Effects of Rituximab and Infliximab Treatment on Carboxypeptidase B and Its Substrates in RA Synovium

Stefan Edginton, Carol Hitchon, Warren Froese, and Hani El-Gabalawy

ABSTRACT. ***Objective.*** We evaluated the synovial effects of 2 potent biologic rheumatoid arthritis (RA) therapies, focusing on their effect on the expression level of carboxypeptidase B (CPB) and its substrates.

Methods. Patients with RA receiving infliximab (IFX; n = 9) or rituximab (RTX; n = 5) had an arthroscopic synovial biopsy at baseline and 16 weeks posttherapy. Expression of CPB, C5a, osteopontin (OPN), CD3, CD20, CD55, and CD68 was assessed by immunohistochemistry and image analysis, and compared with OA synovium. RA disease activity score was assessed at multiple timepoints. Serial serum samples were analyzed for soluble CPB and C5a levels.

Results. The baseline clinical characteristics of patients receiving IFX and RTX were similar. At the time of the second biopsy, 50% of patients had achieved a European League Against Rheumatism good or moderate response. At baseline, expression of CPB, C5a, and OPN was markedly higher in RA compared with OA synovium and correlated with mononuclear cell infiltration. There was an overall reduction in synovial expression of CPB, C5a, and OPN paralleling a reduction in mononuclear cell infiltration, but these changes were not associated with clinical response. After an early reduction in serum C5a levels, these returned to baseline levels at later timepoints.

Conclusion. In response to IFX and RTX treatment, RA synovial expression of CPB, C5a, and OPN decrease independently of the clinical response, reflecting the complex proinflammatory and anti-inflammatory effects of this pathway. (J Rheumatol First Release March 1 2016; doi:10.3899/jrheum.150869)

Key Indexing Terms:
RHEUMATOID ARTHRITIS BIOLOGIC THERAPIES SYNOVIUM CARBOXYPEPTIDASE B

There is an established link between inflammatory pathways and the clotting cascade in rheumatoid arthritis (RA)[1]. Fibrin clots are formed as the endpoint of the coagulation cascade, and accumulation of fibrin in the synovium is a prominent pathologic feature of RA[2,3]. The presence of fibrin within the synovium promotes an inflammatory response[3]. Further, citrullinated fibrin/fibrinogen is a major target of RA-specific autoantibodies[4].

Carboxypeptidase B (CPB), also known as activated thrombin-activatable fibrinolysis inhibitor or carboxypeptidase U, is activated by thrombin in the coagulation cascade. It is produced mainly by the liver as the zymogen pro-CPB[5], but is also found in platelets[6]. *CPB2* (the gene encoding CPB) mRNA has been detected in a spectrum of megakaryoblastic cell lines, monocytoid cell lines, and in endothelial cells[7]. Although either thrombin or plasmin can activate CPB, pro-CPB is most efficiently cleaved to the active enzyme by a complex consisting of thrombin and its cofactor thrombomodulin (TM)[8]. Once activated, CPB hydrolyzes C-terminal lysine on fibrin. This change in structure of fibrin leads to a downregulation in tissue plasminogen activator (tPA)-mediated plasmin generation, thereby lowering the rate of fibrinolysis[9]. In turn, this leads to increased fibrin deposition in tissues such as the synovium, and would thus be expected to have a proinflammatory effect in RA[10].

In contrast to the proinflammatory effects of CPB based on its involvement in fibrinolysis, *in vitro* studies have demonstrated that CPB has other substrates, including osteopontin (OPN) and the anaphylotoxin complement C5a[11]. Cleavage of these proinflammatory molecules would thus likely have an overall antiinflammatory effect. Indeed, a murine study showed that CPB is centrally involved in downregulating C5a-mediated inflammation in inflammatory arthritis, and that CPB deficiency intensifies inflammatory arthritis in a mouse model of RA[1]. The study further demonstrated that a variant of the human *CPB* gene that is associated with a longer half-life is more effective at neutral-

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izing C5a activity *in vitro*, and appeared to have a protective effect against erosive joint damage in RA. Taken together, these observations suggest that the antiinflammatory effects of CPB may outweigh its proinflammatory effects in RA.

In our current study, we evaluated the expression patterns of CPB and its substrates C5a and OPN in RA and osteoarthritis (OA) synovium, and determined the effects of 2 potent RA biologic therapies on their synovial expression levels and on the serum levels of soluble CPB and C5a. We demonstrated that CPB, C5a, and OPN were upregulated in the inflammatory microenvironment of RA synovitis, and were reduced in parallel with decreases in mononuclear cell infiltration, although this was not necessarily associated with a robust clinical response.

MATERIALS AND METHODS

Study subjects. Patients who had failed at least 1 conventional disease-modifying antirheumatic drug (DMARD) and who were initiating either infliximab (IFX) or rituximab (RTX) as a first biologic DMARD were approached at an outpatient ambulatory tertiary referral center to participate in a serial arthroscopic synovial biopsy study. Informed consent was obtained, and the studies received ethics approval from the University of Manitoba Research Ethics Board.

At the baseline visit, prior to initiating the first dose of IFX or RTX, a detailed history and joint examination was performed, subjects completed a modified Health Assessment Questionnaire, and blood and urine samples were obtained. Patients from the IFX-treated cohort had previously failed treatment with 1 or more of the following DMARD: sulfasalazine[4], leflunomide[3], gold[2], hydroxychloroquine (HCQ)[2], cyclosporine[1], and methotrexate (MTX)[1]. Patients from the RTX cohort had previously failed treatment with HCQ[2], azathioprine[1], and MTX[1].

The patients underwent an arthroscopic synovial biopsy procedure of their most inflamed knee joint. All of the arthroscopies were performed by a single orthopedic surgeon (WF) under local anesthesia and conscious sedation. In brief, after synovial fluid was drained from the joint, 15–20 samples (3–5 mm) were obtained from the most macroscopically affected areas within the joint, including the medial, lateral, and suprapatellar aspects of the knee synovial cavity. Study subjects then received IFX (dose 3–5 mg/kg for 0, 2, 6 weeks, then every 8 weeks) or RTX (1000 mg at 0 and 2 weeks) as per standard clinical practice. At the time of each infusion, the patients received 100 mg of methylprednisolone to reduce infusion reactions. The patients were then followed with 3 monthly clinic visits, and then quarterly for 1 year. At each visit, RA activity was assessed using the 28-joint Disease Activity Score (DAS28), and blood samples were obtained. After 12–16 weeks from the baseline assessment, a second arthroscopic biopsy was performed. Clinical response at the time of the second biopsy, at 6 months and 1 year, was calculated based on the European League Against Rheumatism (EULAR) response criteria for the DAS28 scores[12]. For analysis purposes relative to the biomarker data, the responses were dichotomized as responders (EULAR moderate or good response) or nonresponders (EULAR no response).

OA synovial tissue samples were randomly selected from an OA tissue bank obtained at the time of knee arthroplasty and used as controls for the immunohistology experiments. The OA tissues were uniform in their minimal evidence of inflammatory cells.

Tissue processing, immunohistology, and image analysis. Individual tissue samples obtained by arthroscopy were placed in OCT blocks on the same horizontal plane and stored at –80°C until the time of cryostat sectioning and immunohistology. OA samples from arthroplasty were processed in a similar manner. Sequential 6-µm tissue sections were cut using a cryostat and placed on glass slides. Multiple slides were prepared for each tissue block.

One section from each block was used for histological orientation after H&E staining. Immunohistochemistry was performed on sequential sections from each synovial tissue block as previously described[13]. Monoclonal antibodies and dilutions used for immune staining included CPB (anti-CPB, 1:100; Novus Biologicals), C5a (anti-C5a, 1:20; BD Biosciences), OPN (anti-OPN, 1:100; R&D Systems), macrophages (anti-CD68, 1:800; DAKO), T lymphocytes (anti-CD3, 1:50; DAKO), B lymphocytes (anti-CD20, 1:100; DAKO), and fibroblast-like synoviocytes (FLA; anti-CD55, 1:100; Serotec). Briefly, sections were fixed, rehydrated with phosphate/tris buffered saline, and quenched with peroxidase block solution (DAKO). Slides were incubated with normal serum solution, primary antibody solution, secondary antibody solution (DAKO), and streptavidin/horseradish peroxidase solution (DAKO) for 1 h each. Slides were developed with a 3,3'-diaminobenzidine/chromogen solution (DAKO) and counterstained with hematoxylin.

Multiple photographs of each stained slide were identified using microscope photography. Pictures were identified at 20× magnification of multiple areas of interest of the tissue. These included the synovial lining layer and sublining areas, the latter with particular focus on mononuclear cell infiltrates and blood vessels. After identifying multiple pictures per tissue at 20× magnification, the 3 that were most visibly inflamed and demonstrated the highest degree of cellularity and inflammation were selected for quantitative analysis. Using Image Pro Plus 5, 2 investigators (SE and Miranda Ma) independently scored the selected slides, quantitatively scoring each slide for the percentage area positive staining. An average score was calculated from the 3 slides for each tissue, and a final score was obtained from the 2 investigators' scores.

Serum ELISA. Standard commercial ELISA kits were obtained for CPB (Donglin Sci & Tech) and C5a (Hycult Biotech). Following the protocols specific to each kit, we prepared the kits with serum samples that were obtained at the time of the clinical visits. Serum samples from baseline, 1-month, 3-month, and 6-month visits were analyzed. Each sample was run in duplicate to account for the potential variation in sampling and the ELISA plates.

Statistical analysis. Statistical analysis was performed using SPSS statistical software. The absolute and relative change in expression levels was calculated for the study biomarkers. Expression levels between different markers were compared using Mann-Whitney U testing and correlations tested with nonparametric Spearman correlations. Expression levels of the biomarkers were compared in the dichotomized clinical response groups, as described above.

RESULTS

The baseline characteristics of the study patients are shown in Table 1. Overall, the patients receiving IFX and RTX were similar, with both groups having highly active disease as

Table 1. Clinical characteristics of subjects. No differences were statistically significant.

Characteristic	IFX, n = 9	RTX, n = 5
Female, n	9/9	4/5
Age, yrs, mean (SD)	54.7 (14.5)	35.4 (7.5)
Disease duration, yrs, mean (SD)	6.1 (6.7)	3.2 (2.5)
RF-positive, n	5/9	5/5
Baseline DAS28, median (IQR)	6.0 (5.0–7.0)	5.6 (4.0–7.2)
Baseline CRP, median (IQR)	37.4 (8.5–66.3)	42.7 (13.7–71.7)
No. failed DMARD, median (range)	1 (0–2)	2 (1–4)

IFX: infliximab; RTX: rituximab; RF: rheumatoid factor; DAS28: Disease Activity Score at 28 joints; IQR: interquartile range; CRP: C-reactive protein; DMARD: disease-modifying antirheumatic drugs.

reflected by the high DAS28 scores. The patients receiving IFX were somewhat older and had slightly longer disease duration, and 4 out of 9 patients were seronegative for rheumatoid factor, as compared with the patients who received RTX, who were all seropositive. There was no statistically significant difference between any of the characteristics of the groups.

Immunohistological features of RA and OA synovium. Examples of the immunohistological staining patterns seen in RA synovial biopsy samples compared with those seen in an OA tissue obtained at joint arthroplasty are shown in Figure 1 (CPB and C5a). The remainder of the staining can be found in the Supplementary Data (available from the authors on request). CPB and C5a expression was consis-

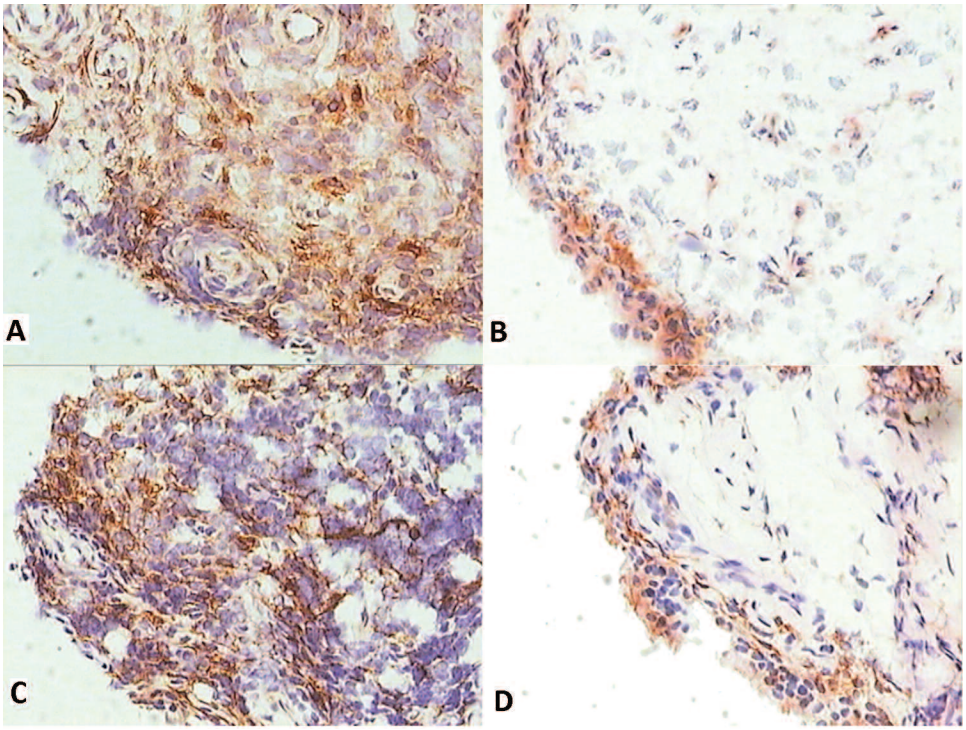


Figure 1. Immunohistochemical detection of biomarkers in RA (A, C) and OA (B, D) synovium. Representative staining for CPB (A, B) and C5a (C, D) is presented. Expression of both CPB and C5a was seen in the lining layer of both RA and OA tissues, but was particularly prominent in perivascular inflammatory cell infiltrates in RA synovium. Immunoperoxidase staining with hematoxylin counterstaining. Original magnification 200×. RA: rheumatoid arthritis; OA: osteoarthritis; CPB: carboxypeptidase B.

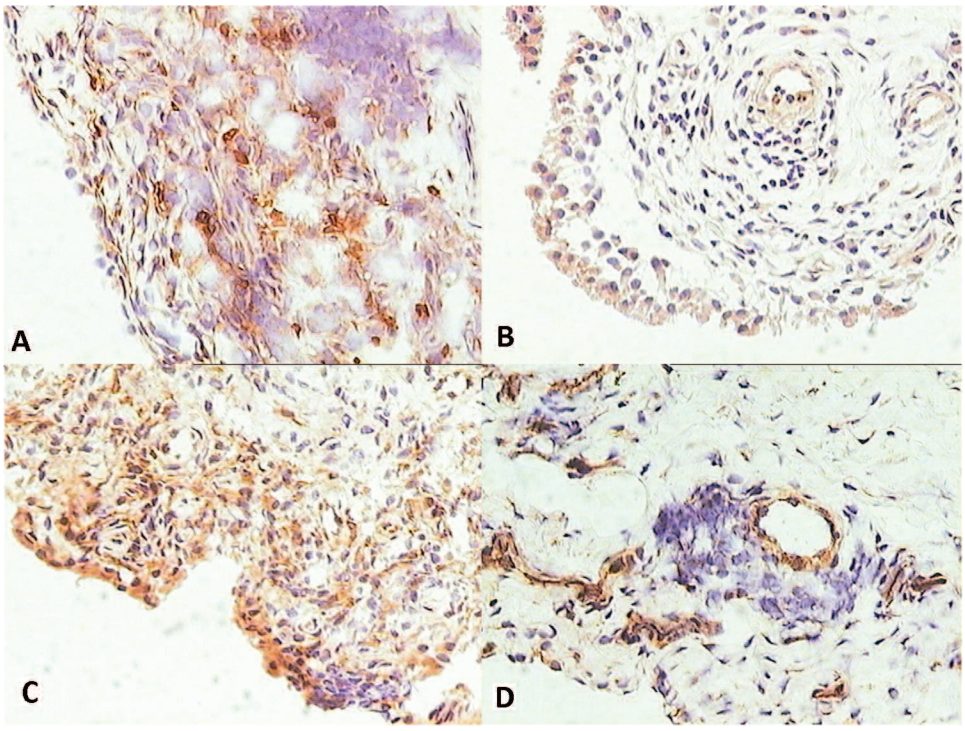


Figure 2. Immunohistochemical detection of CPB in RA synovium before and 16 weeks after treatment with biologics. CPB staining before (A) and after (B) rituximab treatment and CPB staining before (C) and after (D) infliximab treatment are shown. With both agents, CPB staining was reduced after 16 weeks. Immunoperoxidase staining with hematoxylin counterstaining. Original magnification 200×. CPB: carboxypeptidase B; RA: rheumatoid arthritis.

tently detected in the lining layer of RA and OA synovium, but CPB staining was particularly prominent in individual cells present in the inflammatory infiltrate around blood vessels.

In the baseline synovial biopsy samples, image analysis (percent area of tissue positive) of the expression levels for all of the biomarkers showed no significant difference between the IFX and RTX groups (data not shown). Based on this lack of difference between the 2 groups, we analyzed the baseline histological characteristics as a single group (n = 14). Not surprisingly, the baseline expression of the inflammatory cells (CD3, CD20, CD68) was positively correlated and statistically significant. In addition, CPB expression correlated with all 3 inflammatory cells. The strongest association was found with CD68 ($r = 0.758$, $p = 0.002$).

Clinical and synovial effects of biologic therapy. A second synovial biopsy was performed 12–16 weeks after treatment. In comparison to the baseline biopsy, expression levels of CPB, C5a, and OPN all decreased in parallel with decreases in CD3, CD20, and CD68 levels (Figure 2). There were no significant differences in the magnitude of these changes between the 2 biologic treatment groups (Figure 3A). Patients from both treatment cohorts were separated into responders and nonresponders as defined by the EULAR criteria. At 3 months, 1 had a good response and 6 had a moderate response. At 6 months, 3 had a good response and 5 had a moderate response. At 1 year, 3 had a good response and 4 had a moderate response. These 2 responses were categorized as responders versus those who did not respond to treatment. Between these 2 groups at 3 months, no significant difference in biomarker reduction was noted (Figure 3B). At 6 months, the difference in percent reduction between responders and nonresponders was more evident compared with those at 3 months (Figure 3C). However, again the change in biomarker expression between the 2 groups was not statistically significant.

C5a expression levels after treatment correlated with DAS28 scores at 12 weeks and 1 year. OPN expression levels after treatment and B cell expression level at baseline correlated with DAS28 scores at 1 year (Table 2).

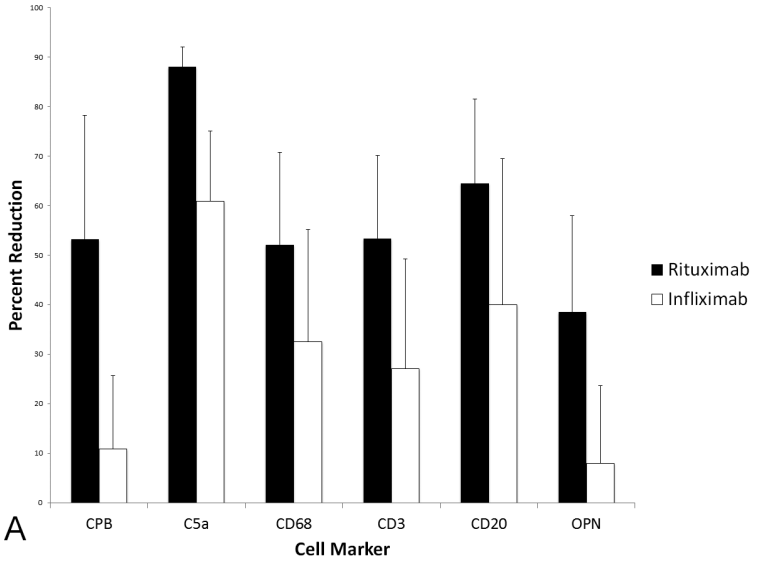
Relationship between synovial expression of biomarkers and serum levels. Using Spearman correlations, CPB and C5a serum levels were compared to their synovial counterparts at baseline and 3 months after treatment (Figure 4). The net reduction in expression was also compared. None of the associations were statistically significant. Using Mann-Whitney U tests, the serum levels of responders and nonresponders were compared at the above timepoints. Again, no statistically significant associations were found.

DISCUSSION

In our current study, we examined the synovial expression of CPB and its substrates in patients with RA and OA, and evaluated the effects of IFX and RTX on the synovial expression and serum levels of these molecules. Our results indicate that synovial expression of CPB and C5a are both correlated with the degree of inflammatory cell infiltration, particularly the presence of macrophages. There was a consistent decrease in the synovial expression of CPB and C5a which paralleled a reduction in inflammatory cell infiltration, although this was not necessarily reflected in clinical responses after 3 months of biologic therapy.

The culmination of the coagulation cascade results in cleavage of fibrinogen by thrombin, forming fibrin[14]. Thrombin not only forms fibrin, but activates factor XIII, which acts by crosslinking fibrin monomers, forming a fibrin clot[15]. Thrombin procoagulant activity is mediated by its formation of a complex with TM, which initiates a series of reactions leading to fibrinolysis[15]. tPA and plasminogen ultimately lead to the degradation of fibrin clots[16]. However, TM is also involved in prolonging fibrin clots, and this is through cleavage of pro-CPB to the activated CPB enzyme[8]. Activated CPB cleaves a carboxyl terminal lysine from degraded fibrin clots[9]. This change in structure reduces binding by tPA and plasminogen, slowing down clot lysis[17]. In this manner, CPB is proposed to have a procoagulant role. Further, fibrin deposition in RA is known to be involved in ongoing inflammation[3]. Through this mechanism, it is hypothesized that CPB exacerbates joint inflammation in RA by prevention of fibrin clot lysis.

CPB is produced as a zymogen by a number of cells. Pro-CPB is expressed primarily by liver cells and subsequently released into the bloodstream[5]. Pro-CPB mRNA has also been found in a number of other cells, including platelets, endothelial cells, and a number of myeloid cell lines including monocytes and megakaryoblasts[6,7]. Figure 1 shows the staining patterns of CPB in the synovium, a tissue with a heterogeneous collection of resident and inflammatory cells in close juxtaposition. Staining in OA synovium (Figure 1B) is essentially confined to the lining layer. The lining layer consists of macrophage-like synoviocytes and fibroblast-like synoviocytes (FLS)[18], but because of the inherent limitations of immunohistology, it is uncertain whether 1 or both cell types express CPB. However, because macrophage-like synoviocytes are of myeloid lineage, it is more likely that it is these cells that account for most of the lining layer staining[19]. In contrast, in RA synovium the CPB staining pattern is more widespread (Figure 1A), with extensive staining evident in both the lining layer and inflammatory infiltrate in the sublining stroma. The sublining infiltrate features several populations of chronic inflammatory cells in close juxtaposition, including B cells, T cells, and macrophages (Supplementary Data is available from the authors on request). Because there is no indication in the literature that lymphocytes express CPB, it is assumed that sublining macrophages are the primary source of the stromal CPB staining. The prominent perivascular staining in some tissues may indicate the presence of recently migrated monocytes into the inflamed RA synovium.



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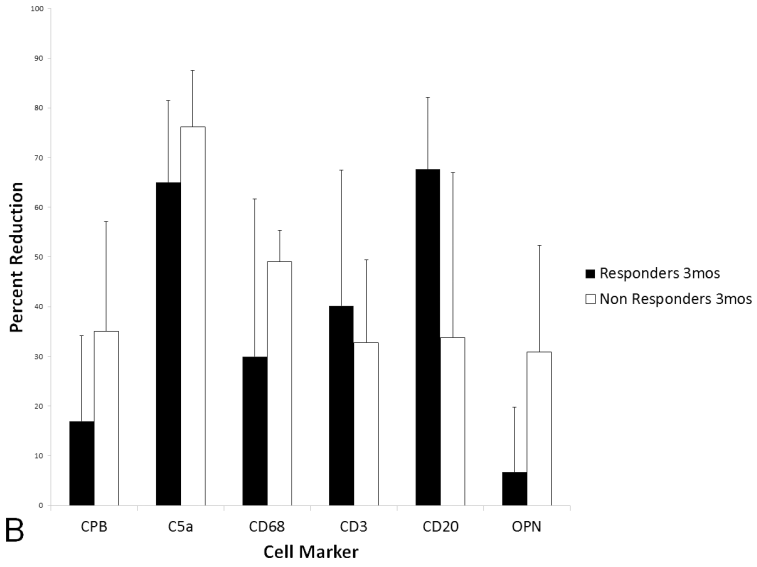

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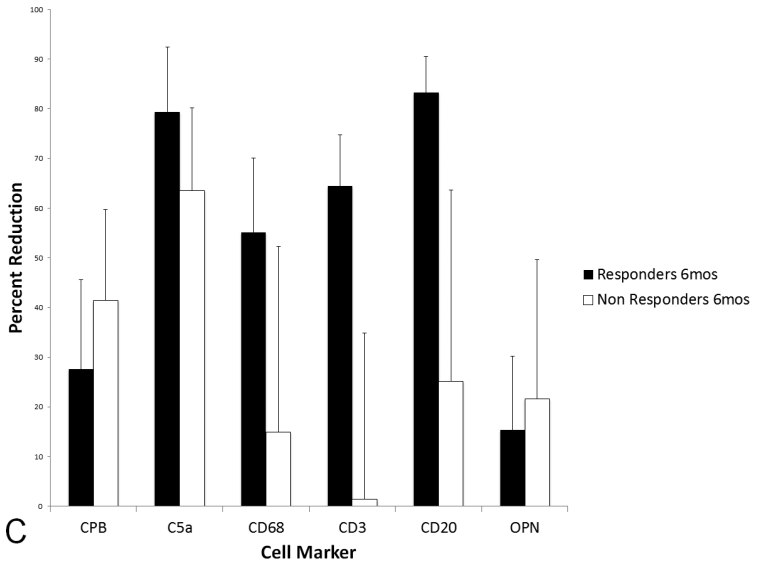

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Figure 3. (A) Relative percent reduction in synovial biomarker expression 16 weeks after initiation of treatment with biologic therapy. Those treated with rituximab (n = 5) showed a greater decrease in biomarker expression as compared with infliximab (n = 9). However, none of the differences were statistically significant. Expression was calculated as percent of area staining positive using image analysis (Image Pro Plus 5). Standard error of the mean is represented by error bars. Analysis was performed independently by 2 researchers (SE and MM). (B) Comparison of relative percent reduction in synovial biomarker expression 16 weeks after initiation of biologic treatment between EULAR-defined responders to treatment and non-responders at 3 months. EULAR response was characterized by improvements in DAS28 from baseline[12]. Between the 2 cohorts, there were no statistically significant differences in change in expression for any of the biomarkers. Expression was calculated as in panel A. (C) Comparison of relative percent reduction in synovial biomarker expression 16 weeks after initiation of biologic treatment between EULAR-defined responders to treatment and nonresponders at 6 months. EULAR response was characterized by improvements in DAS28 from baseline[12]. The differences between the 2 cohorts were more apparent in those who responded at 6 months, but there were no statistically significant differences in change in expression for any of the biomarkers. Expression was calculated as in panel A. EULAR: European League Against Rheumatism; DAS28: Disease Activity Score at 28 joints.

Table 2. Correlations between synovial biomarker expression and clinical measure of disease. All other correlations are not significant.

Comparison	Correlation, r	Significance, p
C5a 16 weeks–DAS28 12 weeks	0.543	0.022
C5a 16 weeks–DAS28 52 weeks	0.539	0.029
CD20 baseline–DAS28 52 weeks	0.478	0.049
CD20 16 weeks–DAS28 12 weeks	0.574	0.016
OPN 16 weeks–DAS28 52 weeks	–0.570	0.022

DAS28: Disease Activity Score at 28 joints; OPN: osteopontin.

We demonstrate a substantial decrease in synovial CPB expression after an average of 3 months of treatment with either IFX or RTX. This decrease roughly paralleled the decrease in the synovial inflammatory infiltrate. There are thus 3 potential explanations for this decrease, which are not mutually exclusive. First, it is likely that the observed reduction in CPB expression primarily reflects the reduced number of macrophages in the synovial microenvironment, as shown in Figure 3. Indeed, it has been proposed that a reduction in synovial macrophages is a key indicator of clinical response in RA, irrespective of the agent used to achieve this[20]. Alternatively, it is also possible that these biologic treatments resulted in a reduction of pro-CPB transcription, particularly by the resident macrophages. Because pro-CPB transcription has been shown to be upregulated by cAMP *in vitro*[21], it is thus possible that either through the inhibition of tumor necrosis factor-α (TNF-α) or through synovial B cell depletion, reductions in intracellular cAMP levels result in decreased transcription of pro-CPB.

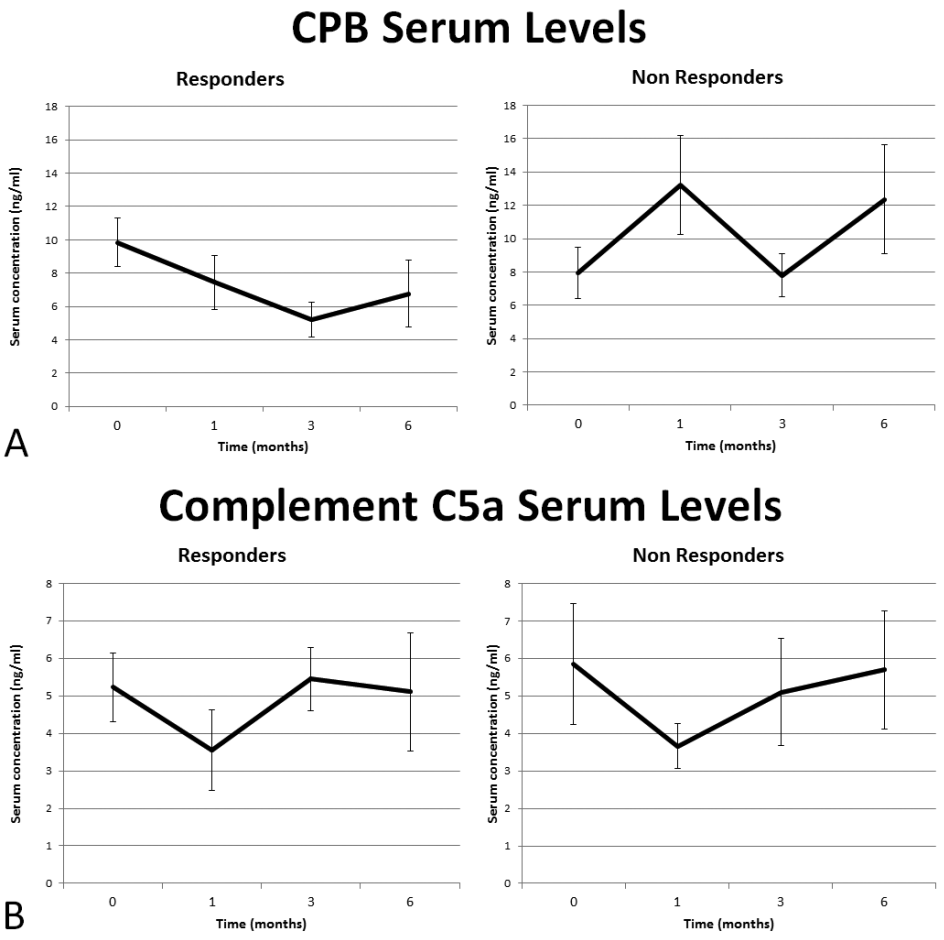

Figure 4. Comparison of concentration of serum biomarkers CPB and C5a using ELISA assay. Responders (n = 7) were compared with nonresponders (n = 7). Error bars represent standard error of the mean. Response to treatment was defined as either good, moderate, or none as per EULAR criteria[12]. The good and moderate responders were grouped together as responders. Measurements were made at 4 timepoints (baseline, 1 mo, 3 mos, and 6 mos) and the averages were placed on the graphs along with the SD. There were no statistically significant variations in biomarker levels between the different groups. It is of note that the CPB serum levels appear to be consistently higher among nonresponders, while C5a serum levels appear unchanged between responders and nonresponders. With both markers, the serum levels after 6 months are not significantly different as compared to baseline. CPB: carboxypeptidase B; EULAR: European League Against Rheumatism.

Finally, it is possible that there is a reduction in the activation of pro-CPB to activated CPB. Because the most effective activator of CPB is the TM-thrombin complex[1,8], and TM is highly expressed by macrophages in RA[22], and effective treatment with biologic DMARD reduces serum TM levels[23], a reduction in the activation of the available synovial pro-CPB is a plausible mechanism. It should be added that the antibody used to detect CPB in the synovium recognizes only the activated form of CPB and not pro-CPB. Thus, studies evaluating the ratio of pro-CPB/CPB may help address the relevance of this potential mechanism.

C5a is a key substrate for activated CPB. This highly proinflammatory molecule is formed in the activation of the complement cascade from cleavage C5 into C5a and C5b by C5 convertase. The C5 convertase complex is formed through either the classical or alternate pathway of the complement cascade[24], both of which may be involved in RA synovitis. Its effects are mediated through binding to the C5a receptor, which is expressed by inflammatory cells in synovial tissues as well as synovial macrophages and fibroblasts[17,22]. C5a levels have been shown to be increased in RA synovial fluid[25]. C5a mediates chemotaxis of phagocytes and serves as an anaphylatoxin, causing degranulation of neutrophils and a number of other inflammatory cells[26]. Importantly, C5a has also been shown to be associated with increased TNF-α expression by FLS[17]. CPB effectively deactivates C5a through cleavage of a terminal arginine from the octapeptide[27]. Thus, the potent proinflammatory effects of C5a are mitigated by CPB. Indeed, using a murine anticollagen antibody–induced inflammatory arthritis model, mice that were CPB-deficient developed more severe arthritis, this being proposed to result from unchecked activation of C5a[28]. Moreover, C5-deficient mice in this model were resistant to the development of inflammatory arthritis[28].

As with CPB, in our current study we observed that synovial C5a expression levels decreased substantially with biologic treatment. This presents a potential paradox in that a decline in CPB levels should result in an increase in C5a levels, and in turn, its proinflammatory biological. Thus, it seems more likely that the decline in C5a synovial expression relates to a reduction in the generation of this complement activation product than to the dynamics of its degradation. In this respect, high concentrations of leukocyte-derived microparticles are found in RA synovial fluid, these having been shown to activate the complement cascade[29], and successful treatment with DMARD may lead to a reduction in the levels of these microparticles and in turn a reduction in complement activation. To date, to our knowledge there are no data regarding the effects of RA therapy on synovial microparticles, although this may represent an important mechanism by which synovial inflammation is ameliorated.

It is now well established that both IFX and RTX are highly effective therapies for RA, although there remains a substantial number of incomplete responders and non-responders to both agents. Moreover, there is considerable variation in the latency of the articular response, with some patients responding within a few weeks while others achieve an optimal response after several months. The mechanisms underlying these differences remain unclear. These 2 agents were chosen for this trial because the mechanism of action by which these 2 monoclonal antibody therapies achieve their clinical benefit differs quite markedly, with the former inhibiting TNF-α and the latter depleting CD20-expressing B cells[30]. Interestingly, it has been shown that IFX was particularly effective in patients exhibiting lymphoid aggregates in the synovium, these being typically populated primarily by T and B lymphocytes[31]. The clinical response to RTX was shown to be associated with depletion of synovial B cells[32,33], and in another study, to a reduction in sublining CD68-positive macrophages[20]. Yet despite the clear targets for each of these monoclonal antibody therapies, it is difficult to attribute the clinical response to a specific biologic effect on the synovium, owing to the complexity and heterogeneity of the synovial inflammatory response in RA.

In our current study, we observed a decline in synovial inflammatory cell infiltration in essentially all patients, and as mentioned, this was broadly associated with a reduction in the synovial expression of CPB and its substrate C5a. Three months after the initiation of these therapies, at the time of the second synovial biopsy, 7 out of the 14 patients had achieved a EULAR-defined moderate or good response[12]. We compared changes in the immunohistological scores from the baseline biopsy in patients achieving a EULAR moderate or good response to the changes seen in those who did not achieve this response, and were not able to demonstrate any clear differences. This may simply reflect the small number of study subjects when compared with the large numbers of study subjects enrolled in the clinical trials that have clearly demonstrated the clinical efficacy of these agents. We speculated that in some cases, the clinical response may lag behind the synovial response, and indeed 2 additional patients transitioned from EULAR moderate response to good response status and 1 more transitioned from no response to moderate response at the 6-month evaluation. Comparing Figure 3B and Figure 3C, it is evident that those who ultimately responded at 6 months showed a greater reduction in biomarker expression, albeit not statistically significant. Nevertheless, these results serve to further demonstrate the complex relationship between the synovial inflammatory processes and the clinical manifestations of RA.

There are inherent limitations to the immunohistological and clinical methodologies used in our study. The potential sampling bias in synovial biopsy studies is well described. It should be noted that the single arthroscopist (WF) who performed all of the study procedures made every attempt to be consistent in biopsying the most macroscopically inflamed areas of each tissue. Further, the selection and number of histological fields chosen for image analysis in each immuno-

histological section was consistent, and based on established protocols used in other similar studies[30,31,32,33]. Nevertheless, the small number of study subjects in each group may compound this inherent sampling bias. Finally, it is acknowledged that the aggregate index (DAS28) used to assess clinical efficacy in RA is better suited for large clinical trials as opposed to small studies such as our current one.

We demonstrate that CPB and its substrate C5a are widely expressed in inflamed RA synovium. CPB has paradoxical proinflammatory and antiinflammatory effects related to its dual involvement in fibrinolysis and regulation of complement activation. The synovial expression of CPB and C5a broadly decrease after 3 months of IFX and RTX therapy in parallel with reductions in inflammatory infiltration, although this is not necessarily reflected in the clinical response.

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