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Impact of Methotrexate upon transdifferentiation and metabolic bioenergetics of neutrophils

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Abstract

Background

Rheumatoid arthritis (RA) is associated with inflammation, oxidative stress along with infiltration of immune cells (~ 90% neutrophils) into inflamed joints. Neutrophils have demonstrated the ability to transdifferentiate into 'neutrophil-dendritic cell hybrids' or N-DCs, acquire antigen presenting properties (HLA-DR/CD80/CD86) and demonstrated oxidative stress. Classical neutrophils rely mainly on glycolysis, but the metabolic bioenergetics of N-DCs remains poorly defined. Accordingly, this study aimed to assess the impact of methotrexate (MTX), upon transdifferentiation and the metabolic bioenergetics of neutrophils.

Methods

The *ex-vivo* effect of MTX on neutrophils sourced from healthy controls was studied (+/- PMA) in terms of $CD83^{+}$, $HLA\text{-}DR^{+}$, generation of ROS, activation (myeloperoxidase) and status of apoptosis (Annexin V, Caspase 3 and Bcl-2) by flow cytometry, while the bioenergetics was assessed using Agilent XFp analyser and expression of key metabolic regulatory enzymes by real time PCR.

Results

In neutrophils, MTX failed to impact on generation of ROS; however, following induction of a pro-oxidant milieu by PMA, MTX demonstrated a synergistic enhancement in the generation of ROS, increased $\%CD83^{high}/HLA\text{-}DR^{high}$ and myeloperoxidase, elevated apoptosis (Annexin V, Caspase 3) along with downregulation of Bcl-2. MTX *per se* did not alter the oxygen consumption rate (OCR), whereas following PMA-induced oxidative stress, MTX enhanced OCR, glycolytic mechanisms remaining unaltered.

Conclusions

MTX, the gold standard for treatment of RA did not alter the redox status of neutrophils, but in a pro-oxidant milieu, MTX facilitated transdifferentiation and activation of neutrophils, altered their apoptotic and metabolic bioenergetics status, indicative of an adverse bystander effect.

Introduction

Rheumatoid arthritis (RA) is a chronic, systemic, inflammatory, autoimmune disorder that mostly affects the peripheral joints i.e. synovial joints leading to destruction of cartilage and bone [Radu and Bungau 2021]. Approximately, 0.5-1% of the global population is affected by RA, along with a female predominance [Ashai and Harvey 2020]. The pathogenesis of RA is associated with infiltration of

immune cells (~ 90% neutrophils) into the sites of inflammation and accompanied by a predominantly pro-inflammatory and pro-oxidant milieu that perpetuates a vicious cycle. Oxidative stress is associated with an intricate cross talk between immune responses and generation of endogenous/exogenous antigens, that results in disease sustenance [Kundu et al. 2012; Datta et al. 2014; Bagchi et al. 2022].

The neutrophil-derived ROS, reactive nitrogen species (RNS) and granule proteases have been strongly implicated in chronic damage and destruction of host tissues, attributed to formation of neo-antigens (e.g. citrullinated histones, cyclic citrullinated peptides or CCP) along with irreversible damage to proteins, lipids and DNA along with bone and cartilage damage [Jing et al. 2023; Pradhan et al. 2019]. Although ROS is considered as a host-defense mechanism of neutrophils [Mocsai 2013; Kruger et al. 2015], it is also a crucial second messenger that determines cell differentiation, maturation, and can cause functional alteration of signaling molecules [Reczek and Chandel 2015]. In the synovial fluid (SF) of patients with RA, a subpopulation of accumulated neutrophils demonstrated the ability to transdifferentiate into neutrophil-dendritic cell hybrids or N-DCs and acquired antigen presenting properties [Bagchi et al. 2022]. Additionally, as compared to canonical neutrophils, these N-DCs demonstrated substantial generation of ROS, potentially contributing to the oxidative stress within synovial joints, and by sustaining the vicious cycle facilitates disease progression [Bagchi et al. 2022].

The first-line disease-modifying anti-rheumatic drug (DMARD) for the treatment of RA is methotrexate (MTX), a stable derivative of aminopterin [Bedoui et al. 2019]. Chronic administration of MTX in patients with RA is associated with hepatotoxicity, which varies from mild hepatitis and cholestasis to acute liver failure and cirrhosis [Pradhan et al. 2023]. In animal models of RA, this MTX mediated hepatic damage was attributed to oxidative stress and was mitigated by the intervention of antioxidants like epigallocatechin 3-gallate, allylpyrocatechol etc. [Pradhan et al. 2023, De et al. 2017]. Furthermore, long-term administration of MTX caused inhibition of free radical scavengers i.e. superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), and thereby by compromising the redox homeostasis caused oxidative stress [Pradhan et al. 2023, Herman et al. 2005, Phillips et al. 2003]. Accordingly, this study was aimed at evaluating the effect of MTX on neutrophils, in a pro-oxidant milieu, in terms of activation, trans-differentiation, and antigen presenting properties, apoptotic status and metabolic bioenergetics.

Materials and methods

Reagents

All reagents were obtained from BD Biosciences (San Jose, CA, USA) except 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester [CM-H_2DCFDA] (Invitrogen, Carlsbad, CA, USA), Phorbol 12-myristate 13-acetate or PMA and Methotrexate (Sigma Aldrich, India), Granulocyte seperating media 1119 and Lymphocyte seperating media 1077 (HiMedia, Mumbai, India). cDNA Reverse Transcription kit, SYBR Green qPCR Master Mix were obstained from Applied Biosystems (Grand Island, NY, USA), TRIzol reagent (Ambion, Austin, TX, USA) and Cell-Tak™ from Corning® (Tewksbury, MA, USA).

For the mitochondrial and glycolytic studies by Extracellular Flux analyzer, XFp Flux pack and XF DMEM medium were obtained from Agilent Technologies (Santa Clara, CA, USA). The study protocol received prior approval from the Institutional Ethics Committee of IPGME&R, Kolkata; written informed consent was obtained from all participants.

Generation of reactive oxygen species in neutrophils

Neutrophils were isolated from peripheral blood using HiSep 1077 and GranuloSep GSM 1119 according to manufacturer's instructions. Briefly, a double gradient was formed by layering an equal volume of HiSep LSM 1077 and GranuloSep 1119 to which blood was carefully layered. Following centrifugation (3000 rpm, 30 min, RT), neutrophils present at the HiSep LSM 1077/ GranuloSep GSM 1119 interphase layer were collected, washed twice in phosphate buffer saline (0.02 M, pH 7.4, PBS), resuspended in 2 ml of PBS, and cell viability (> 95%) confirmed using trypan blue.

Initially, the non-toxic concentration of MTX, was estimated in isolated neutrophils ($5x10^5$ cells/ml) sourced from peripheral blood of healthy donors (n = 5), that after incubation with MTX (0–20 μM, 1hr, 37°C in dark) were washed in PBS, resuspended in 400 μl of PBS, surface stained with propidium iodide (0.01 μg/ml) and acquired in a flow cytometer. The frequency of PI^+ neutrophils was measured to assess cytotoxicity.

The generation of ROS was measured in neutrophils isolated from peripheral blood of healthy controls (n = 5) using, a chloromethyl derivative of 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester or CM-H_2DCFDA [**5**]. Briefly, neutrophils ($5x10^5$ cells/ml), following pretreatment with MTX (2.5–10.0 μM, 1hr, 37°C in dark) ± PMA (1 μg/ml, 15 min, 37°C in dark) were washed and incubated with CM-H_2DCFDA (2 μM, 30 min, 37°C in dark); following two washes with PBS they were resuspended in 400 μl of PBS, and acquired in a flow cytometer [**5**].

Measurement of intracellular myeloperoxidase (MPO)

Neutrophils ($5x10^5$ cells/ml) sourced from peripheral blood of healthy controls (n = 5) were treated with MTX (2.5–10.0 μM, 1hr, 37ºC in dark) ± PMA (1 μg/ml, 15 min, 37ºC in dark). Cells were then fixed and permeabilized with 100 μl of cytofix-perm buffer (BD Biosciences, San Jose, CA, USA) for 20 min. at RT in dark. Cells were then washed, resuspended in perm-wash buffer (100 μl), incubated with MPO-PE (30 min, RT in dark) and after two washes in PBS, resuspended in PBS and acquired in a flow cytometer.

Immunophenotyping of neutrophils

The frequency of CD83 and HLA-DR was measured in neutrophils sourced from peripheral blood of healthy controls (n = 5) by flow cytometry. Neutrophils ($5x10^5$ cells/ml) were treated with MTX (2.5–10.0

μM, 1hr, 37°C in dark), surface stained with CD83-PE and HLA-DR-PerCP, incubated for 30 min, RT in dark and following three washes with PBS resuspended in 400 μl of PBS and acquired in a flow cytometer.

Analysis of apoptosis by phosphatidylserine externalization

To assess the apoptotic status of neutrophils, isolated neutrophils ($5x10^5$ cells/ml) were treated with MTX (2.5 or 5 μM, 1hr, 37°C in dark) and/or PMA (1 μg/ml, 15 min, 37°C in dark). Following centrifugation (5000 rpm, 10 min), and after two washes in PBS, cells were resuspended in annexin V binding buffer [10 mM HEPES/ NaOH (pH 7.4), 140 mM NaCl, 2.5 mM $CaCl_2$] and incubated for 20 min. Annexin V-FITC was added according to the manufacturers' instructions and incubated for 30 min, RT in dark. Finally, after two washes with PBS, cells were resuspended in PBS and acquired in a flow cytometer.

Status of pro-apoptotic Caspase 3 and anti-apoptotic Bcl-2

Neutrophils ($5x10^5$ cells/ml) sourced from peripheral blood of healthy controls (n = 5) were treated with MTX (2.5 and 5 μM, 1hr, 37°C in dark) and/or PMA (1 μg/ml, 15 min, 37°C in dark). Neutrophils were initially surface stained with CD66b-FITC and incubated (30 min, RT in dark), then fixed and permeabilized with 100 μl of cytofix-perm buffer (BD Biosciences, San Jose, CA, USA) for 20 min. at RT in dark. Cells were resuspended in Perm-wash buffer (100 μl) and incubated with Caspase 3 or Bcl-2 for 30 min (RT in dark); after two washes with PBS, resuspended in PBS and acquired in a flow cytometer.

Flow cytometry

Cells were gated based on characteristic linear forward and side scatter morphological gating of neutrophils (events: 5000); Cells were acquired in a BD Accuri™ C6 Plus (BD Biosciences, San Jose, CA, USA) and the fluorescence measured on a biexponential scale using BD Accuri™ C6 Plus analysing software. Frequency and expression (in terms of Geometric mean fluorescence channel, GMFC,) was evaluated in c6 Plus software (BD Biosciences, San Jose, CA, USA).

Impact of MTX on bioenergetics of neutrophils

Real-time measurements of mitochondrial respiration or oxidative phosphorylation (OXPHOS) and cellular glycolytic activity of neutrophils were performed using Seahorse Metabolic Analyzer XFp (Agilent Technologies, Santa Clara, CA, USA) in terms of their oxygen consumption rate (OCR, pmol/min) and extracellular acidification rate (ECAR, mpH/min) respectively, as per manufacturer's instructions [Grudzinska et al. 2023; sarkar et al. 2022]. The glycolytic activities were assessed in neutrophils incubated with MTX (2.5 μM) and/or PMA (1.0 μg/ml) along with glucose (10 mM), oligomycin (10 μM), and 2-deoxyglucose (2-DG, 50 mM). Data was analysed using Seahorse Wave software, version 2.6.1, along with the XF Mito/Glycolysis stress test report generator (Agilent Technologies, Santa Clara, CA,

USA). Similarly, freshly isolated neutrophils from peripheral blood of healthy controls (n = 3), were seeded ($1x10^6$ cells/180 µl/well), and their mitochondrial respiration assessed in cells incubated with MTX and/or PMA along with classical inhibitors, oligomycin (10 µM), carbonyl cyanidep-trifluoromethoxyphenylhydrazone (FCCP, 2 µM) and rotenone-antimycin A (Rot + AA, 1 µM each).

mRNA expression of glycolytic and OXPHOS markers

Neutrophils sourced from healthy controls were treated with MTX (2.5 µM, 1 hr, 37°C) and/or PMA (1.0 µg/ml, 15 min, 37°C), followed by isolation of total RNA by the Trizol method, and concentration measured in a NanodropTM One/OneC Microvolume UV-Vis Spectrophotometer (Thermo Fischer Scientific, MA, USA), and converted to single-stranded cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, MA, USA), according to the manufacturer's instructions. cDNA (1 µg for a 20 µl reaction) for detection of amplicons was done using gene specific primers (sourced from NCBI Primer-BLAST, https://www.ncbi.nlm.nih.gov/tools/primer-blast) whose specificity was confirmed by UCSC In-Silico PCR for human-specific genes (**i**) *HK2* and *LDHA* (for glycolysis) along with (**ii**) *PDK1, SDHA* and *ATP synthase (*for OXPHOS), Table 1.

Table 1
Primers used in this study

	Primers		Primer Sequence (5'-3')
Glycolytic genes	**HK2**	Forward	CGCCTGTGAATCGGAGAGGT
	(Hexokinase 2)	Reverse	GTCAAGGCGCTAACTTCGGC
	LDHA	Forward	AAGCTGTCATGGGTGGGTCC
	(Lactate dehydrogenase)	Reverse	CGGGAAACCATTCCATCCTACTG
TCA cycle and Oxidative Phosphorylation genes	**PDK1**	Forward	TGTGGCTTCTCTAGCGGGAC
	(Pyruvate kinase dehydrogenase)	Reverse	GAGAAGCGCGCGTAGAAGTC
	SDHA	Forward	GATCTTCCTGACTCAGCCTTC
	(Succinate dehydrogenase)	Reverse	GAGACCCTGTCCCTACAATTAC
	ATP synthase	Forward	AAGGTGGGGTAAGGCCAAGC
		Reverse	GCCTACAACTTGGGCAAAGGC

Statistical analysis

Data was checked for normality using by the Shapiro-Wilk test; for parametric data, an unpaired t-test or one-way ANOVA was done followed by post-hoc Tukey's multiple comparison, and for nonparametric data, Mann-Whitney or Kruskal-Wallis test was done, followed by post-hoc Dunn's multiple comparison,

using GraphPad Prism software, version 8.2, (GraphPad Prism software Inc, La Jolla, CA, USA); $p < 0.05$ was considered as statistically significant.

Results

Impact of MTX on generation of reactive oxygen species (ROS) in neutrophils

To assess the non-toxic dose of MTX, neutrophils ($5x10^5$ cells/ml) were initially pretreated with MTX (0-20 µM, 1 hr, 37^oC), surface stained with Propidium iodide (0.01 µg/ml), and the frequency of PI^+ neutrophils assessed by flow cytometry. At baseline, the frequency of PI^+ neutrophils was 1.35(1.30-1.46)% and remained unchanged with MTX (0.625, 1.25, 2.5. 5.0 and 10 µM) being 1.47(1.11-1.68)%, 1.69(1.61-1.76)%, 2.01(1.81-2.08)%, 1.90(1.75-2.07)% and 2.74(2.39-2.94)% respectively (**S1, A i-vi, B**). However, MTX (20 µM) increased the PI positivity to 22.96(19.01-25.17)%, p<0.01 (**S1, A vii, B**); accordingly, the maximum conc. of MTX used was 10 µM.

In terms of generation of ROS, based on CMDCF fluorescence, neutrophils were pretreated with MTX in the presence of PMA, an established pro-oxidant. The cell population was subdivided into ROS^{low} (**R1**) and ROS^{high} generating neutrophils (**R2**). MTX (2.5- 10.0 µM) failed to increase the generation of ROS [frequencies being 1.25(0.74-1.71)%, 1.40(0.58-1.93)%, 1.63(1.16-2.15)% and 1.39(0.95-1.73)% respectively], **Fig. 1A, C**. However, when oxidative stress was induced by PMA, the frequency of **R2** significantly increased to 53.86(51.88-58.22)%, p<0.001, **Fig. 1B,** which following the addition of MTX (2.5-10 µM) increased further to [71.30 (66.36-78.88)%, p<0.01, **Fig. 1B ii, C**], [83.50(79.09-86.45)%, p<0.001, **Fig. 1B iii, C**] and [87.37(84.88-90.75)%, p<0.001, **Fig. 1B iv, C**] respectively**.**

In terms of expression or GMFC of CMDCF in neutrophils from healthy controls, the baseline GMFC of 5717(4772-6972) was comparable with neutrophils treated with MTX (2.5, 5 and 10 µM), being 5235(4867-6722), 6599(5192-7062) and 6787(5616-7117) respectively (**Fig. 1D i-iii**). The addition of PMA enhanced the generation of ROS by 14.06-fold to 58925(53825-62613), p<0.01, which following the addition of MTX (2.5, 5 and 10 µM) increased further to 81871(76289-92063), p<0.01; 101210(97468-107979), p<0.001 and 130221(117020-140035)%, p<0.001 respectively, **Fig. 1D iv**.

Status of myeloperoxidase (MPO) in neutrophils

As oxidative stress is associated with activation of neutrophils, the impact of MTX in terms of MPO was assessed. The baseline frequency of MPO^+ in neutrophils was 5.90(5.13-8.12)%, and remained unchanged with MTX (2.5, 5 and 10 µM), being 12.40(9.27)%, 14.70(11.97-15.24)% and 18.74(16.74-21.51)% respectively **Fig. 2A, C**. In the presence of PMA, the MPO positivity was significantly enhanced to 33.29(30.27-39.97)%, p<0.001, and with the inclusion of MTX (2.5, 5.0 and 10.0 µM), was further enhanced to 41.50(39.09-46.39)%, p<0.05, 63.74(58.17-66.93)%, p<0.001 and 84.75(77.59-94.39)%, p<0.001 respectively, **Fig. 2B, C**.

Status of CD83 and HLA-DR in neutrophils

Oxidative stress in neutrophils is associated with their transdifferentiation into neutrophil-dendritic cell hybrids or N-DCs, features being increased expression of CD83 and HLA-DR [Bagchi et al. 2022]. To assess the impact, if any, of MTX upon transdifferentiation, the frequencies of CD83 and HLA-DR were measured in neutrophils by initially gating them as $CD83^{low}$ (**R3**) and $CD83^{high}$ (**R4**) populations. The baseline frequency of CD83 positivity was 0.97(0.51-1.44)%; with the addition of MTX (2.5-10 μM), the CD83 positivity remained unchanged at 2.90(2.44-4.15)%, 1.97(0.82-2.75)% and 2.90(2.60-3.45)% respectively, (**Fig. 3A, C)**. In PMA treated neutrophils, the frequency of CD83 was significantly enhanced to 23.37(20.79-28.47)%, $p<0.001$, and with co-incubation with increasing doses of MTX (2.5-10 μM) further increased, , the frequencies being 53.24(47.14-61.55)%, $p<0.01$, 71.90(67.13-78.54)%, $p<0.001$ and 84.68(82.75-87.69)%, $p<0.001$ respectively, **Fig. 3B, C**.

Similarly, in terms of HLA-DR, neutrophils were gated as $HLA\text{-}DR^{low}$ (**R5**) and $HLA\text{-}DR^{high}$ (**R6**) populations. The baseline frequency of $HLA\text{-}DR^{high}$ neutrophils [0.60(0.20-0.95)%], was unchanged by MTX (2.5, 5.0 and 10.0 μM), frequencies being 0.80(0.57-1.15)%, 0.59(0.45-1.07)% and 0.69(0.39-1.07)%, respectively **Fig. 3D, F**. The addition of PMA led to an increased frequency of HLA-DR to 26.10 (21.43-30.17)%, and with addition of MTX (2.5, 5 and 10 μM), progressively increased to 49.87(41.24-53.65)%, $p<0.01$,62.70(58.50-69.99)% and 70.20(66.42-75.90)%, $p<0.001$, respectively, **Fig. 3E, F**.

Status of apoptotic markers in neutrophils

As oxidative stress is associated with apoptosis, the impact of MTX upon the apoptotic status of isolated neutrophils sourced from healthy controls was measured in terms of the frequency of annexin V^+ along with a pro-apoptotic marker, caspase 3 and an anti-apoptotic marker Bcl-2. The baseline frequency of annexin V in neutrophils was 15.50(12.41-18.63), which was unaffected by MTX (2.5 and 5.0 μM), frequencies being 18.70(16.51-20.04)% and 39.30(21.99-45.99)% respectively (**Fig. 4A, Di)**. The addition of PMA caused a significant enhancement in the frequency of annexin V to 55.30(49.11-58.92)%, and with MTX (2.5 and 5.0 μM) increased further to 63.90(61.20-68.20)%, $p<0.05$ and 87.73(84.25-91.54)%, $p<0.001$, respectively (**Fig. 4A, Di)**.

Similarly, the baseline status of caspase 3 in neutrophils was [(10.60(7.60-13.52)%, **Fig. 4Bi**]; MTX (2.5 and 5.0 μM), had no impact, frequencies being 12.23(8.38-13.42)% and 14.20(8.85-18.26)%, respectively (**Fig. 4B iii, Dii)**. The addition of PMA significantly elevated the frequency of caspase 3 to 28.30(25.87-33.56)%, that increased further with addition of MTX (2.5 and 5.0 μM) to 69.85(59.28-85.53)%, $p<0.001$ and 85.63(81.32-89.96)%, $p<0.001$, respectively (**Fig. 4B iv-vi & Dii)**.

In terms of Bcl-2, the baseline frequency of $Bcl\text{-}2^+$ neutrophils of 40.70(36.04-44.28)% remained unchanged with the addition of MTX (**Fig. 4C i-iii**), frequencies being 29.27(26.50-32.45)% and 27.35(23.06-28.76)% **(Fig. 4 Diii)**. Following induction of oxidative stress by PMA, the frequency of Bcl-2 was significantly downregulated to 19.45(17.51-20.77)%, $p<0.01$, and co-incubation with MTX (2.5 and 5.0 μM) further decreased the Bcl-2 frequency to 15.63(14.04-17.09)%, $p<0.05$ and 13.30(11.72-14.53)%, respectively (**Fig. 4C iv-vi & Diii)**.'

Status of glycolytic activity in neutrophils

Glucose is converted to pyruvate, and then to lactate in the cytoplasm, or to CO_2 and H_2O in the mitochondria, resulting in a net production of protons; its extrusion into the extracellular medium translates into acidification of the medium, and is measured in terms of the extracellular acidification rate (ECAR), to represent the proportion of glycolysis. To assess specificity, saturating concentration of glucose (10 mM) and 2-DG (50 mM) were used along with oligomycin (10 μM). In terms of non-glycolytic acidification in neutrophils, as compared to baseline, MTX (2.5 μM) had no impact, being 60.25(55.29-69.27) vs. 58.27(57.13-63.29) mPH/min**.** The induction of oxidative stress by PMA had no impact, neither did the addition of MTX (**Fig. 5A i, ii)**; The non-glycolytic acidification in the presence of MTX (2.5 μM) was comparable with baseline being 62.39(59.29-67.61) vs. 61.79(60.13-63.79) mPH/min respectively (**Fig. 5A i, ii)**. A similar trend was observed with regard to their glycolytic status and glycolytic capacity (**Fig. 5A i, iii, iv, Table 2A**). This was substantiated by evaluating the status of hexokinase 2 (*HK2*) and Lactate dehydrogenase (*LDH*); irrespective of the treatment, the glycolytic status remained unaltered (**Fig. 5B i, ii, Table 2C)**.

Table 2A: Effect of MTX on status of glycolysis (mPH/min) in neutrophils

	*Non-glycolytic acidification	*Glycolysis	*Glycolytic capacity
Baseline	60.25(55.29-69.27)	30.37(27.77-33.29)	47.29(40.79-53.27)
MTX (2.5 mM)	58.27(57.13-63.29)	30.73(28.21-37.21)	42.33(37.29-43.27)
PMA (1.0	62.39(59.29-67.61)	50.27(47.29-53.01)	58.29(57.29-60.23)
MTX+PMA	61.79(60.13-63.79)	60.31(58.97-65.37)	67.27(63.78-68.31)

Neutrophils were incubated with MTX (2.5 mM) and PMA (1.0 μg/ml) and the status of glycolysis (mPH/min) measured in neutrophils as described in Materials and methods, and data is stated as *median (IQR).

Table 2B: Effect of MTX on status of oxidative phosphorylation in neutrophils

	@Basal Respiration	@ATP Production	@Spare Respiratory Capacity	@Acute Response
Baseline	16.39 (11.67-21.03)	40.38 (36.93-50.61)	25.37 (21.73-30.27)	8.13 (7.05-12.23)
MTX	16.83 (15.69-22.04)	45.29 (30.37-47.29)	27.79 (22.11-34.25)	9.07 (5.12-12.23)
PMA	210 (192.20-232.30)***	190.60 (179.80-201.70)***	223.20 (210.40-243.1)***	124.9 (101.2-135.10)*
MTX+ PMA	301.60 (289.30-351.30)***#	311.01 (290.80-320.80)***#	359.30 (310.8-370.80)***#	450.80 (430.30-482.90)***##

Neutrophils were incubated with MTX (2.5 mM) and PMA (1.0 µg/ml)and the status of oxidative phosphorylation was evaluated in neutrophils as described in Materials and methods; data is stated as @median (IQR). *p<0.05, **p<0.01, ***p<0.001 as compared to baseline; #p<0.01, ##p<0.001 as compared to PMA.

Table 2C: Effect of MTX in neutrophils on glycolysis and oxidative phosphorylation regulatory genes

$2^{-\Delta\Delta Ct}$	@*HK2*	@*LDH*	@*PDK1*	@*SDH*	@*ATP Synthase*
Baseline	2.17 (1.67-3.40)	2.91 (1.52-3.05)	10.61 (6.81-11.89)	5.41 (4.04-11.01)	16.31 (11.88-18.29)
MTX	2.30 (1.37-2.78)	2.71 (1.85-3.50)	9.52 (6.74-11.74)	11.79 (7.25-23.12)	17.91 (15.92-21.35)
PMA	1.05 (0.06-1.10)	2.34 (1.43-3.47)	2.61 (1.76-3.83)**	101.2 (54.35-304.30)**	66.76 (54.74-70.81)** #
MTX+ PMA	0.10 (0.00-0.28)*	2.37 (1.97-4.15)	0.97 (0.10-1.29)***#	3104 (1848-4541)***#	249.60 (205.7-270)***

Neutrophils were incubated with MTX (2.5 mM) and PMA (1.0 µg/ml) and the expression of glycolysis and oxidative phosphorylation regulatory genes were measured as described in Materials and methods; data is stated as @median (IQR); *p<0.05, **p<0.01, ***p<0.001 as compared to baseline;#p<0.01 as compared to PMA.

Status of mitochondrial bioenergetics in neutrophils

Mitochondrial respiration or oxidative phosphorylation (OXPHOS) was measured using Seahorse XFp extracellular flux analyzer in terms of OCR, wherein the consumption of oxygen occurs by reduction through transfer of electrons in the electron transport chain (ETC). The quantitative changes in the OCR are triggered by oligomycin (inhibiting oxidative phosphorylation by targeting ATP synthase), FCCP (an uncoupler) along with Rot+AA (blockers of complex I and III respectively). In terms of basal respiration in neutrophils, MTX (2.5 μM) did not have any impact, being 16.39 (11.67-21.03) vs. 16.83(15.69-22.04) pmoles/min at baseline [**Fig. 6A i, iv]**. However, PMA significantly enhanced the basal respiration to 210(192.20-232.30) pmoles/min, $p<0.001$, and with addition of MTX, was further increased to 301.60(289.30-351.30) pmoles/min, $p<0.01$ (**Fig. 6A ii-iv)**. The scenario was similar with regard to ATP production, spare respiratory capacity and acute response (**Fig. 6A v-vii, Table 2B).** To assess the specificity of oxidative stress in terms of their potential to drive towards OXPHOS, impact of a lower dose of PMA (0.5 μg/ml) along with MTX was assessed, which demonstrated a comparable acute response in the presence of MTX ± PMA (**Fig. 6B, C)**.

To substantiate the altered energy phenotype of neutrophils by PMA and MTX, the status of regulatory enzymes was assessed in terms of their gene expression. The status of *PDK1*, succinate dehydrogenase (*SDH*) and *ATP synthase* was measured as markers of oxidative phosphorylation. MTX alone had no impact whereas PMA significantly induced the expression of *SDH* and *ATP Synthase.* (**Fig. 6D**) However, when oxidative stress was induced by PMA, the addition of MTX synergistically facilitated further enhancement in the expression of OXPHOS related genes, *PDK1*, *SDH* and *ATP synthase* (**Fig. 6D i-iii, Table 2C)**.

Discussion

RA is a chronic, systemic, inflammatory autoimmune disorder, resulting in progressive joint damage and deformity. The pathogenesis of RA is intricately regulated by a cascade of immune cells [including neutrophils, T and B lymphocytes, macrophages, dendritic cells (DCs), fibroblast-like synoviocytes (FLS) etc.] along with a wide array of inflammatory mediators, wherein oxidative stress consistently plays a crucial role in the initiation and progression [Bagchi et al. 2022, Pradhan et al. 2019]. Elevated levels of ROS along with a compromised antioxidant defense system contributed to the perpetuation of inflammatory responses and joint destruction in patients with RA and a collagen induced arthritis (CIA) model of RA [Datta et al. 2014; De et al. 2017], endorsing that targeted therapies for redox stress and associated cell signaling pathways may have a beneficial impact for treatment of RA.

MTX has the potential to disrupt the redox balance in neutrophils via impacting on their antioxidant defense and ROS scavenging mechanisms [Kaudal et al. 2020]. However, studies regarding the effect of MTX on generation of ROS are contradictory, as depending on the concentration of MTX, duration of exposure, and the inflammatory environment, it can show features of being a pro- or an antioxidant [Phillips et al. 2003]. In cancer cell lines, MTX enhanced the redox imbalance through enhanced release

of H_2O_2 by neutrophils, monocytes, and T lymphocytes, potentially facilitating chemotherapy and causing cytotoxicity [Bedoui et al. 2019; Phillips et al. 2003; Gressier et al. 1994]. Conversely, in patients with RA, low-dose MTX (10–15 mg/week) within circulating neutrophils down-regulated the generation of ROS [Kaudal et al. 2020].

However, most studies till date have been conducted in peripheral blood, and not in the synovial joints which is the main disease focus [Dogru et al. 2019]. Given the predominance of oxidative stress, a pro-inflammatory milieu and an overwhelming presence of neutrophil within the inflamed synovial joint, an *ex-vivo* approach was employed to assess the potential of MTX in modulating generation of ROS. In neutrophils, MTX failed to impact on the generation of ROS; however, when oxidative stress was induced by PMA, MTX synergistically facilitated additional generation of ROS (Fig. 1), suggesting that the impact of MTX is dependent upon the presence of a pro-inflammatory, pro-oxidant microenvironment (Fig. 1). Therefore, as SF has a pro-oxidant milieu [Bagchi et al. 2022; Kundu et al. 2011], it can be proposed that MTX in RA patients have the propensity to have a disease sustaining effect.

In fungal infections, Neutrophil-DC hybrids compared to canonical neutrophils displayed a higher expression of pattern recognition receptors, enhanced phagocytosis, and heightened production of ROS with prominent NETosis, substantiating the intricate association of oxidative stress, phenotypic plasticity and altered functionalities [Fites et al. 2018]. Oxidative stress is intricately associated with the activation of neutrophils, whose key marker of activation is myeloperoxidase (MPO), which is sequestered within azurophilic granules, and plays a pivotal role in the generation of ROS and reactive nitrogen species/RNS [Ndrepepa 2019; Glennon et al. 2018]. MTX did not alter the status of MPO in neutrophils, but in the presence of PMA, MTX synergistically enhanced MPO secretion (Fig. 2), suggesting that under oxidative stress, MTX had the potential to trigger an oxidative burst, that could in turn activate the downstream NETosis signaling pathways, an established contributor in the pathogenesis of RA [Sur Chowdhury et al. 2014].

In patients with RA, neutrophils traditionally viewed as short-lived terminally differentiated cells, can transdifferentiate to neutrophil-dendritic cell hybrids or N-DCs, thereby acquiring the expression of CD83 and MHC class II [Bagchi et al. 2022, Iking-Konert et al. 2001]. These N-DCs can be activated by T cell-derived cytokines, thus potentially perpetuating local inflammation and tissue damage, and forging a link between innate and adaptive immune responses [Iking-Konert et al. 2005]. This priming of neutrophils and their subsequent activation triggers a wide array of phenotypic changes beyond just increased generation of ROS, as for e.g. co-stimulation (CD80, CD86) and enhanced cell adhesion properties (ICAM, VCAM) [Bagchi et al. 2022]. Moreover, transdifferentiated neutrophils demonstrated an enhanced generation of ROS, potentially capable of causing a higher degree of oxidative damage in the inflamed joints [Bagchi et al. 2022]. Although MTX per se did not induce transdifferentiation in neutrophils, creation of an oxidative milieu by addition of PMA led to MTX enhancing the propensity for transdifferentiation, enhanced antigen presentation, thus perpetuating a detrimental cycle, Fig. 3. This clinical simulation demonstrated that MTX under the influence of the microenvironment has the potential to have a disease promoting role.

Neutrophils, being the first line of defense, play a crucial role in innate immune responses against invading pathogens, and undergo apoptosis, necessary for rapid resolution of inflammation and restoration of tissue homeostasis. [Mccracken and Allen 2014; Giese et al. 2019]. Apoptosis of neutrophils is tightly regulated by a complex network of intracellular signaling pathways involving various pro-apoptotic factors [Mccracken and Allen 2014; Giese et al. 2019, Dejas et al. 2023; Geering et al. 2011]. In autoimmune disorders like RA, Inflammatory bowel disease (IBD), psoriatic arthritis, neutrophils can become apoptosis-resistant and survive longer within the localized pro-inflammatory microenvironment [Fresneda et al. 2021; Brannigan et al. 2000]. The synovium of RA patients expresses higher levels of the Bcl-2 family's anti-apoptotic proteins, Mcl-1 and Bcl-2, suggesting delayed apoptosis [Carrington et al. 2021; Chen et al. 2021]. In contrast, in systemic lupus, leukocytes demonstrated an accelerated apoptosis and impaired clearance of apoptotic debris, facilitating formation of auto-antigens that can promote disease progression [Munoz et al. 2008]. In patients with psoriasis, MTX increased the generation of ROS, induced oxidative stress, and enhanced apoptosis through caspase-3 activation [Elango et al. 2014]. It has been previously demonstrated that MTX did not directly induce apoptosis of neutrophils but rather ’primed‘ them for enhanced apoptosis via a JNK-dependent mechanism [Spurlock et al. 2011]. In this study, MTX did not impact on the apoptotic status of neutrophils; however, in a pro-oxidant milieu, as induced by PMA, MTX synergistically enhanced the frequency of pro-apoptotic annexin V and caspase 3, along with downregulation of anti-apoptotic Bcl-2, Fig. 4. Taken together, in a pro-oxidant milieu, MTX demonstrated the potential to enhance cellular apoptosis, which could translate into enhanced release of cellular contents and formation of neo-antigens.

Neutrophils are the most critical immune cell in the initial stages of RA pathogenesis, by virtue of their ability to generate huge amounts of ROS and reactive nitrogen species (RNS), through the activation of NOX2 and iNOS-derived NO respectively, leading to cartilage and bone damage in patients with RA [Mangal et al. 2021]. Additionally, the pro-oxidant milieu could trigger neutrophils to secrete degradative enzymes, pro-inflammatory cytokines and formation of neutrophil extracellular traps or NETs [Mangal et al. 2021, Chen et al. 2018].

In terms of effector functions i.e. phagocytosis, NETosis etc., neutrophils primarily rely on glycolysis and the pentose phosphate pathway (PPP) as their major sources of energy [Rodriguez et al. 2015, Gaber et al. 2017]. In neutrophils, the by-product of PPP is NADPH, a necessary substrate for NADPH oxidase, which facilitates enhanced generation of ROS [Rodriguez et al. 2015, Gaber et al. 2017]. However, oxygen consumption rate or OCR (a measure of oxidative phosphorylation) is also linked with the generation of RO, as; in an *ex-vivo* study, during PMA induced oxidative burst and neutrophil activation, the neutrophils demonstrated an upregulation of oxidative phosphorylation measured in terms of oxygen consumption rate [Grudzinska et al. 2023]. Additionally, during acute inflammation/infections, neutrophils rely on glycolysis and PPP to facilitate the production of the MPO, translating into enhanced antimicrobial functions [Kumar et al. 2019; Hawkins et al. 2021]. However, in chronic inflammation, as in RA, there is a sustained generation of ROS and neutrophils switch to utilizing oxidative phosphorylation (OXPHOS) and PPP for their effector functions [Schuurman et al. 2023; Thind et al. 2024]. This scenario was replicated with the addition of MTX (2.5 μM) as it did not alter the glycolytic phenotype (Fig. 5**)**, but when co-

incubated with PMA, MTX synergistically drove neutrophils towards OXPHOS, Fig. 6 [Chacko et al. 2013; Injarabian et al. 2019].

Conclusions

MTX in a pro-oxidant environment augmented generation of ROS, enhanced transdifferentiation, promoted apoptosis, and shifted the bioenergetics towards mitochondrial oxidative phosphorylation. Therefore, it may be proposed that anti-oxidants be included into the chemotherapeutic regimen, with a view towards preserving the immunomodulatory properties of MTX but curb its ability to enhance disease progression.

Declarations

Competing Interests:

None.

Author Contribution

Designed research: AB, MC; Conducted research: AB, SSG, MC; Provided essential reagents/materials: MC, AG, PG; Data analysis: AB, SSG; Funding acquisition: MC; Supervision and validation: AG, PG, MC; Manuscript writing & primary responsibility for final content: AB, SSG, MC.

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Data Availability

All data supporting the findings of this study are available within the paper and its Supplementary Information.

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Figures

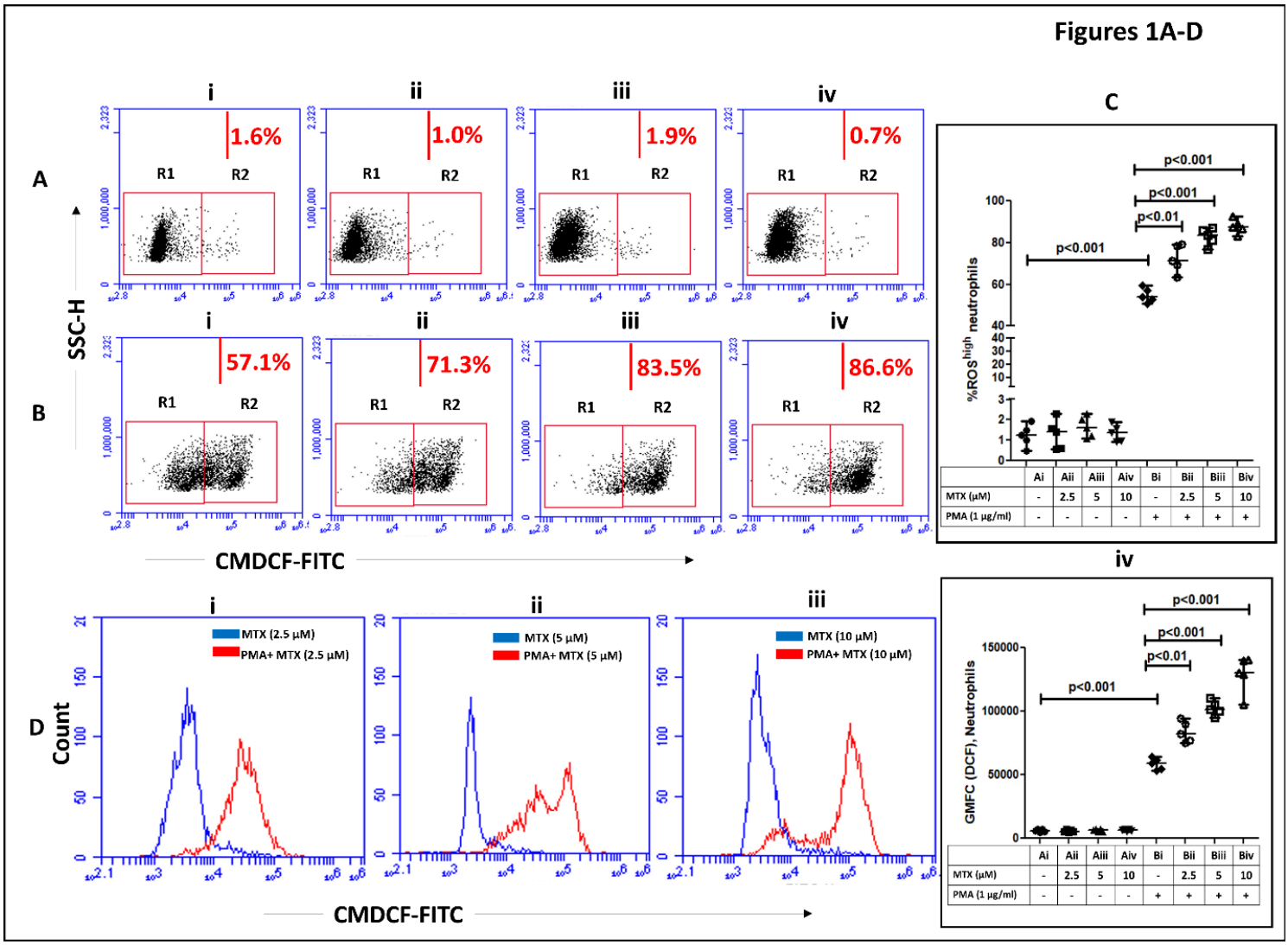


Figure 1

Impact of MTX and PMA upon generation of ROS in neutrophils

A: Representative frequency plots demonstrating the proportion of ROS^{high} generating neutrophils (**R2**) in the absence (**i**) and presence of MTX, 2.5 μM (**ii**), 5 μM (**iii**), 10 μM (**iv**).

B: Representative frequency plots demonstrating the proportion of ROS^{high} generating neutrophils (**R2**) treated with PMA (**i**), PMA+MTX 2.5 μM (**ii**), PMA+MTX 5 μM (**iii**) and PMA+MTX 10 μM (**iv**).

C. Scatter plots demonstrating the frequency of ROS^{high} generating neutrophils (**R2**) treated with MTX ± PMA. Data are indicated in median (IQR).

D i-iii. Representative histogram plots depicting the GMFC of CMDCF in neutrophils treated with MTX (± PMA).

iv. Scatter plots demonstrating the GMFC of CMDCF in neutrophils treated with MTX± PMA. Data are indicated in median (IQR).

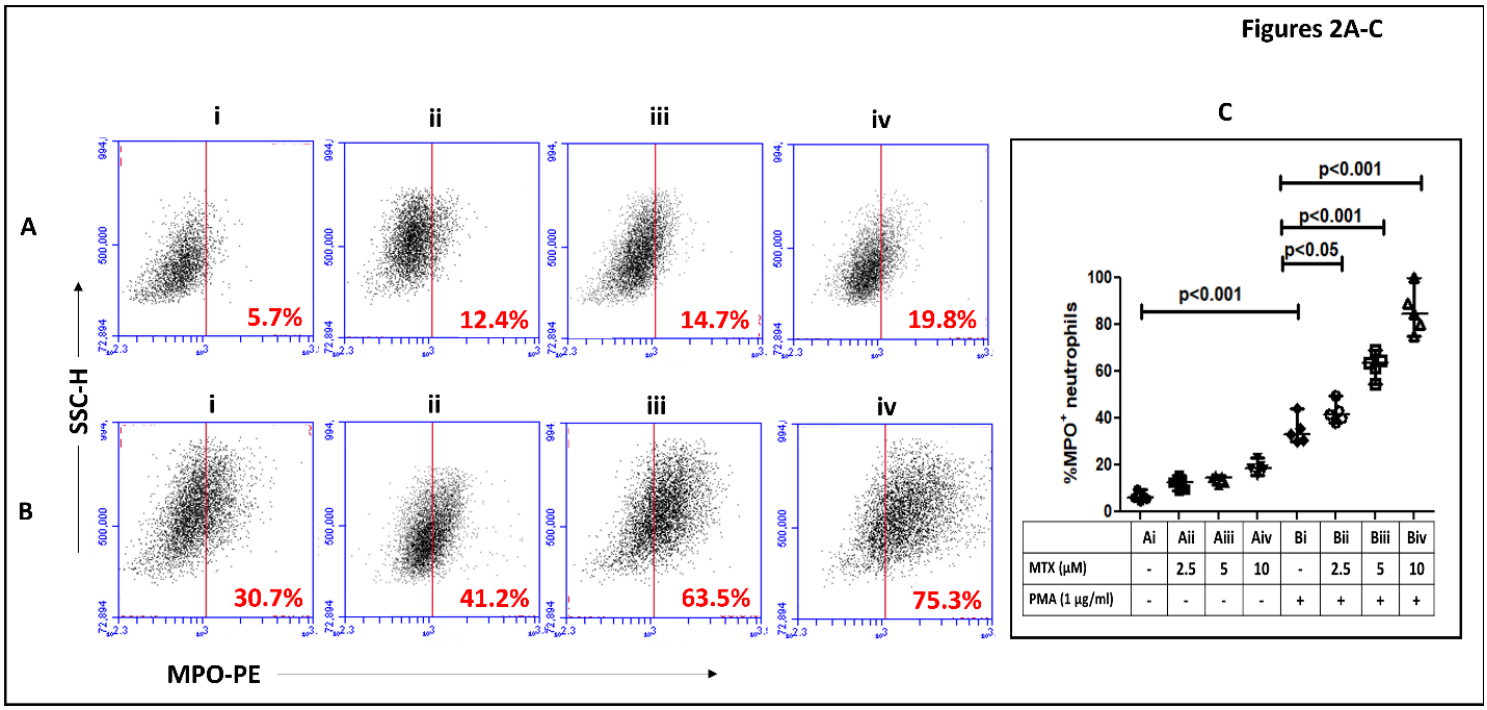


Figure 2

Impact of MTX and PMA on the status of MPO in neutrophils (n=5; 5x10^5 cells/ml)

A i-iv: Representative frequency plots demonstrating the proportion of MPO$^+$ neutrophils (5x10^5 cells/ml, **i**) treated with MTX 2.5 µM (**ii**), 5 µM (**iii**), 10 µM (**iv**)

B i-iv: Representative frequency plots demonstrating the proportion of MPO$^+$ neutrophils (5x10^5 cells/ml, i) treated with PMA (**i**) in the presence of MTX 2.5 µM (**ii**), 5 µM (**iii**), 10 µM (**iv**),

C. Scatter plots demonstrating the frequency of MPO$^+$ neutrophils treated with MTX ± PMA. Data are indicated in median (IQR).

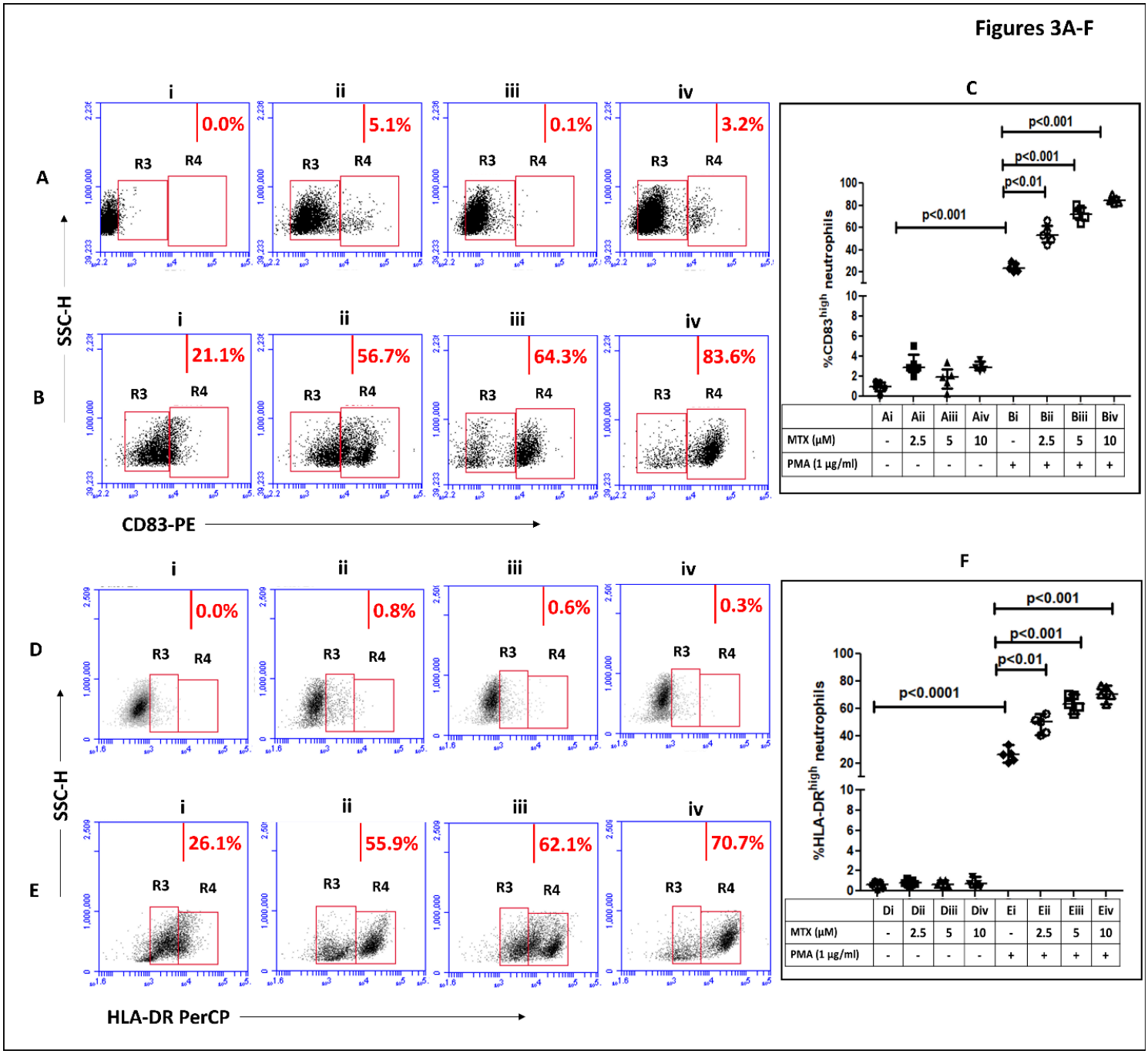


Figure 3

Impact of MTX and PMA on the status of CD83 and HLA-DR in neutrophils

A i-iv: Representative frequency plots demonstrating the proportion of $CD83^{high}$ neutrophils (n=5; $5x10^5$ cells/ml) (**i**), treated with MTX, 2.5 µM (**ii**), 5 µM (**iii**) or 10 µM (**iv**)

B i-iv Representative frequency plots demonstrating the proportion of $CD83^{high}$ neutrophils treated with PMA (**i)** and MTX, 2.5 µM (**ii**), MTX, 5 µM (**iii**) or MTX, 10 µM (**iv**).

C. Scatter plots demonstrating the frequency of $CD83^{high}$ neutrophils treated with MTX ± PMA. Data are indicated in median (IQR).

Di-iv: Representative frequency plots demonstrating the proportion of HLA-DR^{high} in neutrophils (5x10^5 cells/ml, **i**) treated with MTX, 2.5 μM (**ii**), 5 μM (**iii**), 10 μM (**iv**).

E i-iv Representative frequency plots demonstrating the proportion of HLA-DR^{high} neutrophils (5x10^5 cells/ml) treated with PMA, (**i**) and MTX, 2.5 μM (**ii**), MTX, 5 μM (**iii**) or MTX, 10 μM (**iv**).

F. Scatter plots demonstrate the frequency of HLA-DR^{high} neutrophils treated with MTX ± PMA. Data are indicated in median (IQR).

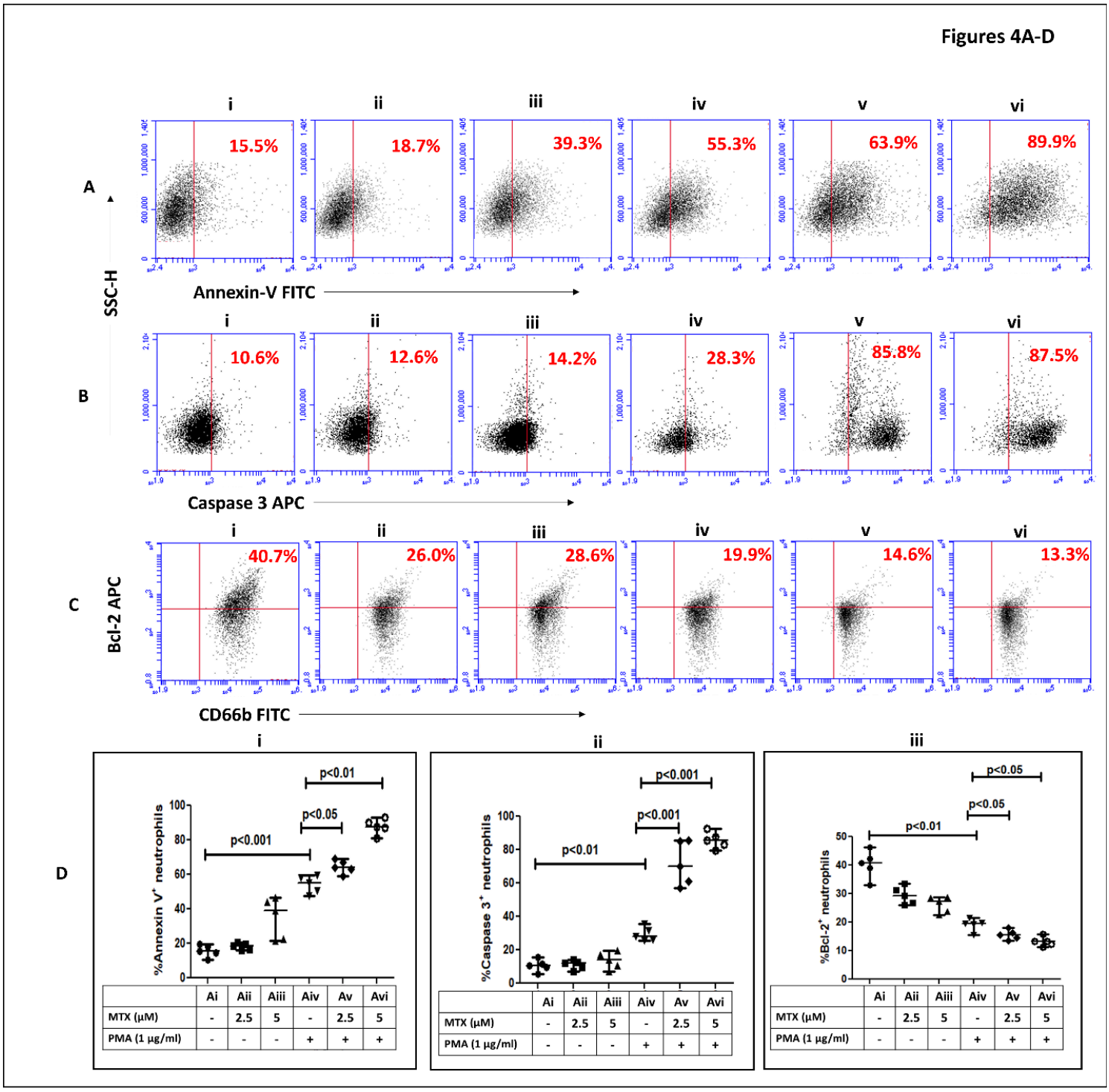


Figure 4

Effect of MTX and PMA on the status of apoptosis in neutrophils

A i-vi: Representative frequency plots demonstrating the proportion of Annexin V^+ neutrophils (n=5; $5x10^5$ cells/ml **i**) treated with MTX, 2.5 μM (**ii**), 5 μM (**iii**), or with PMA (**iv**), PMA + MTX 2.5 μM (**v**) and PMA+MTX 5 μM (**vi**).

B i-vi. Representative frequency plots demonstrating the proportion of Caspase 3^+ neutrophils (**i**) treated with MTX 2.5 μM (**ii**), 5 μM (**iii**), PMA (**iv**), PMA+MTX 2.5 μM (**v**) and PMA+MTX 5 μM (**vi**).

C i-vi. Representative frequency plots demonstrating the proportion of Anti-apoptotic Bcl-2^+ neutrophils (**i**) treated with MTX, 2.5 μM (**ii**), 5 μM (**iii**), PMA (**iv**), PMA+MTX 2.5 μM (**v**) and PMA+MTX 5 μM (**vi**).

D i-iii. Scatter plots demonstrating the frequency of Annexin V^+ (**i**), Caspase 3^+ (**ii**), Bcl-2^+ (**iii**) neutrophils treated with MTX ± PMA. Data are indicated in median (IQR).

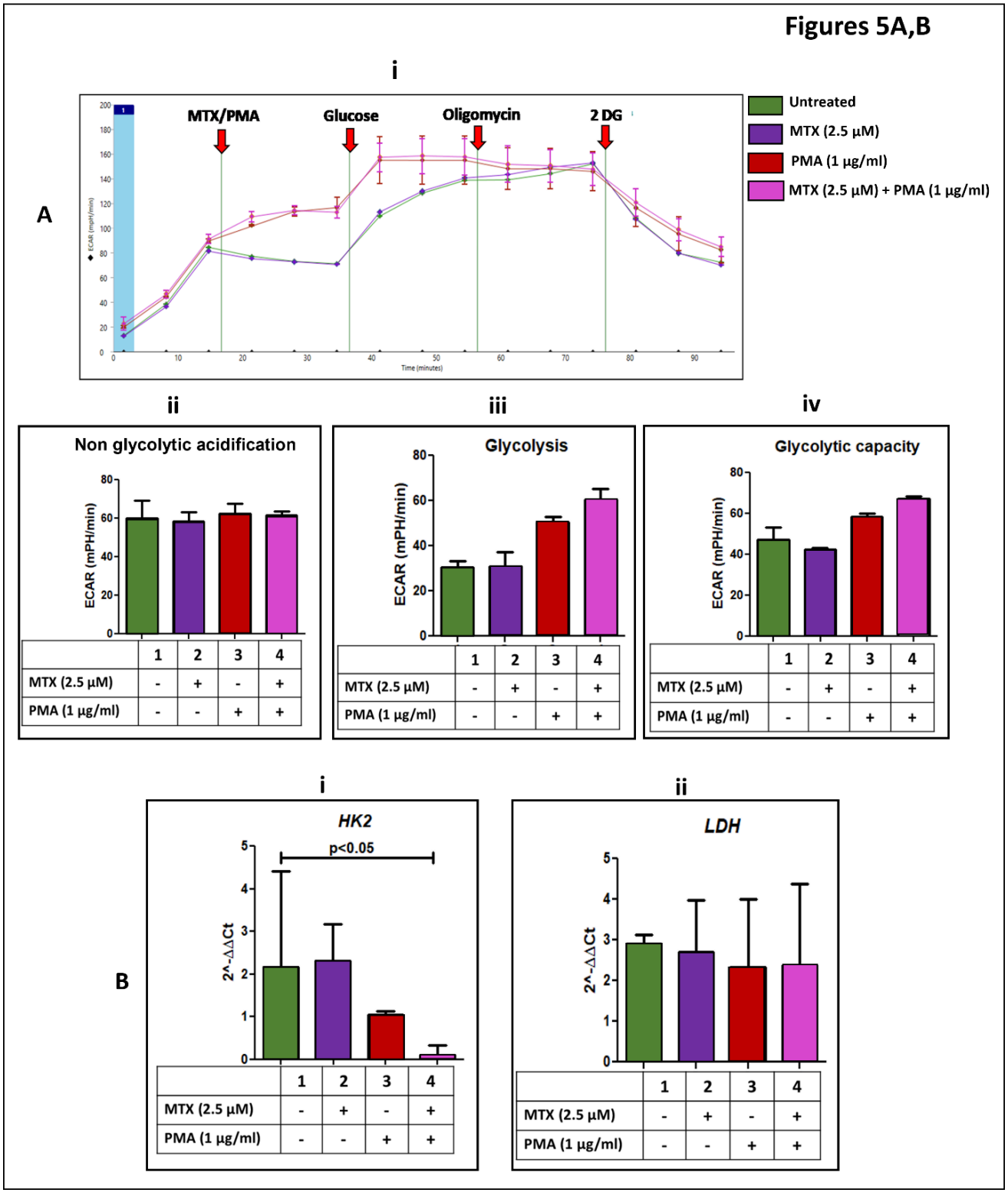


Figure 5

Impact of MTX and PMA upon glycolysis in neutrophils

Ai. Representative plots of extracellular acidification rate or glycolysis in neutrophils ($1x10^6$ cells/ml, n = 3) treated with MTX, PMA or MTX+PMA along with untreated.

Aii-iv: Bar diagrams demonstrating the levels of non-glycolytic acidification (**ii**), glycolysis status (**iii**) and glycolytic capacity (**iv**). Each experiment was done thrice in duplicate. Data are indicated in median (IQR).

Bi,ii: Bar diagram demonstrating the gene level expression of glycolysis regulatory enzymes i.e. Hexokinase or *HK2* (**i**) and Lactate dehydrogenase *LDH* (**ii**) in neutrophils treated with MTX ± PMA. Each experiment was done thrice in duplicates and data are indicated in median (IQR).

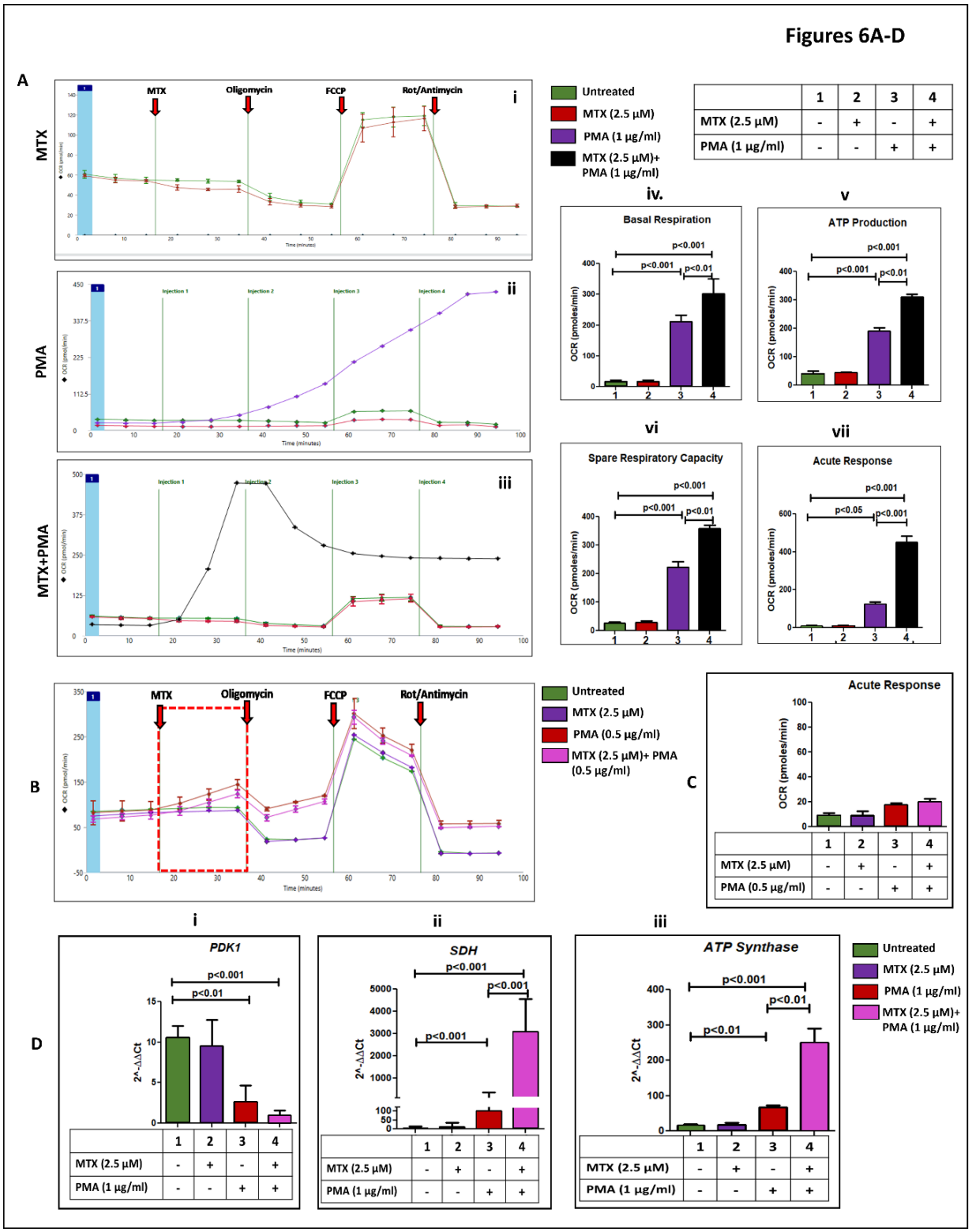


Figure 6

Impact of MTX and PMA upon oxidative phosphorylation in neutrophils:

Ai-iii. Representative plots of oxygen consumption rate (OCR) or oxidative phosphorylation in neutrophils ($1x10^6$ cells/ml, n= 3) treated with MTX (**i**), PMA (**ii**) or MTX+PMA (**iii**) along with untreated.

A iv-vii: Bar diagrams demonstrating the levels of basal respiration (**iv**), ATP production (**v**), Spare respiratory capacity (**vi**) and acute response (**vii**). Each experiment was done thrice in duplicate. Data are indicated in median(IQR).

B. Representative plot of oxygen consumption rate (OCR) or oxidative phosphorylation in neutrophils treated with MTX (**i**), lower dose of PMA i.e. 0.5 µg/ml (**ii**) or MTX+PMA (**iii**).

C. Bar diagrams demonstrating the levels of acute response in neutrophils treated with MTX± PMA. The experiments were done thrice in duplicates. Data are indicated in median (IQR).

Di-iii: Bar diagram demonstrating the gene level expression of oxidative phosphorylation regulatory enzymes i.e. Pyruvate dehydrogenase kinase or *PDK1* (**i**), Succinate dehydrogenase or *SDHA* (**ii**), *ATP synthase* (**iii**) in neutrophils treated with MTX ± PMA. Each experiment was done thrice in duplicates and data are indicated in median(IQR).

Supplementary Files

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- Supplementary1.docx