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# Activation status of peripheral blood neutrophils and the complement system in adult rheumatoid arthritis patients undergoing combined therapy with infliximab and methotrexate

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## Abstract

We examined the functional activity of peripheral blood neutrophils and the complement system activation status in patients with rheumatoid arthritis (RA) undergoing infliximab/methotrexate combined therapy. We studied female RA patients under treatment with infliximab (3–5 mg/kg) and methotrexate (15–25 mg/week) who presented inactive (i-RA; $n = 34$, DAS-28 $\leq 2.6$) or at least moderately active disease (a-RA; $n = 29$, DAS-28 $> 3.2$), and age-matched healthy women ($n = 38$). We measured the levels of reactive oxygen species (ROS) generation (chemiluminescence assay) and membrane expression of FcγRIIa/CD32, FcγRIIIb/CD16, CR1/CD35, and CR3/CD11b receptors (ELISA assay) in neutrophils. We also determined the hemolytic activity of the alternative and classical pathways of the complement system (spectrophotometry), serum levels of C5a and Bb (ELISA assay), and serum chemotactic activity (Boyden chamber). Compared with the control group, i-RA and a-RA patients exhibited: (1) increased neutrophil ROS production and membrane expression of FcγRIIa/CD32, FcγRIIIb/CD16, and CR1/CD35, indicating neutrophil activation; and (2) increased serum chemotactic activity and decreased activity of the alternative complement pathway, indicating systemic complement system activation. The levels of C-reactive protein in a-RA patients were augmented, compared with i-RA patients. Although infliximab/methotrexate combined therapy induced disease remission according to the DAS-28 criteria, both i-RA and a-RA patients still exhibited significant levels of systemic activation of neutrophils and the complement system.

**Keywords** Rheumatoid arthritis · Infliximab · Methotrexate · Neutrophil · Complement system

## Abbreviations

| | |
|---|---|
| Anti-CCP | Anti-cyclic citrullinated peptide antibody |
| CHS | Serum from healthy control subject |
| CL | Chemiluminescence |
| CR | Complement receptor |
| CRP | C-reactive protein |
| DAS-28 | Disease activity score of 28 joints |
| DMARD | Disease-modifying anti-rheumatic drug |
| EULAR | European League against Rheumatism |
| FcγR | Fcγ receptors |
| FITC | Fluorescein isothiocyanate |
| HA | Hemolytic activity |
| IC | Immune complex |
| IFM | Infliximab |
| MFI | Median fluorescence intensity |
| MTX | Methotrexate |
| PE | Phycoerythrin |
| RA | Rheumatoid arthritis |
| a-RA | Active rheumatoid arthritis |

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| | |
|---|---|
| i-RA | Inactive rheumatoid arthritis |
| RAHS | Serum from patient with rheumatoid arthritis |
| ROS | Reactive oxygen species |
| TNF-α | Tumor necrosis factor-α |

## Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease that causes joint damage and long-term disability. Pathogenesis of RA involves a complex interplay among environmental, genetic, and immunologic factors, including dysregulation of innate and adaptive immune function and loss of tolerance to self-antigens [1, 2]. Some key features of RA are swollen joints and the growth of an invasive and inflammatory tissue or pannus across the surface of synovial joints, consisting of activated fibroblasts, macrophages, lymphocytes, and neutrophils. Synovial neutrophils secrete collagen-degrading enzymes and inflammatory mediators that activate osteoclasts to resorb bone and cause irreversible joint destruction [3].

The early diagnosis of RA and early onset of treatment are essential to control disease activity and prevent joint disability [1]. The current drug therapy for RA relies on nonsteroidal anti-inflammatory drugs, glucocorticoids, and disease-modifying anti-rheumatic drugs (DMARD) that should be prescribed to the patients as soon as the disease is diagnosed. Methotrexate (MTX) is the gold-standard DMARD for treating RA and is usually well-tolerated, reduces disease signs and symptoms, and improves joint function. When MTX monotherapy fails, rheumatologists associate other DMARD or biological agents, especially anti-tumor necrosis factor-α (TNF-α) antibodies [4–8]. The combined therapy with MTX and anti-TNF-α antibodies has revolutionized the treatment of RA by providing significant clinical, structural, and functional improvement [9]. Three TNF-α-blocking agents are under clinical use in Brazil: infliximab (IFM; Remicade®, an anti-TNF-α chimeric monoclonal antibody), etanercept (Enbrel®, a soluble TNF-α receptor), and adalimumab (Humira®, an anti-TNF-α humanized monoclonal antibody) [10]. The anti-TNF-α therapy effectively alleviates the inflammation signs and joint damage in about 66% of the patients with RA [11, 12].

TNF-α acts in concert with other chemotactic factors to recruit circulating leukocytes to the synovia. In neutrophils, TNF-α triggers the generation of reactive oxygen species (ROS) that are released to the extracellular milieu together with intracellular proteolytic enzymes and participate in the onset, progression, and perpetuation of joint damage in RA [13]. The active phase of the disease is characterized by increased neutrophil mobilization from bone marrow and a predominant and continuous influx of these cells to the synovia, as demonstrated in animal models and patients with RA [14, 15].

The local and systemic activation of the complement system also contributes to the pathogenesis of RA and other inflammatory human diseases, such as systemic lupus erythematosus [16, 18, 25, 46]. Components of the activated complement system opsonize soluble and tissue-bound immune complexes (ICs), as well as they prime, chemoattract, and activate circulating and synovial neutrophils [18]. Although several studies have reported the complement system activation at the synovial level, just few studies have examined its systemic activation in patients with RA stratified according to disease activity [17].

We have previously reported that neutrophils from MTX-treated patients with active RA exhibit augmented levels of systemic activation, chemotactic capacity, ROS generation, and Fcγ and complement receptor (FcγR and CR, respectively) expression [19]. To continue investigating the impact of drug therapy on patients with RA, we examined the functional responsiveness of neutrophils (respiratory burst, chemotaxis, and expression of membrane receptors) and the activation status of the complement system (classical and alternative pathway) in MTX-non-responder patients treated with combined therapy with IFM and MTX.

## Materials and methods

### Patients and healthy subjects

This study enrolled 63 women aged 27–58 years (mean = 45 years), with established RA—34 with inactive disease (i-RA) and 29 with active disease (a-RA)—who were followed-up from 2 to 28 years (mean = 7 years) in the Rheumatology Outpatient Clinic at Ribeirão Preto Medical School Hospital of the University of São Paulo (HC-FMRP-USP, Ribeirão Preto, SP, Brazil), from April 2011 to April 2014. The patients were diagnosed according to the American College of Rheumatology criteria [20]. RA duration varied from 2 to 27 years (mean = 8 years) and all patients exhibited some degree of bone erosion in hands and/or feet radiographs. Among the 29 a-RA patients, 9 were double-positive for rheumatoid factor (RF) and anti-cyclic citrullinated peptide (anti-CCP) antibody, 10 were single-positive for RF, 16 were single-positive for anti-CCP, and 12 were double-negative for RF and anti-CCP. Among the 34 i-RA patients, 18 were double-positive for RF and anti-CCP, 22 were single-positive for RF, 24 were single-positive for anti-CCP, and 6 were double-negative for RF and anti-CCP.

All the patients had undergone previous treatment with MTX for at least six months, but they had not reached inactivity or low activity disease status, according to the Disease Activity Score of 28 joints (DAS-28) criteria [4, 21]. At the

time of enrollment, the patients were under treatment with IFM (3–5 mg/kg every eight weeks) in combination with MTX (15–25 mg/week) and prednisone (5–10 mg/day); after six months of treatment, they were classified as responders or non-responders to the therapy according to the DAS-28 criteria. The patients treated with this combined therapy for more than six months who remained with active disease (at the time of enrollment) had secondary failure (loss of efficacy) and their therapy was adjusted. However, the further therapeutic adjustments were out of the scope of the present study.

Age- and sex-matched healthy subjects ($n = 38$) who fulfilled the criteria reported by Paoliello-Paschoalato et al. [22] were also recruited for this study. The Research Ethics Committee of the Ribeirão Preto Medical School Hospital of the University of São Paulo (HC-FMRP-USP, Ribeirão Preto, SP, Brazil) approved the study protocol (HCRP n. 10097/2002). All the patients and healthy subjects signed the informed consent form to participate in this study.

## Clinical and laboratory parameters of patients with RA

The level of disease activity was determined using the DAS-28 score [23]. Blood samples were collected and patient assessment was performed just before each IFM infusion. The serum levels of RF, anti-CCP antibody, and C-reactive protein (CRP) were determined using immunological assay kits according to the manufacturer's instructions, as reported previously [24].

## Neutrophil isolation

Venous blood was collected from the antecubital vein of each subject into vacutainer tubes, and immediately diluted into the same volume of Alsever's solution as anticoagulant. Neutrophils were isolated by the gelatin method as described previously [22].

## Preparation of immune complexes (ICs)

Immune complexes composed of ovalbumin and rabbit polyclonal anti-ovalbumin IgG were prepared at equivalence, and further opsonized with sera from patients with RA (RAHS) or healthy control subjects (CHS), as described previously [19]. Non-opsonized IC stimulates neutrophils via FcγR alone, while opsonized IC stimulates neutrophils via FcγR + CR cooperation [25].

## Neutrophil ROS generation

The overall neutrophil ROS production was measured as reported by Paoliello-Paschoalato et al. [19]. Briefly, neutrophils ($1 \times 10^6$ cells/mL) were incubated with luminol (280 µM; Sigma–Aldrich, St. Louis, MO, USA) for 3 min, at 37 °C, and further stimulated with IC (80 µg/mL)—either non-opsonized or opsonized with autologous serum—for 30 min, at 37 °C. The luminol-enhanced chemiluminescence (CL) production was measured using the AutoLumat LB 953 luminometer (EG&G Berthold, Bad Wildbad, Germany).

## FcγR and CR expression in neutrophils

The levels of FcγRII (CD32), FcγRIII (CD16), CR1 (CD35), and CR3 (CD11b) expression in neutrophils were measured as reported previously [19], using the following mouse monoclonal antibodies acquired from BD Pharmingen™ (BD Biosciences, San Jose, CA, USA): fluorescein isothiocyanate (FITC)-conjugated anti-human CD32 (clone FLI 8.26, IgG2b,k), FITC-conjugated anti-human CD35 (clone E11, IgG1,k), phycoerythrin (PE)-conjugated anti-human CD16 (clone 3G8; IgG1,k), PE-conjugated anti-human CD11b/Mac-1 (clone ICRF 44; IgG1,k), FITC-conjugated non-specific isotype control (clone 27–35; IgG2b,k), PE-conjugated non-specific isotype control (clone MOCPC-21; IgG1,k), and BD Simultest™ Control γ1/γ1. The cell samples were analyzed using the BD FACSCanto flow cytometer (Becton, Dickinson and Company; Franklin Lakes, NJ, USA). Data from 10,000 events were collected and analyzed using the BD FACSDiva software. Neutrophils were identified on the basis of the forward- and right-angle light scattering, while the relative number of labeled neutrophils and the median channel fluorescence intensity (MFI) for each antibody indicated the receptor density.

## Serum chemotactic activity

Neutrophil migration towards RAHS and CHS was assayed following the procedure described by Boyden [26] and modified by Paoliello-Paschoalato et al. [19]. Briefly, the lower compartment of the 48-well modified Boyden chamber (Neuro Probe, Cabin John, MD, USA) was filled with serum, pre-treated or not with zymosan (Sigma–Aldrich, St. Louis, MO, USA), and covered with a polycarbonate membrane filter (13-mm diameter and 3-µm pore size). The upper compartment was filled with neutrophil suspension ($6 \times 10^5$ cells). After a 30-min incubation at 37 °C in a humidified chamber, the membranes were fixed and stained with appropriate solutions. Neutrophil migration was determined by means of the leading front technique [27].

## Hemolytic activity of the complement system

Hemolytic activity of the classical and alternative pathways of the complement system in RAHS and CHS was assessed as reported by Landi-Librandi et al. [28]. Briefly,

erythrocytes—rabbit erythrocytes (for the alternative pathway) or sheep erythrocytes sensitized with rabbit anti-sheep erythrocyte antibodies (for the classical pathway)—were suspended in appropriate buffers and the absorbance value of the suspension was adjusted to 0.7–0.8 at 700 nm. Diluted sera were added to the erythrocyte suspension and kinetics of hemolysis was monitored at 700 nm for 10 min, at 37 °C. The time required for sera to lyse 50% of the erythrocyte suspension ($T_{1/2}$) was calculated.

### Analysis of complement activation fragments

The complement activation fragments Bb and C5a were quantified in RAHS and CHS samples using the MicroVue™ Complement Bb Plus Fragment EIA and the MicroVue™ Complement C5a EIA assay kits, respectively, according to the manufacturer's instructions (Quidel, San Diego, CA, USA).

### Data analysis

Experimental data were processed and analyzed with the aid of the GraphPad Prism software (version 5.01 for Windows, GraphPad Software, San Diego, CA, USA).

The normality of distribution of data was assessed using the Shapiro–Wilk test. The groups were compared using the following non-parametric tests: (1) the Mann–Whitney test for two independent variables or (2) the Kruskal–Wallis test followed by the Dunn's post-hoc test for three independent variables, as indicated in the legends. $p < 0.05$ was considered significant.

## Results

### Inflammatory markers

The patients were stratified into two groups according to their disease activity status (a-RA and i-RA) based on their DAS-28 score and serum levels of some inflammatory markers. Compared with i-RA patients, a-RA patients exhibited higher DAS-28 score and serum CRP levels, but similar levels of RF and anti-CCP antibodies (Table 1).

### Functional responsiveness of neutrophils

First, we examined the neutrophil ROS-generating capacity elicited via FcγR alone or in combination with CR. The FcγR + CR cooperation elicited ROS generation more strongly than FcγR alone in i-RA and a-RA patients and control subjects (Fig. 1). Compared with the respective controls for stimulation via FcγR or FcγR + CR, neutrophils from both i-RA and a-RA patients produced increased levels of ROS (Fig. 1).

Next, we analyzed the pattern of FcγR and CR expression in neutrophils, especially the FcγRIIa (CD32a), FcγRIIIb (CD16b), CR1 (CD35), and CR3 (CD11b/CD18) expression levels. Compared with the control group, neutrophils from i-RA and a-RA patients exhibited increased levels of FcγRIIa, FcγRIIIb, and CR1 expression; their expression levels in both groups of patients were statistically similar ($p > 0.05$) (Fig. 2a–c). The level of CR3 expression was similar among the three groups studied (Fig. 2d). Hence,

**Table 1** Clinical and laboratory data of patients with inactive and active rheumatoid arthritis (i-RA and a-RA, respectively)

| Data | i-RA (n = 34) | a-RA (*n* = 29) | Reference values |
|---|---|---|---|
| DAS-28 | < 2.0 | > 4.5 | < 2.6: disease remission<br>2.6–3.2: low disease activity<br>3.2–5.1: moderate disease activity<br>> 5.1: high disease activity |
| CRP (mg/dL)[a] | 0.47 (0.26/0.93) | 1.59 (1.23/1.92)* | < 0.3–1.0: minimal inflammation<br>> 1.0: intense inflammation<br>> 4.0: sepsis |
| Anti-CCP (U/mL)[a] | 171.0 (6.0/233.7) [*n* = 24][b] | 29.6 (6.0/217.0)** [*n* = 16][b] | < 7: negative<br>7–10: undetermined<br>> 10: positive |
| RF (UI/mL)[a] | 227.5 (87.5/506.0) [*n* = 22][c] | 140.5 (55.2/603.8)*** [*n* = 10][c] | 0–29: non-reactive<br>30–70: weakly reactive<br>> 80: reactive |

Statistics: i-RA vs a-RA, two-tailed Mann–Whitney test: *$p < 0.0001$; **$p = 0.2504$; ***$p = 0.5897$

*anti-CCP* anti-cyclic citrullinated peptide antibody, *CRP* C-reactive protein, *DAS-28* disease activity score of 28 joints, *RF* rheumatoid factor

[a]Results are expressed as median and range

[b]Number of patients positive for anti-CCP antibodies

[c]Number of patients positive for RF

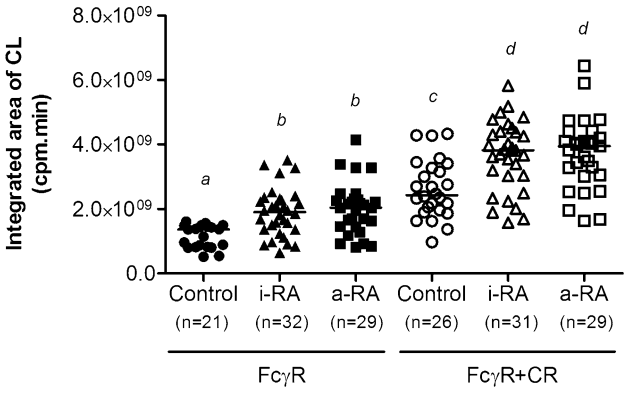


**Fig. 1** Neutrophil ROS generation in patients with active (a-RA) or inactive (i-RA) rheumatoid arthritis treated with infliximab in combination with methotrexate and prednisolone. The cells were stimulated via FcγR or FcγR + CR with non-opsonized IC and IC opsonized with autologous serum, respectively, and overall ROS generation was measured using the luminol-enhanced chemiluminescence (CL) assay. The control group was composed of age- and sex-matched healthy subjects. Groups not sharing the same letter were significantly different from each other ($p < 0.05$; Kruskal–Wallis test followed by the Dunn's multiple comparison test). Bars represent median values

the increased levels of membrane receptors expression were directly associated with the enhanced ROS production in neutrophils from patients with RA treated with IFM + MTX.

## Activation status of the complement system

We examined the activation status of the classical and alternative pathways of the complement system in sera from i-RA and a-RA patients. Compared with the control group, both groups of patients displayed slower hemolytic activity of the alternative pathway of the complement system (Fig. 3a), which indicated an increased systemic activation. The three groups also did not differ with respect to the rate of hemolytic activity of the classical pathway and serum levels of the activation fragments Bb and C5a (Fig. 3b–d).

We examined the chemotactic activity of sera from patients with RA using neutrophils from healthy subjects to indirectly determine the presence of activated complement system fragments (Fig. 4). Before treatment with zymosan, sera from a-RA patients induced the strongest neutrophil migration, which was significantly different from the migration induced by sera from i-RA patients; both samples induced neutrophil migration over two times more effectively than sera from control subjects. Pre-treatment with zymosan significantly enhanced the chemotactic activity of sera from the three groups studied, indicating that they contained intact complement system proteins.

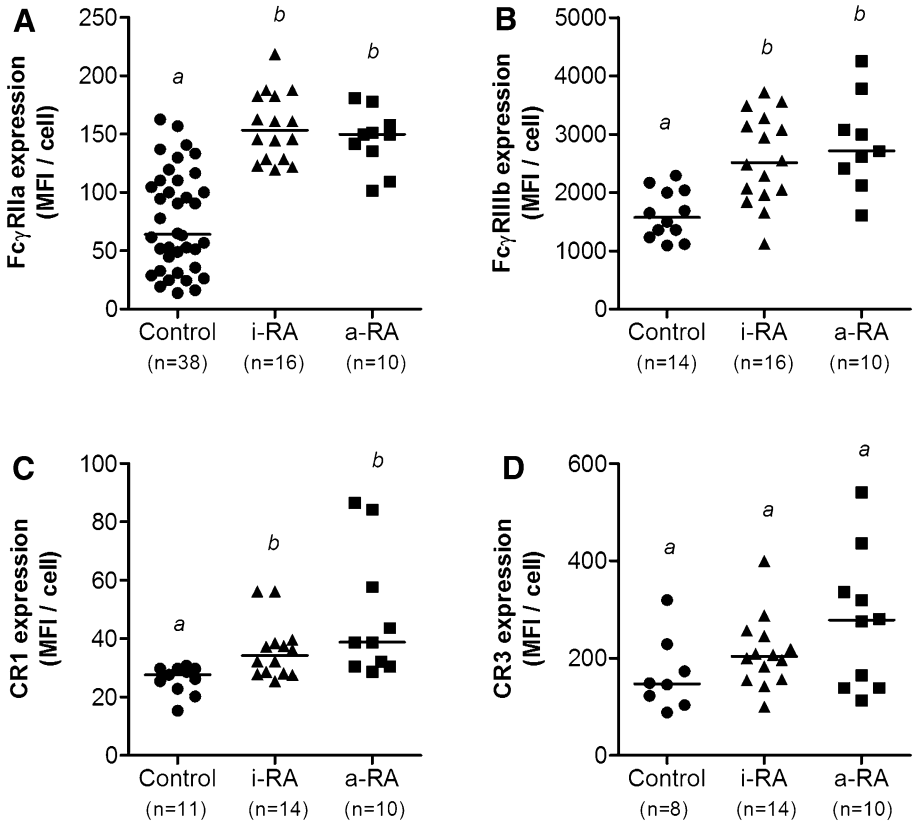


**Fig. 2** Neutrophil FcγRIIa (**a**), FcγRIIIb (**b**), CR1 (**c**), and CR3 (**d**) expression in patients with active (a-RA) or inactive (i-RA) rheumatoid arthritis treated with infliximab in combination with methotrexate and prednisolone. The control group was composed of age- and sex-matched healthy subjects. *MFI* median channel fluorescence intensity. Groups not sharing the same letter were significantly different from each other ($p < 0.05$; Kruskal–Wallis test followed by the Dunn's multiple comparison test). Bars represent median values

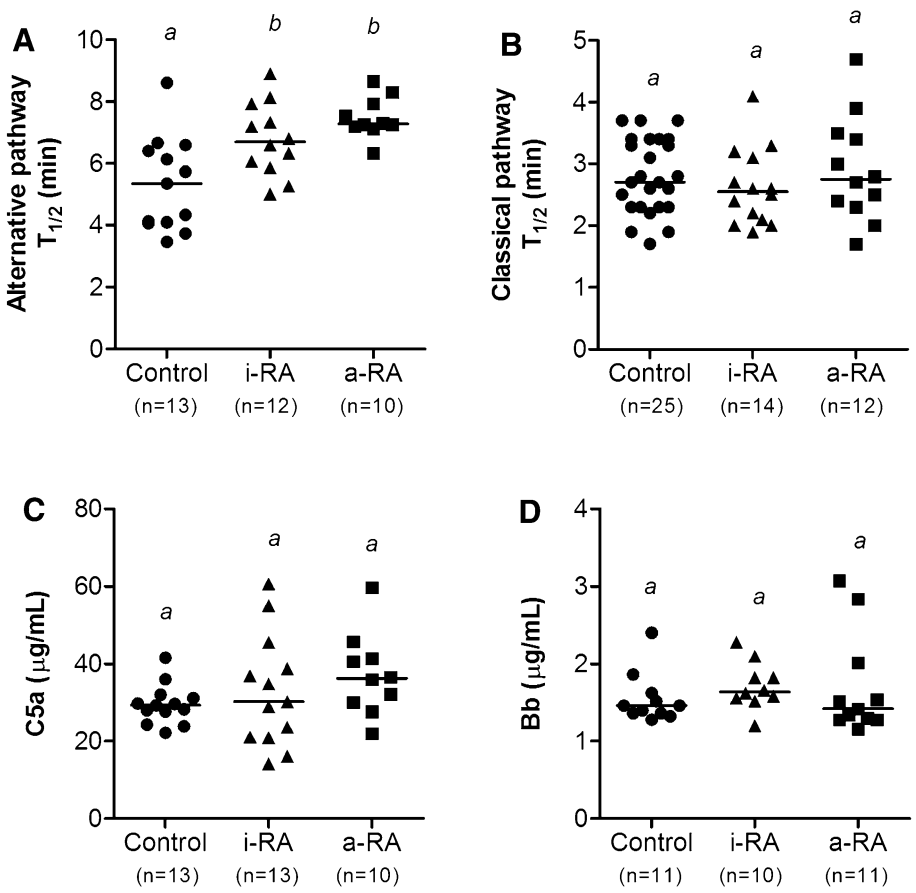


**Fig. 3** Activation status of the complement system in patients with active (a-RA) or inactive (i-RA) rheumatoid arthritis, treated with infliximab in combination with methotrexate and prednisolone. The control group was composed of age- and sex-matched healthy subjects. Hemolytic activity of the alternative (**a**) and classical (**b**) pathways of the complement system was assayed in serum samples diluted 1:40 and 1:12, respectively. Concentration of the complement activation fragments C5a (**c**) and Bb (**d**) in serum samples was determined by the ELISA. Groups not sharing the same letter were significantly different from each other ($p < 0.05$; Kruskal–Wallis test followed by the Dunn's multiple comparison test). Bars represent median values

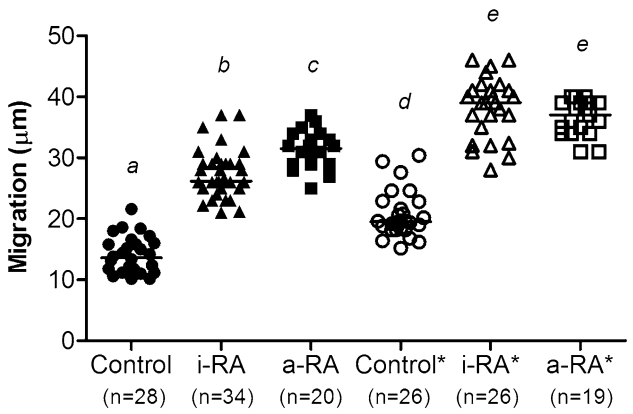


**Fig. 4** Neutrophil migration induced by sera from patients with active (a-RA) or inactive (i-RA) rheumatoid arthritis treated with infliximab in combination with methotrexate and prednisolone. Serum samples from each subject were tested before and after treatment with zymosan (treated samples were marked with *). Groups not sharing the same letter were significantly different from each other ($p < 0.05$; Kruskal–Wallis test followed by the Dunn's multiple comparison test). Bars represent median values

### Influence of autoantibodies on the studied variables

Considering that RF and anti-CCP antibodies are essential elements of the pathogenesis of RA—the former is associated with disease activity while the latter is associated with structural outcomes that not directly impact on disease activity [29]—we further stratified the a-RA and i-RA patients into four subgroups according to the presence of these autoantibodies: double-positive for RF and anti-CCP, single-positive for RF, single-positive for anti-CCP, double-negative for RF and anti-CCP. Although stratification reduced the number of subjects in each group, the results of statistical data analyses for all the variables tested were similar to those found for the whole data set (data not shown), as reported in the previous sections. This finding confirms the lack of influence of the autoantibodies RF and anti-CCP on the neutrophil and complement system parameters assessed in patients with RA.

## Discussion

In the present study, we examined the functional responsiveness of neutrophils and the activation status of the complement system in patients with RA treated with IFM + MTX

combined therapy. We also compared the main results obtained with those previously reported for patients with RA who responded to MTX monotherapy [19], as summarized in Table 2. We found that neutrophils from the three groups studied—i-RA and a-RA patients and control subjects—stimulated via FcγR + CR produced greater amounts of ROS than those stimulated via FcγR alone, corroborating literature reports [19, 30].

In contrast to neutrophils from patients with RA undergoing MTX monotherapy, which exhibit increased ROS production only when the disease is active [19], we found that neutrophils from both i-RA and a-RA patients treated with IFM + MTX combined therapy produced increased levels of ROS (Fig. 1). In agreement with our findings, peripheral blood neutrophils from patients with RA treated with either the anti-TNF-α monoclonal antibody adalimumab or non-steroidal anti-inflammatory drugs exhibit increased ROS-producing and chemotactic capacity [31]. In addition, treating RA patients with a chimeric TNF-α-blocking monoclonal antibody does not suppress the peripheral blood neutrophil capacity to produce ROS and phagocytose *Staphylococcus aureus* [32].

Human neutrophils constitutively express the low-affinity FcγRIIa and FcγRIIIb receptors, CR1 (CD35), CR3 (CD11b/CD18, Mac-1), and CR4 receptors [18]. We have previously reported that peripheral blood neutrophils from a-RA but not i-RA patients undergoing MTX monotherapy express increased levels of FcγRIIa, CR1, and CR3 [19]. The levels of FcγRIIa, FcγRIIIb, and CR1 expression were augmented in both i-RA and a-RA patients treated with IFM + MTX (Fig. 2), which may explain, at least in part, their increased neutrophil ROS-producing capacity. A two-week treatment with IFM reverses the FcγRI expression back to the basal levels, but does not change the levels of FcγRIIa and FcγRIIb expression in peripheral blood monocytes from patients with RA [33]. IFM also lowers the levels of FcγRIIa expression after the first and/or second administration cycle without changing the levels of FcγRIIIb expression in neutrophils from patients with RA [34].

In addition to the increased levels of membrane receptors expression, the activation status of the complement system can directly affect neutrophil activation in peripheral blood [18]. The literature reports that synovia of patients with RA usually exhibits low levels of the complement proteins C3, C4, and factor B, associated with increased levels of complement fragments [35]. Hemolytic activity of the classical pathway of the complement system in sera from i-RA and a-RA patients during treatment with MTX alone [19] or in combination with IFM was similar to that detected in sera from control subjects (Fig. 3b). Hemolytic activity of the alternative pathway was slowed down in sera from both i-RA and a-RA patients during treatment with IFM + MTX (Fig. 3a). In contrast, only sera from a-RA patients undergoing MTX monotherapy exhibit slower hemolytic activity [19]. The serum levels of Bb were increased in both a-RA and i-RA patients undergoing MTX monotherapy [19], but they were not significantly different from the levels detected in the control group in a-RA and i-RA patients undergoing IFM + MTX combined therapy (Fig. 3d). a-RA patients under treatment with MTX alone [19] or in combination with IFM (Fig. 3c) displayed increased serum levels of C5a; the difference between the latter group and the control group

**Table 2** Comparative analysis of neutrophil functional responsiveness and complement system activation status in patients with rheumatoid arthritis treated with methotrexate alone (MTX) or in combination with infliximab (IFM)

| Parameter | i-RA vs control | | a-RA vs control | | a-RA vs i-RA | |
|---|---|---|---|---|---|---|
| | MTX[a] | IFM + MTX | MTX[a] | IFM + MTX | MTX[a] | IFM + MTX |
| ROS generation | | | | | | |
| FcγR | = | > | = | > | = | = |
| FcγR + CR | = | > | = | > | = | = |
| Receptor expression | | | | | | |
| FcγRIIa | = | > | = | > | = | = |
| FcγRIIIb | = | > | = | > | = | = |
| CR1 | = | > | > | > | > | = |
| CR3 | = | = | > | = | > | = |
| Complement system | | | | | | |
| HA alternative pathway ($T_{1/2}$) | = | > | > | > | > | = |
| HA classical pathway ($T_{1/2}$) | = | = | = | = | = | = |
| Serum level of C5a | = | = | > | = | > | = |
| Serum level of Bb | > | = | > | = | > | = |
| Serum chemotactic activity | = | > | > | > | > | > |

*HA* hemolytic activity, *IFM* infliximab, *MTX* methotrexate, *a-RA* patients with active rheumatoid arthritis, *i-RA* patients with inactive rheumatoid arthritis

[a]Data from a previous study from our research team [19]

was near the limit of statistical significance ($p = 0.0586$). The stronger ability of sera from a-RA patients to induce neutrophil chemotaxis, as compared with sera from i-RA patients (Fig. 4), indicates the presence of activated complement system fragments. The increased neutrophil chemotactic activity of zymosan-treated sera also indicates activation of the complement system. Together, these findings demonstrated that the complement system remained activated in a-RA and iRA patients, probably associated with the alternative pathway, which was not inhibited by IFM + MTX combined therapy.

Circulating and deposited ICs, RFs, anti-CCP antibodies, and CRP are some activators of the complement system in RA [36, 37]. Anti-CCP antibodies from RA patients may activate the complement system via both the classical and alternative pathways [38]. In our study, the serum levels of anti-CCP antibodies and RF did not significantly differ between a-RA and i-RA patients (Table 1). The acute-phase protein CRP activates the complement system both in vitro and in vivo, mainly via the classical pathway [39, 40]. We found that i-RA patients had lower serum levels of CRP than a-RA patients, which was associated with the DAS-28 score (Table 1), and probably mediated systemic activation of the complement system. This finding corroborates literature reports [41–43]. Most of the patients with RA display augmented plasma levels of activated complement and CRP-complement complexes, which correlate with the parameters of disease activity [40]. The synovial levels of TNF-α in patients with RA are over twofold greater than in patients with other degenerative joint diseases, and correlate positively with the levels of the complement activation markers C3a and Bb [44]. The plasma levels of the C3 and C4 activation fragments C3b/c and C4b/c, respectively, the DAS-28 score, and the plasma level of CRP markedly lowered two weeks after IFM administration to patients with RA [41].

The most common complication of the long-term therapy with TNF-α-blocking agents is the high incidence of infections, mainly caused by intracellular pathogens [45]. The set of results reported herein suggests that the increased susceptibility to infections is not related to impairment of neutrophil ROS generation and expression of the phagocytic receptors FcγR and CR. In both i-RA and a-RA patients, their levels remained significantly higher than those detected in the control group during the course of IFM + MTX combined therapy (Figs. 1, 2).

Although patients with RA who responded to IFM + MTX combined therapy (i.e. i-RA patients) exhibited decreased disease activity (DAS-28) and lowered levels of inflammatory mediators (CRP) when compared with non-responders (i.e. a-RA patients), both i-RA and a-RA patients presented functional alterations in neutrophils and complement system that indicated that they were activated and the disease activity was not completely subsided. Considering that: (1) neutrophils are one of the major actors of pathogenesis of RA [3]; (2) the main autoantibodies (anti-CCP and RF) detected in sera from patients with RA activate the complement system [35, 37]; and (3) treatment of RA with MTX alone diminishes neutrophil ROS production and complement system activity in MTX-responder patients [19]; further studies are required to examine the efficacy of the IFM + MTX combined therapy for MTX-non-responder patients.

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**Authors contribution** LFM and ABPP devised the project, designed the study, analyzed and interpreted the results, and drafted the manuscript. LFM conducted all the experiments. RDRO followed-up and selected the patients, and determined their clinical parameters. AECSA assisted in all the experiments, and in data processing and analysis. LMK assisted in statistical data analyses, and drafted and proofread the manuscript. EAD selected the patients and supervised the study. YMLV devised the project, searched for funding, and supervised the study. All authors discussed the results, and revised and approved the final version of the manuscript.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflicts of interest.

**Ethical approval** The Research Ethics Committee of the Ribeirão Preto Medical School Hospital of the University of São Paulo (HC-FMRP-USP, Ribeirão Preto, SP, Brazil) approved the study protocol (HCRP n. 10097/2002). All the procedures conducted in humans complied with Resolution 466/12 of the Brazilian National Health Council, which follows the recommendations of the 1964 Helsinki declaration and its later amendments.

**Informed consent** All the patients and healthy subjects signed the informed consent form to participate in this study.

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