Methotrexate Blocks Proliferation Not Inflammation to Modulate Immunity

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BACKGROUND

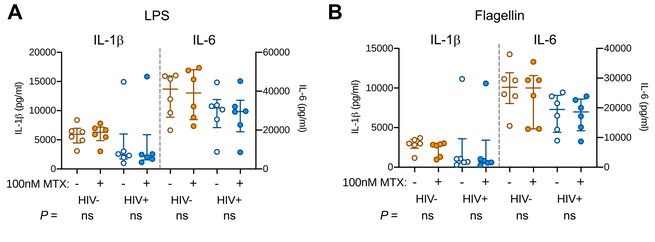
- Inflammation associated with increased risk of comorbidities persists in people living with HIV (PLWH) on combination antiretroviral therapy.
- Low-dose methotrexate (MTX) is an anti-inflammatory treatment for rheumatological disorders.
- A recent placebo-controlled trial (ACTG A5314) of low-dose MTX in PLWH found no changes in plasma inflammatory indices, but numbers of total and cycling (Ki-67+) CD4 and CD8 T cells decreased in the low-dose MTX arm[1].

[1] Hsue, et al. 2019. *Clin. Infect. Dis.* 68:1877-1876.

HYPOTHESIS

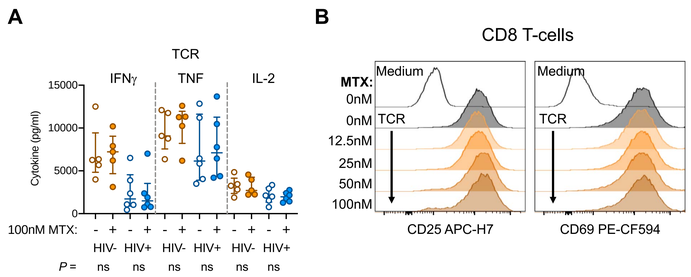
- In ART-treated HIV infection, the *in vivo* anti-inflammatory effects of low-dose methotrexate (MTX) may be mediated more by blocking T-cell homeostasis than by effects on monocyte cytokine expression or T cell activation and cytokine production.
- We test this hypothesis by treating cells with MTX *in vitro* in the presence of homeostatic and pro-inflammatory stimuli and then measuring cytokine release and indices of activation and proliferation.

MTX DOES NOT INHIBIT MONOCYTE CYTOKINE PRODUCTION



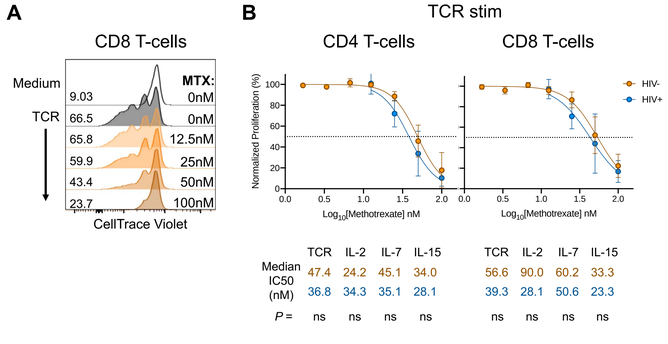
Monocytes were enriched from peripheral blood from HIV-uninfected (n=6) and HIV-infected (n=6) donors and stimulated overnight with or without 100nM MTX and with (A) LPS (10ng/ml) or (B) flagellin (500ng/ml). Supernatants were harvested and measured for IL-1β and IL-6 by ELISA. Significance determined by Wilcoxon signed rank test.

MTX DOES NOT INHIBIT T-CELL ACTIVATION IN VITRO



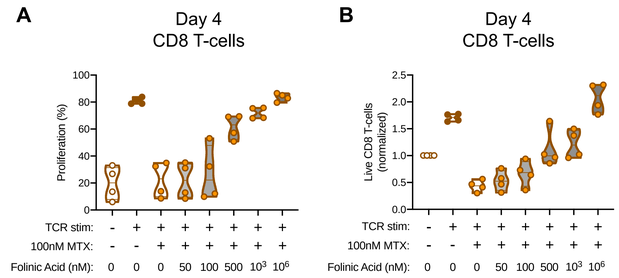
(A) T-cells were enriched from peripheral blood of HIV-uninfected (n=5) and HIV-uninfected (n=6) donors and stimulated overnight with anti-CD3 (10μg/ml) and anti-CD28 (5μg/ml), with or without 100nM MTX. Supernatants were harvested and measured for IFNγ, TNF, and IL-2 by ELISA. (B) PBMCs from HIV-uninfected (n=8-11) and HIV-infected (n=5-6) donors were stimulated for 4 or 7 days with anti-CD3/anti-CD28 (TCR), IL-2 (180U/ml), IL-7 (10μg/ml), or IL-15 (25ng/ml) in various concentrations of MTX. Expression of CD25 and CD69 on CD8 T-cells from an HIV-uninfected donor after 7 days of TCR stimulation is shown. Significance determined by Wilcoxon signed rank test.

T-CELL PROLIFERATION INDUCED BY T-CELL RECEPTOR OR CYTOKINE STIMULATION IS BLOCKED BY MTX IN VITRO



(A) PBMCs from HIV-uninfected (n=8-11) and HIV-infected (n=5-6) donors were labeled with CellTrace Violet and stimulated for 4 or 7 days with anti-CD3/anti-CD28 (TCR), IL-2, IL-7, or IL-15 in various concentrations of MTX. CellTrace Violet levels in CD8 T-cells from an HIV-uninfected donor after 7 days of TCR stimulation is shown. (B) Half maximal inhibitory concentration (IC50) of MTX for TCR stimulation and median IC50s are shown for CD4 and CD8 T-cells after 7 days of the indicated stimulations. Significance determined by Mann-Whitney test.

FOLINIC ACID TREATMENT CAN RESTORE PROLIFERATION



PBMCs from HIV-uninfected (n=4) donors were labeled with CellTrace Violet (CTV) and stimulated for 2 or 4 days with anti-CD3/anti-CD28 (TCR) with or without 100nM MTX and various concentrations of folinic acid. (A) Proportion of CD8 T-cells that diluted CTV (proliferation). (B) The number of live CD8 T-cells, normalized to medium-only control. (C) The proportion of CD8 T-cells from HIV-uninfected donors (n=8) that were undergoing apoptosis (LiveDead+FLICA^{hi}) was enumerated. FLICA, fluorescently-labeled inhibitor of caspase activation.

CONCLUSIONS

- MTX at concentrations up to 100nM does not inhibit expression of IFNγ, TNF, and IL-2 in response to TCR stimulation or IL-1β and IL-6 after stimulation with the TLR4 and TLR5 agonists LPS and flagellin *in vitro.*
- Proliferation of CD4 and CD8 T cells in response to TCR and common γ-chain cytokine stimulation is profoundly reduced by MTX and is associated with cell death *in vitro*.
- Folinic acid could restore T cell proliferation, but did not fully rescue cell death.
- Our data are fully consistent with the *in vivo* effects of MTX in PLWH suggesting that the major effect of MTX on immune function is an inhibition of cellular proliferation.
- *In vivo* folic acid supplementation (1mg/day) during low-dose MTX may not sufficiently rescue dihydrofolate reductase activity.

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