of rat adjuvant-induced arthritis [21].

Subtype selective adenosine receptor inhibitors modified the response of cell cultures exposed to low-dose MTX, while no effect was found when these same adenosine receptor inhibitors were given alone, an intriguing finding for the generation of adenosine in U937 cell cultures on exposure to MTX. Exposure to MTX plus selective inhibitors, however, resulted in both monocyte differentiation-propagating and differentiation-inhibiting constellations. Based on these divergent receptor effects, we cannot state any relation of specific adenosine receptor-mediated effects and the differentiation of promonocytic cells.

The molecular basis and a suggested unidirectional or mutual relationship of secreted cytokines and monocyte differentiation are yet unknown. Here we investigated IL-18 regulation by MTX and other, more potent monocyte-differentiating conditions. The presented results indicated IL-18 secretion from monocyte progenitor cells in the course of differentiation by 1,25-OH-D_3 + TGF-β and MTX + thymidine combinations, but not on exposure to MTX alone. The IL-18 shift from the intracellular compartment into the supernatants may to some extent be related to cytotoxicity of MTX + thymidine stimulation, but this consideration can be definitely denied for the 1,25-OH-D_3 + TGF-β experiments. Interleukin-18 underlies a complex regulation, with a more constitutive rate of transcription and translation, but with stimulus-dependent activation of the stored protein through interleukin-1 converting enzyme (ICE or caspase-1), prior to secretion of either activated or uncleaved IL-18 [37]. At present, the enhanced IL-18 release appears to be limited to preformed and stored IL-18 protein. Enhanced IL-18 release along with monocyte differentiation seems controversial to the reduced IL-8 secretion and lowered IL-1beta and IL-1Ra ratio by MTX [24, 26] and in respect to the IL-1beta- and IL-8-inducing properties of IL-18 [29]. This induction cascade in peripheral blood mononuclear cells depended on the presence of T lymphocytes not present in our experiments. Nevertheless, IL-18 is inferred to have a proinflammatory role in rheumatoid arthritis [28], and IL-18 release from maturating monocyte progenitor cells thus provides additional evidence that monocyte differentiation may be beneficial for arthritis treatment in respect of some [24, 26] but not all cytokines.

For future experimental approaches addressing the molecular action of low-dose MTX, folate supplementation experiments might be helpful in estimating the therapeutic relevance of obtained results in respect of the clinical experiences. Interpreting monocyte differentiation by low-dose MTX treatment in light of its low intensity, its absolute folate dependence, and in addition some proinflammatory aspects, it is unlikely to be the most relevant anti-inflammatory effect of this important compound for RA treatment.

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