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Student Name: Stefan Edginton **Date:** August 8, 2014

Project Title: Effects of rituximab and infliximab on carboxypeptidase B and its substrates in RA synovium

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Department: Rheumatology

SUMMARY:
Objective: Carboxypeptidase b (CPB) promotes coagulation and may have an anti-inflammatory role in arthritis through its ability to cleave pro-inflammatory mediators osteopontin (OPN) and complement C5a. We evaluated synovial expression of CPB, C5a and OPN at baseline and post-treatment with biologics and explored associations with clinical response.
Methods: RA patients receiving infliximab (n=9) or rituximab (n=5) had a synovial biopsy at baseline and 16 weeks post therapy. Expression of CPB, C5a, OPN, the macrophage marker CD68, B-cell marker CD20 and T-cell marker CD3 was assessed using immunohistochemistry and image analysis. Two blinded investigators independently calculated expression. Clinical disease activity scores (DAS28) were obtained at baseline, the second arthroscopy, and one year. Synovial expression and associations between biomarkers with clinical activity were evaluated using non-parametric tests.
Results: The patients receiving infliximab and rituximab were clinically similar. CPB staining was most intense in the synovial lining layer, around blood vessels and in some lymphocytic infiltrates. OPN and C5a staining was more diffuse throughout the synovium. At baseline, CPB expression correlated with macrophages as well as T and B lymphocytes. C5a staining correlated with B lymphocytes. Synovial expression did not correlate with baseline DAS28. At 16 weeks C5a expression correlated with DAS28 and with DAS 1 year post treatment.
Conclusion: CPB expression may be linked to macrophages and B&T lymphocytes. Of these, it is likely that synovial macrophages play the greatest role in CPB expression.

Supervisor Signature

[redacted]

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Introduction

There is an established link between inflammatory pathways and the clotting cascade in chronic inflammatory disorders such as Rheumatoid Arthritis (RA)(1). Accumulation of fibrin in the synovium is a prominent pathologic feature of RA (2,3). Fibrin clots are formed as the endpoint of the coagulation cascade. The presence of fibrin within the synovium promotes an inflammatory response (3). Further, citrulline substituted fibrin/ fibrinogen is a major target of RA-specific autoantibodies (4).

Thrombin-activatable plasma carboxypeptidase B (CPB, also known as activated thrombin-activatable fibrinolysis inhibitor [TAFIa] or carboxypeptidase U) plays a role 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 megakaryoblastic cell lines, monocytoid cell lines and in endothelial cells (7). Pro-CPB can be cleaved to the active enzyme by thrombin and plasmin, but the most effective activator of CPB is a complex consisting of thrombin and its cofactor thrombomodulin (1). This complex can remove the activation peptide of pro-CPB approximately 1000 times faster than plasmin or thrombin alone (8). Once activated, CPB hydrolyzes C-terminal lysines on fibrin. This change in structure of fibrin leads to a downregulation in tPA-mediated plasmin generation, thereby lowering the rate of fibrinolysis (9). This function of CPB leads to increased fibrin deposition in the synovium and would ultimately be expected to increase inflammation in synovium.

However, studies have demonstrated that CPB has other substrates in vitro, including proinflammatory mediators osteopontin (OPN) and the anaphylotoxin complement C5a (10). Cleavage of these molecules would likely have an anti-inflammatory effect, the opposite of its effect via cleavage of fibrin. A murine study showed that CPB plays a central role in downregulating C5a-mediated inflammation in inflammatory arthritis and that CPB deficiency intensifies inflammatory arthritis in a mouse model of RA (1). The study went further to demonstrate that the effects could be extrapolated to human RA by showing that the gene encoding a CPB variant with a longer half-life is protective for erosive joint damage in RA. Additionally, this longer half-life CPB molecule is more effective at neutralizing C5a activity in vitro (1). This study suggests that the anti-inflammatory function of CPB may have a greater effect than its pro-inflammatory function in inflammatory arthritis (1).

In this study, we further explored the role CPB plays in rheumatoid arthritis. We looked at synovial tissues from RA patients before and after first-time treatment with a biologic DMARD (infliximab or rituximab). Using immunohistochemistry, we stained for CPB within RA synovium as well as staining for some of its substrates (C5a & OPN) along with common cell markers for T-cells, B-cells and macrophages. In addition, we compared the changes in these markers to changes in clinical disease activity scores (DAS28 using CRP) and other clinical markers.
We found that CPB expression levels were associated with those of B-cells, T-cells and macrophages in RA synovium. Treatment with biologics led to a decrease in expression of all markers in the synovium. We also found that C5a expression after treatment with biologics correlated with DAS28 short-term and longterm.

Materials and Methods

Study Subjects

Patients who had failed at least one conventional DMARD and who were starting their first biologic were approached at an outpatient ambulatory tertiary referral centre. Informed consent was obtained and the studies received ethics approval from the University of Manitoba Research Ethics Board.

Prior to initiating first biologic therapy (rituximab or infliximab), a detailed history and examination of the joints was performed (HEG), and subjects completed a questionnaire asking about symptoms and functional status (modified health assessment questionnaire [mHAQ]). Blood and urine samples were obtained. Synovial biopsy samples were obtained by arthroscopy prior to biologic and 3 months post biologic. The procedure was performed by a single orthopedic surgeon (WF) under local anesthetic and conscious sedation. Once excess fluid was drained from the joint, the surgeon took 15-20 samples (3-5mm) from the most macroscopically affected areas within the joint, usually from both sides of the joint. The samples were then snap frozen in OCT and stored at -70ºC. Subjects received infliximab (dose 3-5mg/kg, 0, 2, 6 weeks then every 8 weeks) or rituximab (1000 mg at 0 and 2 weeks with 100mg methylprednisolone) as per standard clinical practice.

Patients were followed every four weeks for three months. At each visit, joint exams were performed and blood samples were taken. At three months a second arthroscopic biopsy was performed. After this, patients were followed up at six months and one year from the initiation of treatment. Clinical response was measured by European League Against Rheumatism (EULAR) DAS28 scores (11). There was no statistically significant difference between the two cohorts (See Table 1).

Patients from the infliximab cohort had previously failed treatment with sulfasalazine (4), leflunomide (3), gold (2), hydroxychloroquine (2), cyclosporine (1), and methotrexate (1). Patients from the rituximab cohort had previously failed treatment with hydroxychloroquine (2), azathioprine (1), and methotrexate (1).

Tissue Acquisition and Processing

Tissue samples were obtained by arthroscopy and placed individually in OCT blocks and stored at -70ºC. Tissues were from before treatment, as well as 3 months after treatment with either rituximab or infliximab. 6um tissue slices were cut using a cryostat and placed on slides. Multiple slides were prepared for each tissue block. An additional set of slides was prepared using synovial tissue from osteoarthritis (OA) patients. These samples were obtained at the time of joint replacement surgery.

Image Analysis

One slide from each block was stained using H&E as previously described (12). Immunohistochemistry was performed, staining for CPB (anti-CPB, 1:100, Novus Biologicals, USA), C5a (anti-C5a, 1:20, BD Biosciences, Canada), OPN (anti-OPN, 1:100, R&D Systems, USA), macrophages (anti-CD68, 1:800, DAKO, Canada), T lymphocytes (anti-CD3, 1:50, DAKO, Canada), B lymphocytes (anti-CD20, 1:100, DAKO, Canada) and fibroblast-like synoviocytes (anti-CD55, 1:100, Serotec, USA) Sections were fixed, rehydrated with P/TBS, and quenched with peroxidase block solution (DAKO). Slides were incubated with normal serum solution, pri-

mary antibody solution, secondary antibody solution (DAKO) and Streptavidine/HRP solution (DAKO) for one hour each. Slides were developed with a DAB/chromogen solution (DAKO) and counterstained with hematoxylin.

Multiple photographs of each stained slide were captured using microscope photography. Each slide was first viewed under low power to assess the integrity of the tissue. Pictures were captured at 20X magnification of multiple areas of interest of the tissue. These included the synovial lining layer, sublining layer, around blood vessels, and lymphocytic infiltrates. After capturing at least ten pictures per tissue at 20X magnification, the three that were most visibly inflamed and demonstrated the highest degree of cellularity and inflammation were selected for quantitative analysis.

Using Image Pro Plus 5, two investigators (SE/Miranda Ma) independently scored the selected slides, quantitatively scoring each slide for %area positive staining. An average score was calculated from the three slides for each tissue, and a final score was obtained from the two investigators' scores.

Statistical analysis

The absolute and relative change in expression levels was calculated for the different biomarkers. Expression levels between different markers were compared using mann whitney U testing and correlations tested with non-parametric spearman correlations. p values less than 0.05 were considered significant. Statistical analysis was performed using SPSS. Expression levels were compared to patients' clinical data, including DAS28 as well as other inflammatory markers.

Results

Baseline Immunohistological Characteristics

The immunohistological staining patterns are shown in Figure 1.
CPB staining was most intense in the lining layer and to a lesser extent in the sublining and perivenular areas. Expression was co-localized with fibroblast-like synoviocytes (FLSs) as well as CD68 positive macrophages in serial sections. C5a staining distribution was focused primarily to the lining layer. Perivenular areas and some synoviocytes also stained positively. Osteopontin stained positively diffusely throughout the synovium. Its expression was co-localized with many cells, including macrophages, FLSs and activated T cells.

Baseline histological data showed no statistically significant differences in scores (%area positive staining) between the infliximab and rituximab groups for all biomarkers. Because of this lack of difference between the two groups, we analyzed the baseline histological characteristics in one group. The expression of macrophages (CD68), T-cells (CD3) and B-cells (CD20) were all correlated. CPB expression also correlated to all three cell types and C5a was correlated with B-cell expression (see Table 2). All other correlations at baseline were not significant.

Effects of infliximab/rituximab therapy on synovial immunohistochemical features and clinical parameters

16 weeks after treatment, expression levels of all biomarkers decreased. There was no significant difference in expression change between the two treatment groups (See Figure 2). Patients from both drug cohorts were separated into responders and non-responders as defined by EULAR criteria. At 1 year, 3 had a good response and 4 had a moderate response. These two responses were categorized as responders versus those who did not respond to treatment. Between these two groups, no significant difference in biomarker reduction was noted (See Figure 3). 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 (see Table 3).

Discussion

In our study we looked at the synovium of patients with RA as well as some with OA. In those with RA we obtained synovial samples before and after treatment with a biologic DMARD (either rituximab or infliximab). We used immunoperoxidase staining to assess levels of CPB, C5a, OPN along with other common cell types such as macrophages and B and T lymphocytes. We looked for associations between staining of the aforementioned molecules as well as the above cells. We also compared the histological data of the patients with clinical data. An association was found between CPB and macrophages, B-cells and T-cells at baseline. C5a staining correlated to baseline B-cell staining, and C5a levels after treatment correlated with DAS in the short term (12 weeks) and long term (1 year).

The culmination of the coagulation cascade results in cleavage of fibrinogen by thrombin, forming fibrin (23). Thrombin not only forms fibrin, but activates Factor XIII, which acts by cross-linking fibrin monomers, forming a fibrin clot (24). Thrombin's procoagulant activites are mediated by its formation of a complex with thrombomodulin (TM), which initiates a series of reactions leading to fibrinolysis (24). Tissue plasminogen activator (tPA) and plasminogen ultimately lead to the degradation of fibrin clots (25). However, TM also plays a role in prolonging fibrin clots, and this is through cleavage of pro-CPB (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 (16). In this manner, CPB has a procoagulant role. Furthermore, fibrin deposition in RA is known to play a role in ongoing inflammation (3). Therefore, it would be reasonable to assume that CPB exacerbates inflammatory joint disease through its 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. The OA synovium (Figure 1j) appears to be confined to the lining layer. The lining layer consists primarily of macrophage-like synoviocytes (MLS) and fibroblast-like synoviocytes (FLS) (26). Due to the inherent limitations of immunohistology and the analysis of heterogenous tissues, we can not say for certainty which cells in the lining layer are expressing CPB. However, MLSs are of myeloid lineage, thus it is more likely that it is these cells that are staining positively (27). In RA synovium (Figure 1i), CPB is staining positively in the lining layer,

the sublining layer as well as in infiltrates. Again, we can not say with certainty which cells are expressing CPB in these regions. However, in comparing staining to that of B-cells, T-cells and macrophages (Figure 1 c,e,g) it is apparent that these cells are very abundant in many of the same regions. There is no evidence in the literature of lymphocytes producing CPB, so again it would be reasonable to assume it is the macrophages (or MLSs) that are primarily responsible for the CPB. There are also some perivenular regions that are staining positively, and it is possible that endothelial cells play a role in those regions.

Figure 2 demonstrates the considerable decrease in CPB staining after treatment with both rituximab and infliximab. There are three potential explanations for this decrease. First we must consider pro-CPB transcription by cells. Pro-CPB transcription has been shown to be up-regulated by increases in cAMP in vitro (28). It is possible that with effective treatment levels of cAMP and other inducers decrease in the synovium. Next we must consider the activation of pro-CPB to CPB. There are a number of molecules that play this role including thrombin and plasmin, but by far the most effective activator is the TM-thrombin complex (1,8). TM is expressed by macrophages in RA (20), and effective treatment with biologic DMARDS has been shown to reduce serum TM levels (21). A third possibility is that CPB expression and activation haven't changed, but simply the number of cells producing pro-CPB and its activators (likely macrophages) have decreased. Figure 2 shows the decrease in all of the inflammatory cells with treatment. It is likely that a combination of the above factors is responsible for this decrease, but further research is required to answer these questions.

Besides fibrin, another of CPB's substrates is the complement protein C5a (10). C5a is formed from the cleavage of complement protein C5 into C5a and C5b by C5 convertase. C5 convertase can be formed through either the classical or alternate pathway of the complement cascade (29). C5a plays an important role in chemotaxis of phagocytes and is an anaphylotoxin, causing degranulation of a number of inflammatory cells (15). C5a has also been shown to be associated with increased TNFa expression by synoviocytes (16). An anti-collagen antibody-induced inflammatory arthritis murine model found that CPB-deficient mice developed more severe arthritis, and it was believed this was due to unchecked proliferation of C5a (17). Additionally, C5 deficient mice in this model were protected from arthritis development, further supporting this idea (17). C5a levels have been shown to be increased in RA synovial fluid (18). Its effects are mediated through binding to the C5a receptor (C5aR), which is expressed by inflammatory cells in synovial tissues as well as synovial macrophages and fibroblasts (16,19).

CPB effectively inactivates C5a through cleavage of a terminal arginine from the octapeptide (14). C5a staining also decreased substantially with biologic treatment. Again, there could be a number of explanations for this change. It could be that increased cleavage of C5a by CPB leads to this large decrease in staining after treatment. However, this idea would be more feasible if CPB increased in the synovium. Since both CPB and C5a decreased after treatment, it is less likely that inactivation due to CPB is the primary cause of C5a's reduction. Another possibility is that the synovial environment changes with treatment in such a way that less C5a is formed. High concentrations of leukocyte-derived microparticles are found in RA synovial fluid, which have been shown to activate the complement cascade (22). Treatment with DMARDS may lead to a drop in the production of these microparticles and other complement activators by leukocy-

tes. On the other hand, this drop could simply be attributed to the reduction in leukocytes, as was shown in Figure 2.

Another substrate of CPB is osteopontin (OPN). It is a glycosylated phosphoprotein that has proinflammatory functions as well as playing a role in bone remodelling (30). It is expressed by a number of different cells, including macrophages, natural killer cells and T-cells (30). Thrombin cleaves OPN forming OPN-R (31). This newly-formed molecule is important in macrophage recruitment and in the production of cytokines during cell-mediated immunity (32). CPB cleaves OPN-R, preventing its binding to other molecules that mediate the above effects (30). As with C5a, OPN staining decreased with treatment. Since both CPB and OPN decreased, it is unlikely that OPN's decrease can be primarily attributed to its inactivation by CPB. Since macrophages and T-cells also decreased after treatment, it is more likely that the drop in immune cells explains the lower expression of this molecule.

It is well established that both infliximab and rituximab are highly effective therapies for rheumatoid arthritis. The mechanism of action by which these two monoclonal antibody therapies achieve their clinical benefit differs quite markedly, with the former inhibiting TNFa and the latter depleting CD20 expressing B cells. Interestingly, it has been shown that infliximab was particularly effective in patients exhibiting lymphoid aggregates in the synovium, these being typically populated primarily by T and B lymphocytes (33). The clinical response to rituximab was shown to be associated with depletion of synovial B cells (34), and in another study, to a reduction in sublining CD68 positive macrophages (35). Thus, 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 the current study, there was no significant difference in the clinical response between those treated with rituximab and those with infliximab. Both drugs were effective at lowering DAS28 after one month as well as one year. Seven out of the fourteen patients had a EULAR-defined moderate or good response (11). When we compared the immunohistochemical results against responders and non-responders, there was no significant difference at baseline or after treatment. Thus, we could not identify immunohistological predictors of response, although the number of patients studied was modest. Substantial reduction in macrophages, T cells, and B cells were seen with both infliximab and rituximab, but these did not necessarily predict clinical response.

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Figure 1

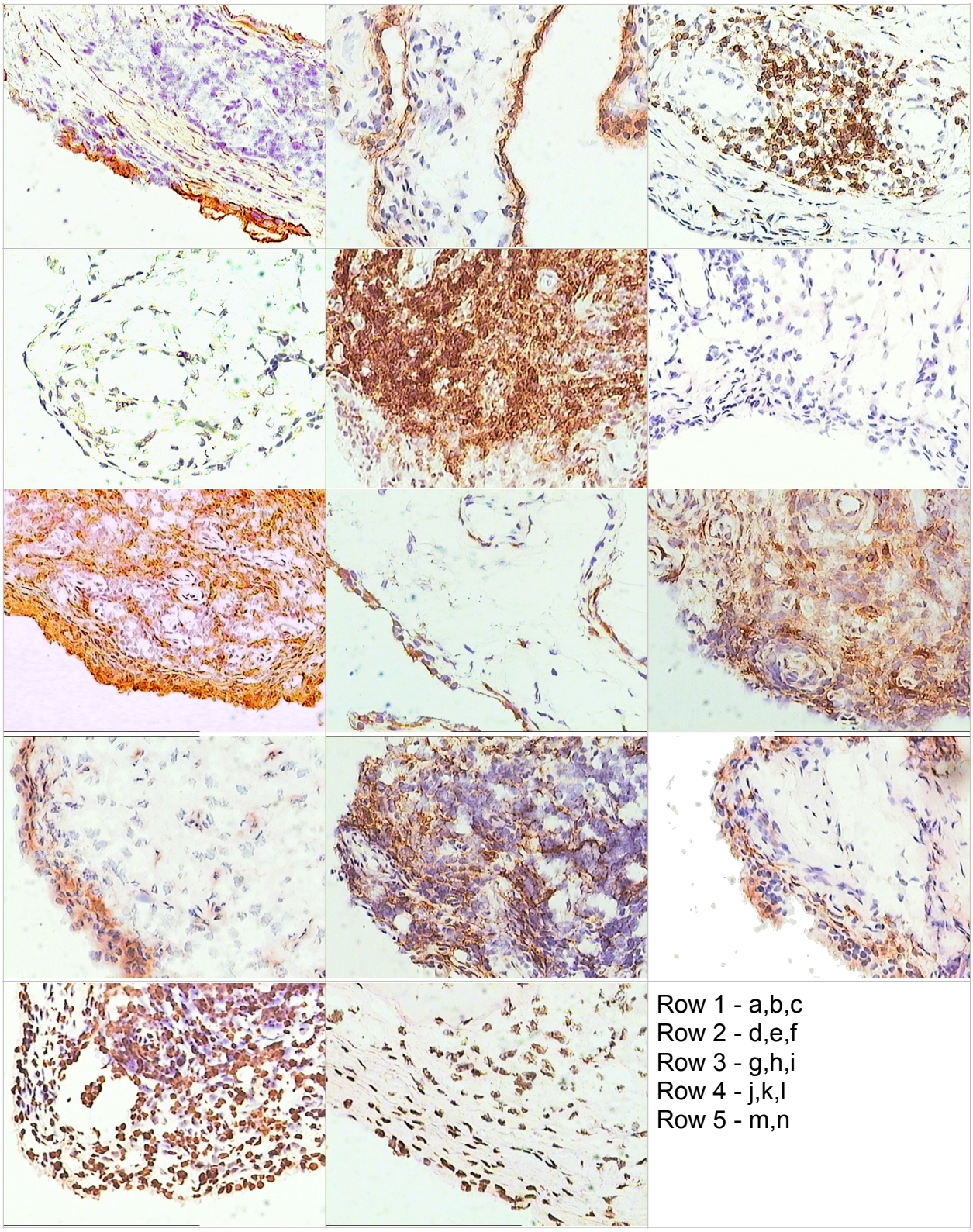

Figure 2

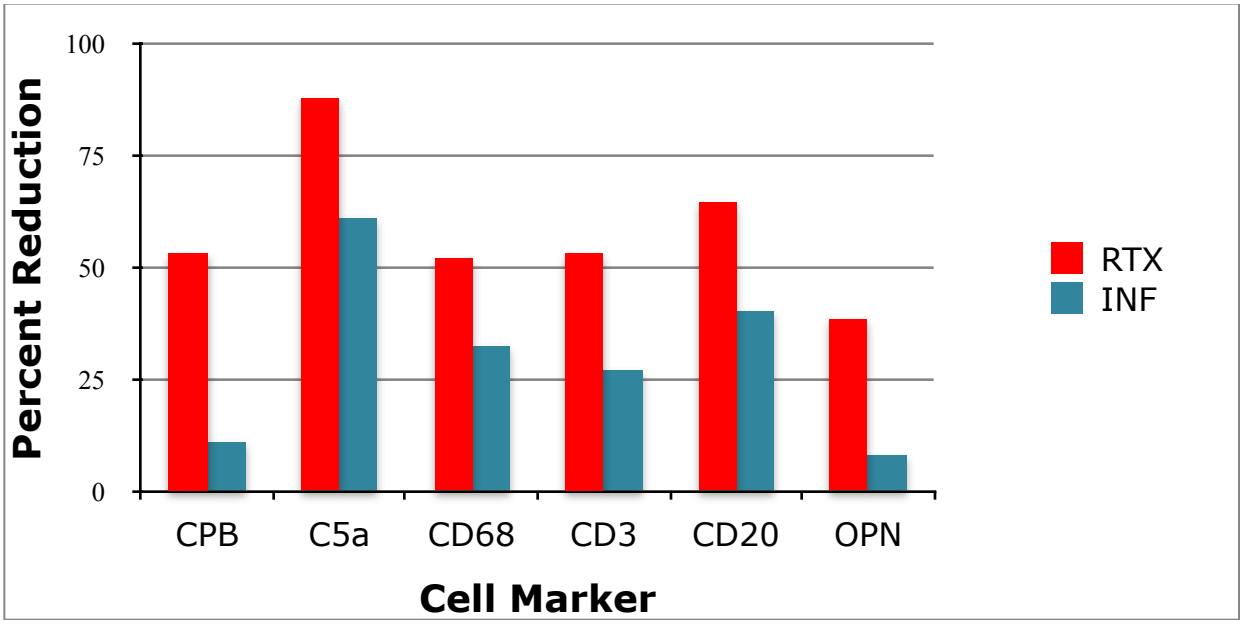

Figure 3

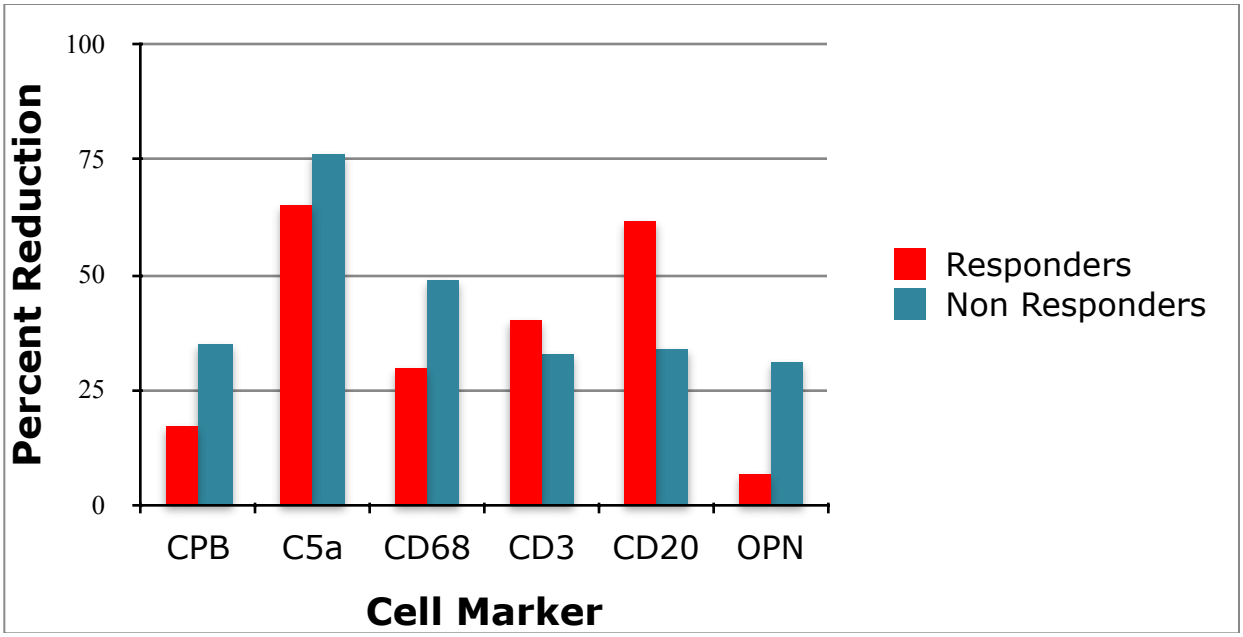

Figure Legend

Figure 1 - Immunohistochemical detection of biomarkers in RA synovium (a,c,e,g,i,k,m) and OA synovium (b,d,f,h,j,l,n). Tissues stained to express CD55 (a,b), CD3 (c,d), CD20 (e,f), CD68 (g,h) CPB (i,j), C5a (k,l), OPN (m,n). Brown color indicates positive staining. In RA tissues, CPB staining was concentrated in the lining layer and next to blood vessels, and co-localized with macrophages (CD68) and fibroblast-like synoviocytes (CD55). CPB also co-localized with some

cells present in cellular infiltrates. C5a staining was primarily seen in the lining layer and perivenular regions in RA tissues as well. In OA tissues, CPB and C5a staining was limited to the lining layer. In both RA and OA, osteopontin staining was diffusely positive for many cells throughout the tissue, but RA was more cellular overall and thus showed a higher degree of staining. Immunoperoxidase staining with hematoxylin counterstaining; original magnification X200.

Figure 2 - Relative percent reduction in synovial biomarker expression 16 weeks after initiation of treatment with biologic therapy. Those treated with rituximab (RTX, n=5) showed a greater decrease in biomarker expression as compared to infliximab (INF, n=9). However, none of the differences were statistically significant. Expression was calculated as % area positive staining using image analysis (Image Pro Plus 5). Analysis was performed independently by two researchers (SE & MM).

Figure 3 - 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. EULAR response was characterized by improvements in DAS28 from baseline (11). Between the two cohorts, there were no statistically significant differences in change in expression for any of the biomarkers. Expression was calculated as in Figure 2.

Table 1 - Clinical characteristics of subjects

	Rituximab (n=5)	Infliximab (n=9)
Gender (%F)	80	100
Age (SD)	35.4 (7.5)	54.7 (14.5)
% RF positive	100	55.6
Median baseline DAS28 (IQR)	5.6 (1.6)	6.0 (1.0)
Median baseline CRP (IQR)	42.7 (29)	37.4 (28.9)
Median #failed DMARDS (range)	2 (1-4)	1 (0-2)
Prednisone before Tx (n)	3	0

Table 2: Correlations between baseline expression

Comparison	Correlation (r)	Significance (p)
CPB – CD68	0.758	0.002
CPB – CD3	0.648	0.012
CPB – CD20	0.547	0.021
C5a – CD20	0.521	0.028
CD68 – CD20	0.644	0.006
CD68 – CD3	0.802	0.001
CD3 – CD20	0.820	0.000

All other correlations NS

Table 3: Correlations between synovial biomarker expression and clinical measure of disease

Comparison	Correlation (r)	Significance (p)
C5a 16wks – DAS28 12wks	0.543	0.022
C5a 16wks – DAS28 52wks	0.539	0.029
CD20 Baseline – DAS28 52wks	0.478	0.049
CD20 16wks – DAS28 12wks	0.574	0.016
OPN 16wks – DAS28 52wks	–0.570	0.022

All other correlations NS