

## Delayed neutrophil apoptosis in very early rheumatoid arthritis patients is abrogated by methotrexate therapy

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**Key words:** Rheumatoid arthritis, neutrophils, apoptosis, methotrexate.

### Abbreviations:

FCS: foetal calf serum  
mAb: monoclonal antibody  
PBS: phosphate-buffered saline  
PMN: polymorphonuclear leukocytes  
VERA: very early rheumatoid arthritis  
MTX: methotrexate  
RA: rheumatoid arthritis

Competing interests: none declared.

### ABSTRACT

**Objectives.** To analyse the activation state and apoptosis of circulating neutrophils in untreated very early rheumatoid arthritis (VERA) and after exposure to low dose corticosteroids and methotrexate (MTX).

**Methods.** Neutrophils were isolated from the peripheral blood of VERA patients at 3 different times: before any treatment was started, 2 weeks after starting a low dose of prednisone (5-10 mg) and 4 months after reaching more than 20mg/week of MTX. The expression of different activation markers (CD11b, CD64, CD86 and CD69) in freshly isolated neutrophils was analysed by flow cytometry. Apoptosis was measured by the loss of DNA content, which was analysed by flow cytometry using propidium iodide.

**Results.** Compared to neutrophils from healthy controls, we have found a delayed neutrophil apoptosis within 6 h and 22 h of cultured polymorphonuclear leukocytes (PMN) derived from VERA patients without any treatment or treated with corticosteroids. The delay of PMN apoptosis was restored to control levels after treatment with MTX.

**Conclusion.** The treatment of VERA patients with corticosteroids did not affect the delay of neutrophil apoptosis. However, delayed apoptosis was restored to control levels after treatment with low dose MTX, which highlights the importance of early RA treatment with MTX.

### Introduction

Rheumatoid arthritis (RA) is a severe, chronic inflammatory disease characterized by an inflammatory immune response that leads to joint damage and destruction, which has been mainly explained as a T cell centred disorder, with tight interactions between these cells, macrophages and B lymphocytes (1, 2). However, there is also evidence for a relevant role of neutrophils in the early stages of RA pathogenesis. In fact, neutrophils can produce cytokines, chemokines and complement and also express Fc receptors, all critical players for inflammation onset and persistence (3). Neutrophils and their products contribute to pannus formation, destruc-

tion of the collagen matrix (4) as well as the polysaccharides of the cartilage (5) by production of reactive oxygen species (6) and proteolytic enzymes (7). Of particular relevance for a hypothetical early role of neutrophils in RA pathophysiology is the result of the depletion of neutrophils in a mice model of arthritis, which causes resistance to induced inflammation (8). On the other hand, circulating neutrophils rapidly die by spontaneous apoptosis and, consequently, their potential deleterious effect is largely dependent on the balance between neutrophil survival and their clearance (3), controlled by the expression of apoptosis associated proteins, which are affected by the cytokine milieu (9). In accordance with this the suppression of neutrophil viability by enhancement of apoptosis blocks their susceptibility to immune-complex-mediated arthritis and to thioglycol-induced peritonitis (10).

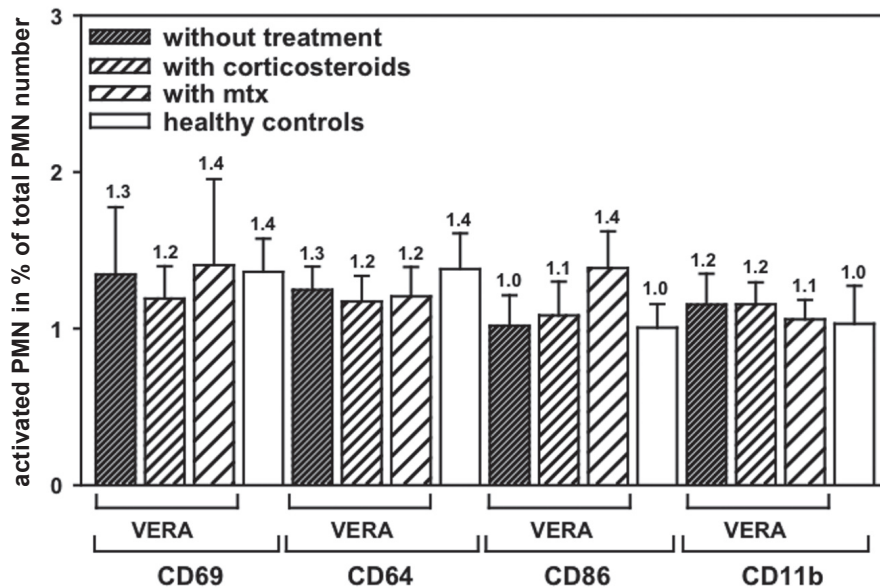
In this study, we have characterized the activation state and apoptosis of circulating neutrophils from very early RA (VERA) patients. We have found a delayed neutrophil apoptosis which was normalised after methotrexate (MTX) treatment.

### Materials and methods

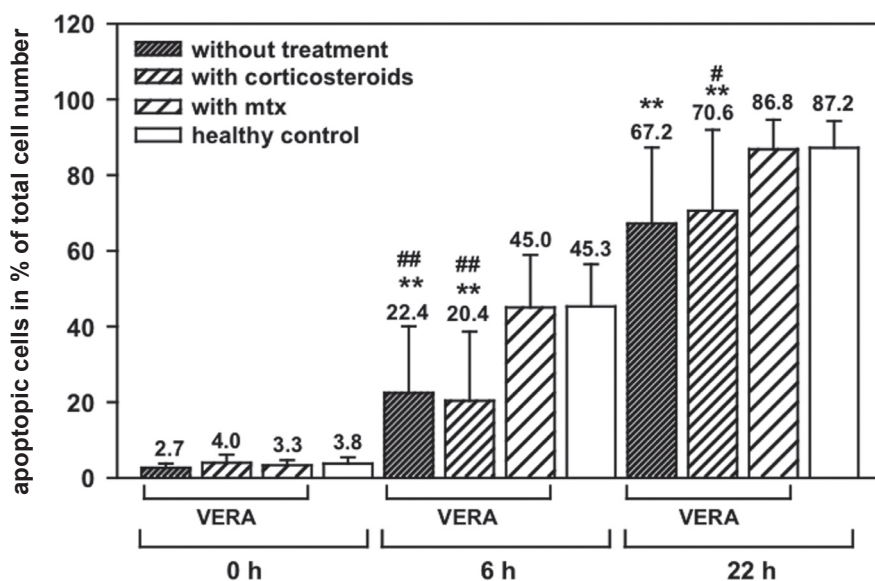
#### Patients

Neutrophils were isolated from peripheral blood of 14 polyarthritis patients with less than 6 weeks of disease duration, who latter fulfilled the American College of Rheumatology criteria for RA (VERA), at 3 different times: before any treatment was started, 2 weeks after starting a low dose of oral prednisone (5-10 mg) and, for those who had an unremitting course, 4 months after reaching more than 20mg/week of oral MTX. Disease activity was assessed by the Disease Activity Score (DAS28) and the functional status was evaluated with the Health Assessment Questionnaire (HAQ).

Neutrophils from 15 healthy donors were used as controls. The local ethics committee approved the study and patients signed an informed consent. Patient management was done in accordance with the standard practice and the study was conducted in accordance with regulations governing clinical tri-



**Fig. 1.** Activated PMN from peripheral blood. Flow cytometry analysis of the activation state of whole blood PMN from healthy controls and very early rheumatoid arthritis (VERA) patients. PMN activation was measured by detecting the expression of CD69, CD64, and CD86 and over expression of CD11b. No differences were found in the percentage of activated PMN between patients at the different time-points and controls. Data represent mean values of positive PMN  $\pm$  standard deviation.



**Fig. 2.** Delayed apoptosis in blood PMN from VERA patients. Analysis of DNA content of isolated blood PMN from healthy controls ( $n = 15$ ) and very early rheumatoid arthritis (VERA) patients before ( $n = 12$ , due to technical reasons, two of the samples were not available) and after treatment with corticosteroids ( $n = 14$ ) and MTX ( $n = 9$ ). Data represent mean values of apoptotic PMN  $\pm$  standard deviation. Symbols used: ## ( $p < 0.01$ ) and # ( $p < 0.02$ ) represent the comparison between VERA patients before and after treatment with corticosteroids vs. VERA patients treated with MTX; \*\* ( $p < 0.01$ ) represents the comparison between VERA patients before and after treatment with corticosteroids vs. healthy controls.

als such as the Declaration of Helsinki as amended in Edinburgh (2000).

#### Isolation and culture of PMN

Blood PMN was isolated after spontaneous erythrocyte sedimentation on a

discontinuous Percoll<sup>™</sup> gradient as previously described (11). PMN viability was  $> 99\%$  as assessed by the trypan blue exclusion test; purity was  $> 98\%$  as analysed by flow cytometry. PMN were cultured in RPMI-1640 medium sup-

plemented with 10% foetal calf serum (FCS) and antibiotics (50 U/ml penicillin, 50  $\mu$ g/ml streptomycin) in 5% CO<sub>2</sub> at 37°C.

#### Analysis of PMN activation

PMN were incubated for 1 h at 4°C in the dark with the FITC-conjugated anti-CD11b mAb, anti-CD64 mAb and anti-CD86 (indirect labelled with goat anti mouse PE-conjugated), FITC-conjugated anti-CD66b and PE-conjugated anti-CD69. Subsequently, samples were treated for 10 min in the dark with 500  $\mu$ l of FACS<sup>™</sup> lysing solution (Becton Dickinson), washed twice with PBS/1 mM EDTA and subjected to flow cytometry. In each sample, 10<sup>4</sup> cells were counted, and analysed using Cell Quest software (Becton Dickinson, USA).

#### Analysis of DNA content

DNA content was analysed by flow cytometry (FACSCalibur, Becton Dickinson, USA) using propidium iodide. PMN ( $5 \times 10^5$ ) were suspended in 70% ethanol. PMN were permeabilized over night at -20°C, washed with PBS/1 mM EDTA and suspended in 300  $\mu$ l of the same buffer. After addition of 20  $\mu$ g/ml RNase and 50  $\mu$ g/ml propidium iodide (final concentrations), samples were incubated for 15 min at room temperature and kept at 4°C until flow cytometric analysis. In each sample, 10<sup>4</sup> cells were counted and analysed using Cell Quest software (Becton Dickinson, USA).

#### Antibodies

PE/FITC-conjugated anti-human mAb: CD69, CD11b, CD66b and isotype controls were obtained from Immunotools, (Friesoythe, Germany). Purified anti-CD64 and anti-CD86 mAb was obtained from Chemicon (Germany) and the goat F(ab')<sub>2</sub> anti-mouse immunoglobulins/RPE-conjugated was obtained from DAKO (Denmark).

#### Reagents

Propidium iodide was obtained from Calbiochem, United Kingdom. RNase was obtained from Roche, Germany. Percoll was obtained from Amersham Biosciences, Sweden. Buffers, cell culture media, antibiotics and FCS were obtained from Gibco, United Kingdom.

### Statistical analysis

The data shown represent mean  $\pm$  standard deviation (SD) where applicable. Statistical significance was determined using student's *t*-test and  $p < 0.05$  was considered significant. Unpaired student's *t*-test was calculated between control and patients at each time-point and paired student's *t*-test between the same patients at different time-points.

### Results

The patients enrolled in this study showed a significant improvement in disease activity as measured by DAS28 and in physical function as assessed by the HAQ score. In fact, mean ( $\pm$  SD) baseline DAS28 was  $5.4 \pm 1.6$ , which was reduced to  $4.3 \pm 1.9$  after prednisone treatment and to  $3.4 \pm 2.1$  after maximum MTX effect. Additionally, the HAQ score also improved from a mean baseline of  $1.2 \pm 0.6$  down to a final score of  $0.7 \pm 0.7$ .

We analysed the different leukocyte population counts of whole blood samples from VERA patients and controls by flow cytometry. We found no differences in leukocyte populations between VERA patients and healthy controls (data not shown).

To find out whether neutrophils in VERA patients are primed in the peripheral blood, we analysed the expression of different activation markers (CD11b, CD64, CD86 and CD69) in freshly isolated neutrophils which were analysed by flow cytometry. The results of PMN activation state and the amount of activated PMN were similar in controls and patients with and without treatment (Fig. 1).

We analysed the spontaneous neutrophil apoptosis to find out whether the ongoing inflammation could affect this process. Apoptosis of freshly isolated neutrophils (0h) was similar in controls and patients. However, spontaneous neutrophil apoptosis in culture at 6 h and 22 h was significantly delayed in patients without treatment compared to healthy controls. After 6h, only 22.4%

apoptotic PMN were found in VERA patients, whereas in healthy controls there were 45.3%. After 22 h VERA patients PMN were 67.2% apoptotic compared to 87.2% in the control group. The treatment with low doses of corticosteroids did not induce significant changes in neutrophil apoptosis as compared to the behaviour of PMN from untreated patients (Fig. 2). Interestingly, there was an abrogation of the delayed neutrophil apoptosis at 6 h and 22 h after treatment for 4 months with MTX (Fig. 2).

### Discussion

We have shown a decrease of spontaneous apoptosis in circulating neutrophils from VERA patients as compared to healthy controls. This is in accordance with a delayed synovial leukocyte apoptosis that was recently described in RA patients with less than 12 weeks of disease duration (12). In addition, we have found that this effect was abrogated by MTX, but not by a low dose of corticosteroids. These results are also corroborated by previous reports suggesting that MTX, *in vitro*, increases apoptosis of peripheral blood leukocytes from healthy donors, without a synergistic effect from the addition of corticosteroids (13, 14).

The delay observed in PMN apoptosis can be the result of proinflammatory cytokines, such as tumour necrosis factor- $\alpha$  (15), and may create the appropriate conditions for an inflammatory vicious cycle that might contribute to the self-perpetuation of an acute arthritis episode. The ability of MTX to restore the normal survival time of circulating neutrophils, which paralleled the improvement in disease activity and functional impairment, highlights the importance of early MTX introduction to maximize the chance of disease remission.

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