The effect of methotrexate on neutrophil reactive oxygen species and CD177 expression in rheumatoid arthritis

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Abstract

Objective

Neutrophils are found in abundance in the synovial fluid of patients with rheumatoid arthritis (RA), where they are activated and show high reactive oxygen species (ROS) production. However, there is limited data on circulating neutrophils in peripheral blood of patients with RA in terms of ROS production, expression of activation markers and the effect of treatment with methotrexate (MTX) on ROS.

Methods

This single-centre prospective study recruited patients of RA classified as per the 2010 ACR/EULAR criteria. In the cross-sectional arm, we included three groups, treatment-naïve RA (naïve-RA), MTX-treated RA (MTX-RA) and healthy controls, and compared ROS production and surface markers of neutrophil activation. In the longitudinal arm, we studied the change in neutrophil ROS production after 8 weeks of MTX treatment in naïve-RA patients. Neutrophil ROS production was measured by flow cytometry using dihydrorhodamine-123 (DHR) and by chemiluminescence using luminol. Surface expression of CD177, CD11b and CD64 was measured by flow cytometry.

Results

This study included 103 patients (50 naïve-RA, 53 MTX-RA) and 20 controls. Both naïve-RA and MTX-RA patients showed higher ROS production than healthy controls in unstimulated neutrophils in the DHR assay ($p<0.001$ and $p=0.004$). MTX-RA patients showed significantly lower ROS production than naive-RA, in both unstimulated ($p=0.004$) and PMA-stimulated neutrophils in the DHR assay ($p=0.03$). On longitudinal follow-up of 24 naïve-RA patients, there was a significant reduction of neutrophil ROS production (by 55% from baseline) ($p<0.001$) after 8 weeks of MTX. Neutrophil CD177 expression was higher in both naïve-RA and MTX-RA (trend) than controls ($p=0.001$ and $p=0.09$). MTX-RA neutrophils showed lower expression of CD177 than naïve-RA ($p=0.01$). CD11b expression was higher in MTX-RA compared to controls ($p=0.01$).

Conclusion

Circulating neutrophils in RA showed higher ROS production and higher expression of CD177 and CD11b compared to controls. MTX treatment was associated with a reduction in ROS production and CD177 expression, which may be one of the mechanisms by which MTX works in RA.

Key words

rheumatoid arthritis, methotrexate, reactive oxygen species, neutrophils, CD177, CD11b, dihydrorhodamine, longitudinal study

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Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterised by a predominant affliction of joints in the form of synovial inflammation (synovitis) (1). During the early stages of RA, neutrophils are among the first cells to be recruited to the synovial cavity (2), where they accumulate in the synovial fluid (3). These synovial neutrophils are derived from neutrophils circulating in peripheral blood, from where they migrate to joints driven by chemokines and expression of adhesion molecules (4).

In the synovial cavity, neutrophils become activated in response to the inflammatory microenvironment and immune complexes (5). These produce high amounts of reactive oxygen species (ROS) and cytokines (6). ROS and granular degradative enzymes cause damage to DNA, proteins and lipids and stimulate inflammation-associated tissue damage (7). Their association with the degree of joint inflammation, and huge abundance in synovial fluid suggest a pivotal role for neutrophils in the pathogenesis of RA (8, 9).

However, data on the characteristics of neutrophils circulating in the peripheral blood of patients with RA is less robust. Some studies have reported higher ROS production by circulating neutrophils in RA, however, others have failed to find any difference compared to controls (10). In addition, surface expression of activation markers like CD11b, CD177 and CD64 on neutrophils in RA has not been studied. Both, CD11b (an integrin subunit) and CD177 are crucial in adhesion of neutrophils to endothelial cells and their subsequent migration into the tissues. CD64 is FcγRI and may be responsible for immune-complex related activation of neutrophils.

Methotrexate (MTX) is an analogue of folic acid and was initially proposed for the treatment of RA in view of its anti-proliferative properties. However, it soon became clear that the mechanism of action of low-dose MTX in RA was anti-inflammatory rather than cytotoxic (11). One of the proposed targets of its anti-inflammatory action was neutrophils. *In vitro* data demonstrated reduced neutrophil adherence (12) and reduction of ROS production (13) with MTX. One study reported reduced neutrophil chemotaxis and migration with MTX (13). However, there are only limited studies that have evaluated the effect of MTX on circulating neutrophils in patients of RA; and even these have reported conflicting results (14-16). Furthermore, there is no prospective study that has been done in this regard

This study examined differences in neutrophil ROS production and surface expression of activation markers (CD11b, CD177 and CD64) between treatment naïve-RA, methotrexate treated RA and healthy controls. Further, we assessed the longitudinal change in neutrophil ROS production after treatment with MTX in treatment-naïve patients of RA.

Methodology

Study design

This single-centre prospective study was carried out in the Rheumatology Clinic of a University hospital in India. Patients with rheumatoid arthritis (RA) who fulfilled the 2010 American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) criteria (17) were recruited, along with healthy controls. This study followed procedures as laid down by the declaration of Helsinki for experimentation in human subjects and was approved by the Institutional Ethics Committee (PGI IEC-04/2017-624, dated 24.04.2017). An informed written consent was taken from included subjects.

In the cross-sectional arm of the study, we recruited three groups, treatment-naïve RA patients (only on low dose steroids or NSAIDs) (naive-RA), methotrexate treated patients (MTX ≥15 mg/week for ≥6 months, no other DMARD except hydroxychloroquine) (MTX-RA) and healthy controls (HC). Patients with renal insufficiency, liver disease or any recent infection were excluded. For the longitudinal arm of the study, naive-RA patients were treated with MTX, started at 15 mg/week and increased by 5 mg after 2–4 weeks.

Clinical parameters

Demographic and relevant clinical and laboratory data including age, disease

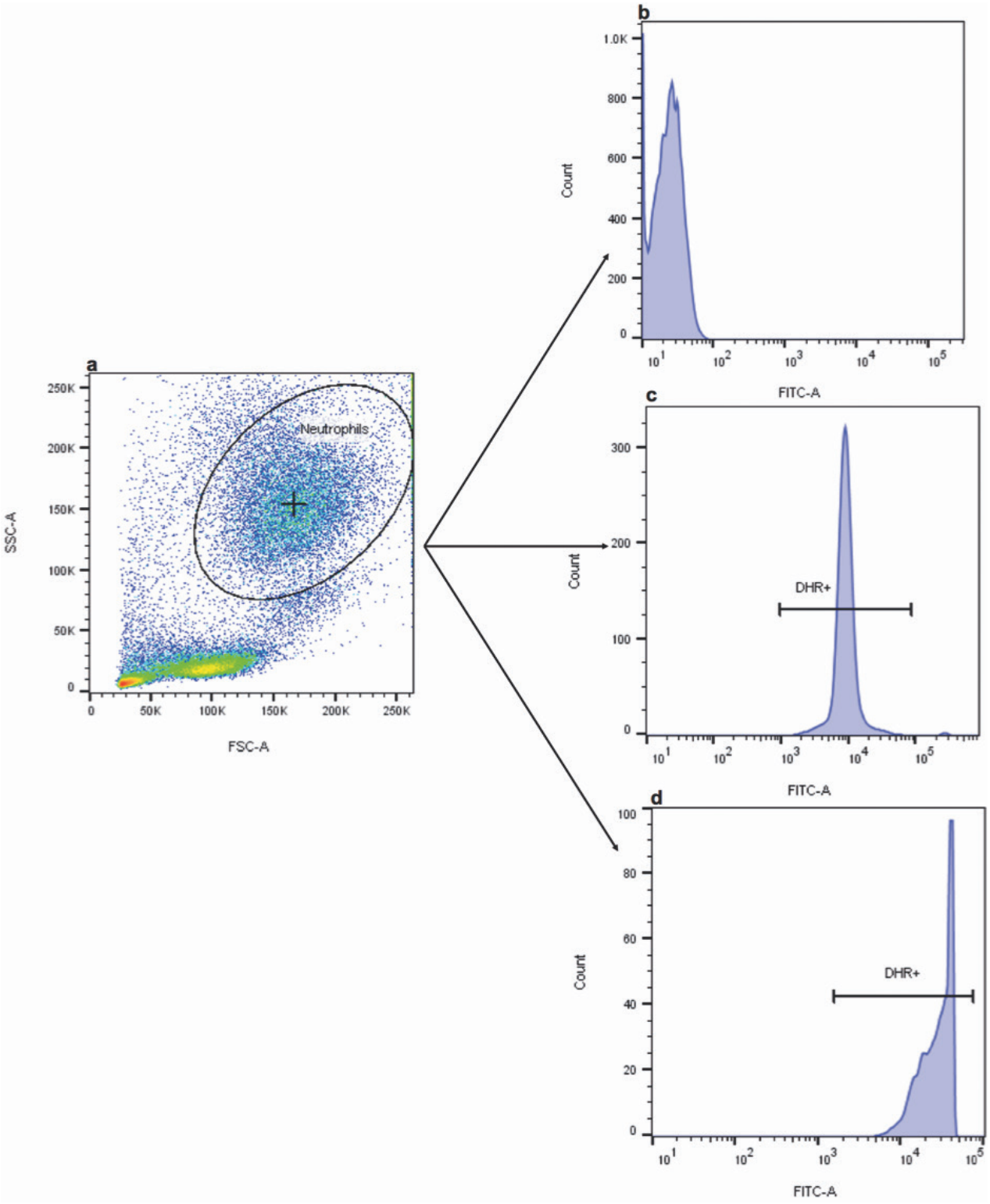

Fig. 1. Representative plots indicating gating strategy for Dihydrorhodamine 123 (DHR) staining. (**a**) Forward and Side scatter plots were used to identify Neutrophils. Median fluorescence intensities of DHR were calculated then in FITC channel after gating for (**b**) Unstained neutrophils (**c**) Unstimulated neutrophils (**d**) PMA-Stimulated neutrophils.

duration, rheumatoid factor, anti-cyclic citrullinated peptide (anti-CCP) and 28-joint counts were recorded in a predesigned pro forma. Disease activity was determined using the modified disease activity score using three variables [DAS28 (3)] (18). Functional status was assessed using the Indian validated version of the Health Assessment Questionnaire (HAQ) (19).

Sample collection and neutrophil isolation

A single-time, non-fasting, morning blood sample (7–8 mL) was collected from all recruited RA patients

and healthy controls. A second blood sample was obtained from patients in the longitudinal arm of the study after completing 8 weeks of MTX treatment. Blood samples were used for determining erythrocyte sedimentation rate (ESR) and processed for laboratory assays. In the laboratory, blood samples were subjected to red blood cell (RBC) depletion by incubating blood with HetaSep™ (STEMCELL Technologies Inc., Vancouver, Canada) for 30 minutes at 37°C (Blood: HetaSep 5:1). A part of this sample was used for Dihydrorhodamine-123 (DHR) assay and surface staining for flowcytometry. The remaining part was further processed for neutrophil isolation. For this purpose, the RBC depleted sample was layered on an equal volume of Ficoll-Paque (HiMedia Laboratories, Mumbai, India) and centrifuged at 400g for 30 minutes. After that, the cell-pellet (containing neutrophils) was separated, washed and resuspended in ammonium chloride lysis buffer (for RBC lysis) for 4 minutes at 37°C. After washing the pellet, cells were resuspended in RPMI and counted using haemocytometer to adjust the cell count to 5 x 10^6/ml. The purity of the neutrophils obtained was >95%.

ROS detection using Dihydrorhodamine 123 (DHR)

After washing RBC depleted blood samples, DHR (Sigma-Aldrich, St Louis USA) was added at a concentration of 5µg/mL and cells incubated for 5 min at 37°C. Subsequently, Phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, St Louis USA) was added at a concentration of 250 ng/ml (0.4 µM) and cells were further incubated for 15 minutes at 37°C. Cells were then washed and centrifuged. The cell pellet was finally resuspended in FACS buffer and acquired within 30 min on BD LSRFortessa™ (BD Biosciences, USA) flow cytometer. Unstained cells and unstimulated cells were used as negative controls. The gating strategy is shown in Figure 1.

ROS detection using luminol

After neutrophil isolation, 100ul of Hank's balanced salt solution (HBSS) was added to 100 ul of RPMI containing neutrophils (5 x 10^6/ml). 50µM luminol (Sigma-Aldrich, St Louis USA) was added and cells were incubated for 10 min at 37°C. Stimulation was done by adding 500 ng/ml (0.8µM) PMA or 0.25µM N-formylmethionyl-leucyl-phenylalanine (fMLP) and the plate was put in the chemiluminesce reader, Tecan infinite M200 instrument (Tecan Group Ltd, Mannedorf, Switzerland) immediately. Readings were acquired every minute for 60 minutes using the Magellan software (Tecan Group Ltd, Mannedorf, Switzerland). Area-under-curve was calculated after exporting the readings to the Microsoft Excel (Microsoft Corporation, Redmond,

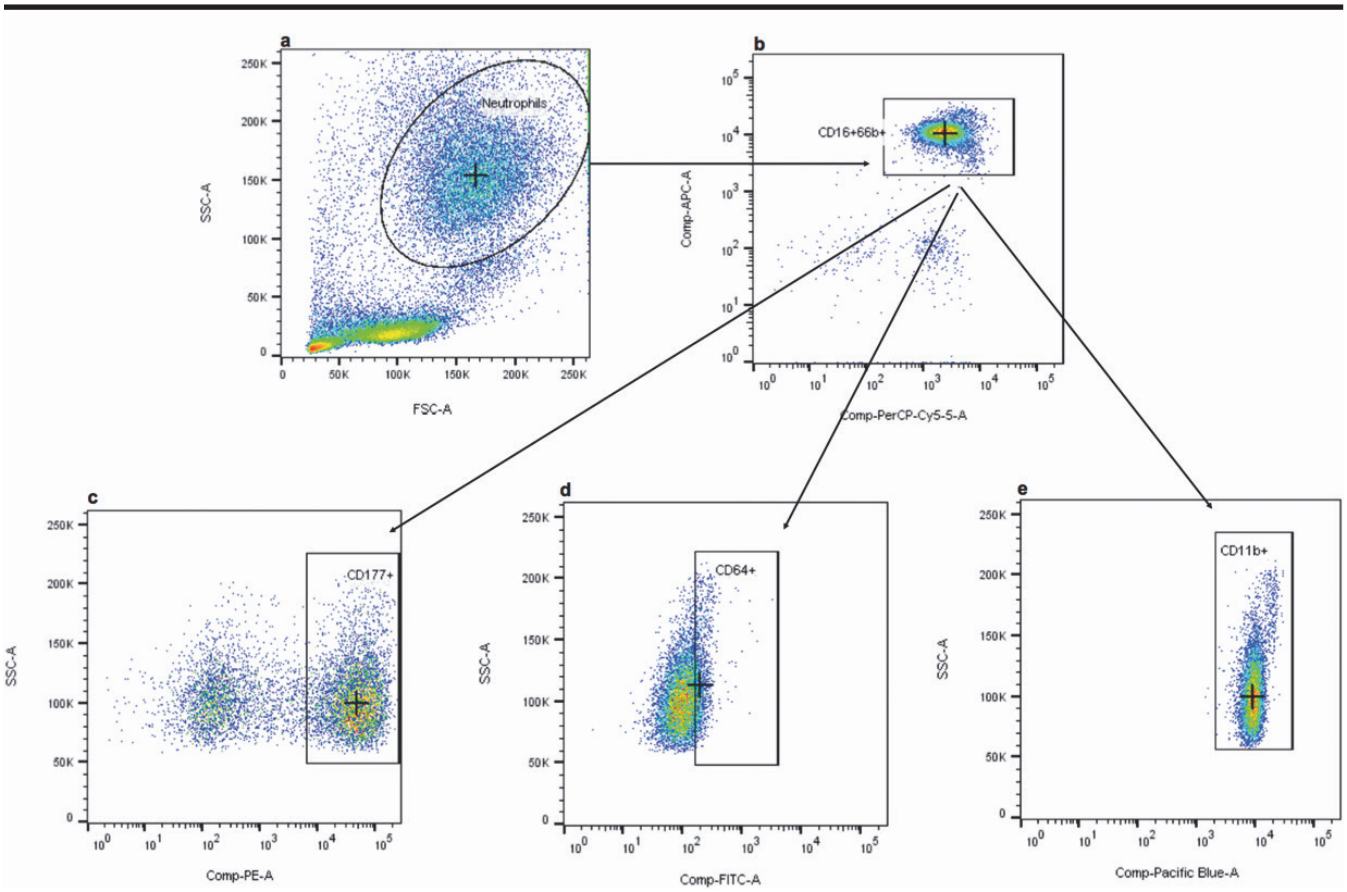

Fig. 2. Representative plots indicating gating strategy for neutrophil activation markers. Neutrophils were first identified by the (**a**) FSC and SSC profile and then validated as (**b**) $CD16^+CD66b^+$cells. Dual positive neutrophils were then gated for the presence of cell surface markers (**c**) CD177 (**d**) CD64 and (**e**) CD11b. **+** indicates median of gated population.

USA). Cells alone and those with only luminol (no PMA or fMLP) served as controls. Chemiluminescence kinetic profile is shown in Figure S1.

Assessment of activation markers on neutrophils

RBC depleted blood samples (0.1 x 10^6 cells in 100µl of PBS) were surface stained with PerCP-Cy5.5 labelled CD66b, APC labelled CD16, Pacific blue labelled CD11b, FITC labelled CD64 and PE labelled CD177 (all antibodies from BioLegend, California, USA). These were then incubated at 4°C for 20 min in the dark. Cells were washed, resuspended in FACS buffer and acquired using BD LSRFortessa™ (BD Biosciences, San Jose, USA). Single colour controls were run for compensation and fluorescence minus one controls (FMOs) were used to determine cut-off for positive events. The gating strategy is shown in Figure 2. For CD177, which is expressed on only a sub-population of neutrophils, the positive population was gated and its median fluorescence intensity (MFI) was used for analysis.

Statistical analysis

Data distribution for individual variables was examined using the Kolmogorov–Smirnov test and if significant, non-normality was accepted. Otherwise, the histogram was visually examined and skewness and kurtosis (should be between -1 to +1) were reconfirmed before assuming normality (Supplementary Table S1). Categorical variables were summarised as proportions. Continuous variables were expressed as median (interquartile range) or mean (standard deviation), as appropriate. Continuous variables were compared between groups using the two-tailed Mann-Whitney U-test (non-normal distribution) or the independent samples *t*-test (normal distribution). For paired samples, the paired t test (normal distribution) or Wilcoxon Signed Rank (non-normal distribution) test was used to determined statistical significance. Data were analysed using GraphPad Prism (GraphPad Software, CA, USA). A *p*-value <0.05 was considered statistically significant.

Table I. Baseline characteristics of the patients of rheumatoid arthritis recruited in the study.

Parameter[a]	Naïve-RA n=50	MTX-RA n=53	*p*-value
Age, years	43.8 ± 12.3	47.6 ± 10.1	0.1
Disease duration, years, median (IQR)	2 (1–4)	4 (2–8)	0.06
Tender joint count (0-28)	15 ± 8	8 ± 6	<0.001
Swollen joint count (0-28), median (IQR)	4 (2–10)	2 (0–3)	<0.001
ESR (0-180), mm 1st hour	83 ± 46	58 ± 37	0.003
I-HAQ (0-3)	1.2 ± 0.6	0.7 ± 0.6	<0.001
RF positive, n (%)	36/41(88)	40/51 (78)	0.3
Anti CCP positive, n (%)	28/35 (80)	24/42 (57)	0.4
DAS-28 (3)	6.3 ± 1.2	4.9 ± 1.3	<0.001
Methotrexate dose mg/week	-	18.7 ± 4.7	-

[a] Mean±SD except where specified; Naïve-RA: treatment naïve RA patients (not on MTX), MTX-RA: methotrexate treated RA patients (≥15 mg/week at least 6 months); ESR: erythrocyte sedimentation rate, westegren; I-HAQ: Indian health assessment questionnaire disability; RF: Rheumatoid factor; CCP: cyclic citrullinated peptide; DAS-28 (3): disease activity score using 28 joints and three variables.

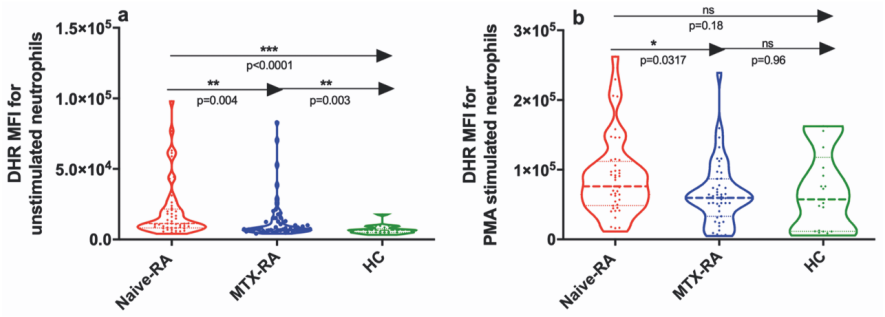

Fig. 3. Representative violin plots showing the ROS production in neutrophils of different groups using Dihydro-rhodamine (DHR). Flow cytometry analysis was performed on DHR stained (**a**) unstimulated and (**b**) PMA stimulated neutrophils for naive patients (Naive-RA), methotrexate treated patients (MTX-RA) and healthy controls (HC). The median in the graph is represented by the bold horizontal dashed line within the box, and the non-bold horizontal dotted lines indicate interquartile range. Two-tailed Mann-Whitney U-test was used for analysis; *p<0.05; **p<0.01; ***p<0.001.

Results

Patient characteristics

A total of 103 patients with RA and 20 healthy controls were recruited in this study. This included 50 patients of naive-RA (F:M=41:9) and 53 patients of MTX-RA (F:M=43:10). Baseline characteristics of these patients have been summarised in Table I. There was no significant difference in the mean age of patients between the two groups. DAS28(3) was significantly higher in naïve-RA compared to the MTX-RA patients (p<0.001). Among the naïve-RA patients, one patient was on low-dose prednisolone and hydroxychloroquine respectively.

Assessment of neutrophil ROS production

Both naïve-RA and MTX-RA patients showed higher ROS production (median MFI) than healthy controls in unstimulated neutrophils in the DHR assay (11290, 7301, 6555; p<0.001 and p=0.004 respectively) (Fig. 3a). MTX-RA patients had significantly lower ROS production (median MFI) than naive-RA, in both unstimulated (p=0.004) and PMA-stimulated neutrophils in the DHR assay (59637, 76133 p=0.03) (Table II, Fig. 3b). There was no significant difference in ROS production as measured by the chemiluminescence assay (Table II, Fig. S2).

Assessment of neutrophil activation markers

Neutrophil CD177 expression (median MFI) was higher in both naïve-RA and MTX-RA (trend-to) compared to controls (49385, 33505, 26855, p=0.001 and p=0.09 respectively). It was significantly lower in MTX-RA compared to naive-RA (p=0.01, Fig. 4a). Neutrophil CD11b expression (median MFI) was significantly higher in MTX-RA compared to controls (5535, 3710, p=0.03),

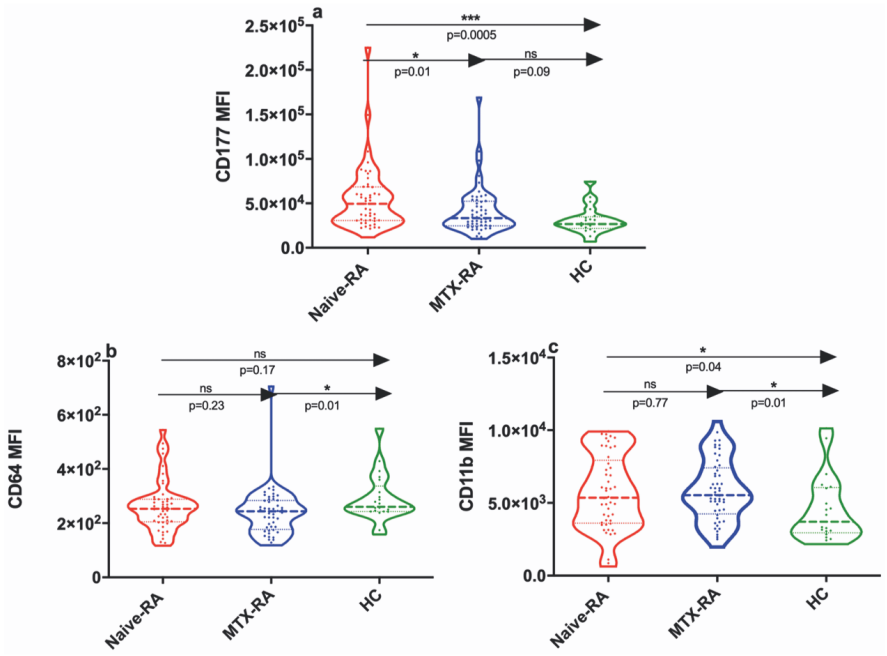

Fig. 4. Representative violin plots showing neutrophil activation marker profile of different groups. Flow cytometry analysis indicating (**a**) CD177 MFI (**b**) CD64 MFI and (**c**) CD11b MFI in neutrophils of naive patients (Naive-RA), methotrexate treated patients (MTX-RA) and healthy controls (HC). The median in the graph is represented by the bold horizontal dashed line within the box, and the non-bold horizontal dotted lines indicate interquartile range.
Two-tailed Mann-Whitney U-test was used for analysis; *p<0.05; **p<0.01; ***p<0.001.

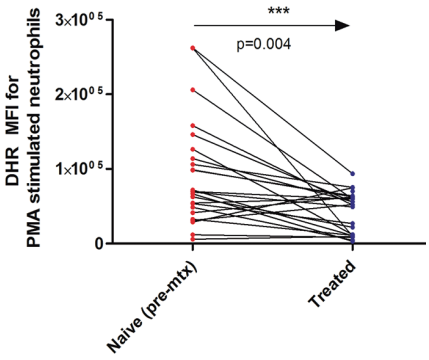

Fig. 5. Before-after dot-and-line plots showing the change in ROS production in neutrophils after treatment with methotrexate. Flow cytometry analysis was performed on DHR stained PMA stimulated neutrophils of naïve-RA patients before methotrexate treatment (pre-MTX) and after 8 weeks of methotrexate treatment (Treated). Two-tailed Wilcoxon-signed rank test was used for analysis; *p<0.05; **p<0.01; ***p<0.001.

and showed a trend-to-significance in naïve RA compared to controls (p=0.10). However, the expression of CD11b (median MFI) was not significantly different between naïve-RA and MTX-RA patients (p=0.66) (Fig. 4c). There was no significant difference in CD64 expression (median MFI) between naïve-RA and controls (p=0.17), however, MTX-RA had a lower expression compared to controls (240, 260, p=0.02) (Table II, Fig. 4b).

Longitudinal change in ROS production with MTX treatment

In the longitudinal arm, 24 naïve-RA were included. After 8-weeks of MTX treatment, mean (SD) DAS28(3) declined from 6.1 (1.0) to 5.1 (1.0) (p<0.001). Correspondingly, there was a significant decline in neutrophil ROS production post PMA stimulation in the DHR assay. ROS production declined by 55% from mean (SD) MFI of 92781 (70870) to 41536 (27496) (p=0.001) (Fig. 5). There was also a trend-to decline in ROS production in unstimulated neutrophils in DHR assay (p=0.09). However, there was no significant change in the ROS production detected in the luminol assay (data not shown).

Discussion

This study found higher ROS production in unstimulated neutrophils of patients with RA compared to healthy controls. This is consistent with findings of Eggelton *et al*. who found circulating neutrophils in RA to be in a 'primed state of readiness' with greater superoxide production upon fMLP stimulation compared to controls (10). In addition, we found higher surface expression of CD177 and CD11b in neutrophils of RA patients than controls. Studies pertaining to surface activation markers in circulating neutrophils in RA are very limited. CD11b is an integrin subunit, essential for chemotaxis and emigration of neutrophils and has been shown to be increased by other studies as well (20), probably mediated through high cytokines or NET (21) and may be a potential target in RA, as shown in animal models. (22) CD177 has been shown to modulate neutrophil migration by interacting with integrins like CD11b (23). A previous study also reported higher expression of CD177 in RA (24). We also studied CD64 (FC-γR1), but found lower levels in MTX-RA than healthy controls. This is consistent with a previous study which found that CD64 expression was increased in only synovial and not circulating neturophils in RA (25).

The reasons for activation and high ROS production in circulating neutrophils in RA may be related to the elevated levels of cytokines (like GM-CSF and TNF-α) or stimulation by circulating immune complexes (rheumatoid factor) through FcgammaRIIIb (26). These circulating neutrophils migrate to the synovial cavities and continue to produce high amount of ROS that may be responsible for cartilage and bone destruction (27). Thus, measures to reduce ROS may be important in reducing inflammation and preventing joint damage.

MTX is the bench-mark for efficacy and safety among all disease-modifying anti-rheumatic drugs (DMARDs) including conventional, biological and small molecule DMARDs (28). Its mechanism of action in RA remains to be fully elucidated, but is thought to be mainly anti-inflammatory that may be mediated by an effect on neutrophils (29). This study found neutrophils obtained from the circulating peripheral blood of MTX-treated RA patients showed lower ROS production than those of treatment-naïve patients. This is consistent with an initial report by Laurindo *et al*. that showed a reduction in superoxide production, as measured

Table II. Summary measures of the various laboratory tests done in the study.

	Naïve-RA (n=50)	MTX-RA (n=53)	Healthy controls (n=20)	*p*-value naïve-RA *vs*. HC	*p*-value MTX-RA *vs*. HC	*p*-value naïve-RA *vs*. MTX-RA
DHR unstimulated, MFI, median (IQR)	11290 (8222–21617)	7301 (6513–14054)	6555 (4997–7747)	***p***<0.001	***p***=0.004	***p***=0.004
DHR PMA stimulated, MFI, median (IQR)	76133 (48705–111833)	59637 (33229–86923)	57322 (11559–117524)	0.180	0.963	0.032
Luminol unstimulated, mean (SD) (x 10^3)	177 (182)	161 (182)	117 (74)	0.154	0.724	0.493
Luminol PMA stimulated, ALU, mean (SD) (x 10^3)	1880 (1336)	1703 (995)	2029 (1034)	0.480	0.219	0.668
Luminol fmlp stimulated, ALU, mean (SD) (x 10^3)	329 (356)	322 (340)	281 (177)	0.650	0.577	0.807
CD177, MFI, median (IQR)	49385 (30750–68400)	33505 (24755–52405)	26855 (21815–34965)	0.001	0.093	0.013
CD11b, MFI, mean (SD)	5630 (2630)	5840 (2130)	4540 (2230)	0.085	0.013	0.664
CD64, MFI, median (IQR)	250 (205–290)	240 (180–280)	260 (240–340)	0.172	0.016	0.228

by ferricytochrome c reduction, with MTX treatment (14). Some early studies had failed to detect this effect of MTX possibly due to the lower dose of MTX used in patients at that time (16). A similar reduction in neutrophil ROS production with MTX has also been reported in adjuvant-induced arthritis in rats (30). The current study extends these findings and also corroborates them in the longitudinal arm of the study, where the neutrophil ROS production was found to decline by more than 50% after 8 weeks of MTX monotherapy.

This reduction in ROS by MTX does not seem to be a by-stander effect of general dampening of inflammation *per se*. Okuda *et al*. demonstrated that *in-vitro* MTX exposure reduced superoxide generation in both unprimed neutrophils and those primed by tumour necrosis factor-alpha (TNF-α) or bacterial lipopolysaccharide (31). The exact mechanism is not established, but seems to be mediated through adenosine. In a seminal study in 1983, Cronstein *et al*. showed that adenosine was a modulator of neutrophil ROS (32). Subsequently, they demonstrated *in-vitro* that methotrexate led to diminished adherence of neutrophils that was mediated by adenosine (12). Due to technical problems and its short half-life, adenosine could not be measured in this study. Interestingly, the anti-TNF-α biological adalimumab does not reduce neutrophil ROS production (33). This may be one of the reasons underlying the synergistic efficacy of anti-TNF-α drugs and MTX in RA.

The merits of this study include an analysis of key surface markers of neutrophil activation, measurement of ROS production by two standard methods and having a longitudinal arm in the study. However, this study suffers from some limitations. We could not demonstrate any changes in ROS by luminol chemiluminescence assay. This may relate to the inherent differences between the DHR and the luminol assays. DHR is a membrane permeant dye, which preferentially reacts with peroxynitrities intracellularly, and gets oxidised to rhodamine (in many steps) to give off fluorescence (34). On the other hand, luminol preferentially reacts with hydrogen peroxide; and also with a host of free radicals. Unlike DHR, luminol detects both extracellular and intracellular ROS (35). Finally, the higher PMA concentration used in the luminol assay could have overwhelmed any differences. Another limitation of the study was that changes in surface activation markers were not studied in the longitudinal arm.

To conclude, this study found higher ROS production, CD11b expression and CD177 expression in circulating neutrophils of patients with RA compared to healthy controls. Importantly, MTX treated patients showed lower ROS production and CD177 expression. These changes could underlie one of the ways by which MTX acts in RA.

Acknowledgements

Methotrexate tablets were obtained as a gift from IPCA laboratories, Mumbai, India.

Key messages

- Circulating neutrophils from patients of rheumatoid arthritis showed higher production of reactive oxygen species (ROS) and higher expression of CD177 and CD11b compared to healthy controls.
- Methotrexate treatment was associated with a significant reduction in ROS production and expression of CD177 in circulating neutrophils of patients with RA, and may be one mechanism by which it works in RA.

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