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# The evolving systemic and local biomarker milieu at different stages of disease progression in rat collagen-induced arthritis

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**Abstract**

Rats with collagen-induced arthritis (CIA) were necropsied on 14 occasions from 4 days after induction to 27 days after disease onset to evaluate the kinetics of local (joint protein extracts) and systemic (serum) levels of inflammatory and pro-erosive factors. Systemic increases in α1 acid glycoprotein and KC/GRO together with systemic and local enrichment of interleukin (IL)-1β, IL-6, CCL2, transforming growth factor (TGF)-β and elevated IL-1α and IL-18 in joint extracts preceded the onset of clinical disease. Systemic upregulation of IL-1β, IL-6, TGF-β CCL2, RANKL and prostaglandin $E_2$ ($PGE_2$) during acute and/or chronic CIA coincided with systemic leukocytosis and a CD4+ T-cell increase in blood and spleen. In contrast, progression of joint erosions during clinical CIA was associated with intra-articular increases in IL-1α/β, IL-6, IL-18, CCL2, KC/GRO and RANKL, and a dramatic decline in osteoprotegerin (OPG). These data indicate that systemic and local events in inflammatory arthritis can be discrete processes, driven by multiple cellular and humoral mediators with distinct temporospatial profiles.

**Keywords:** *rat collagen-induced arthritis, cytokines, biomarkers, inflammation, bone resorption*

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## Introduction

Immune-mediated arthritis is initiated and maintained by interacting cascades of proinflammatory cytokines (Arend & Dayer 1990, Taylor 2003). Tumour necrosis factor (TNF)-α, interleukin (IL)-1 and IL-6 are thought to be the leading mediators in patients with rheumatoid arthritis (RA) or spondyloarthritides (Brennan et al. 1991, Arend & Dayer 1995, Keller et al. 2003, Roberts & Butler 2005). Their fundamental role is reflected by neutralizing antibodies and receptors against these cytokines to control structural and clinical aspects of disease in arthritic patients (Bresnihan et al. 1998, Richard-Miceli & Dougados 2001) and in rodents with experimentally induced

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arthritis (Wooley et al. 1993a,b, Kuiper et al. 1998, Joosten et al. 1999, Feige et al. 2000, Coxon et al. 2002). As a result, anticytokine biologics particularly those targeting TNF-α are regularly employed to combat rheumatoid arthritis and other immune-mediated inflammatory diseases (Breedveld 1999, Calabrese 2003, Kourbeti & Boumpas 2005).

Despite major progress in the understanding of the role of cytokines in inflammatory arthritis, several key questions remain to be addressed. First, patients differ in their response to cytokine blockade, suggesting that the respective role of a specific cytokine in triggering the arthritic disease process may differ among individuals. Such differences are likely to be relevant for experimental arthritis, as shown by models solely based on the overexpression of TNF or IL-1. Second, systemic and local cytokine expression may profoundly differ from each other. Thus, synovial inflammatory tissue might not be the only place where these mediators are produced, as lymph nodes, spleen and liver are sources contributing to systemic cytokine expression. While peripheral blood provides an expedient source to assess systemic levels of these factors, joint tissue can also provide important information on local changes (Arend 2001, Andreakos et al. 2002, Stolina et al. 2005). Data comparing local versus systemic levels of markers and mediators of RA progression are rare in the literature. However, some of the existing reports describe divergent levels when comparing local and systemic compartments (Steiner et al. 1999, Rosengren et al. 2003, Stolina et al. 2005). These reports highlight the likelihood that systemic markers and mediators of arthritis might not fully reflect the underlying local disease progression. Third, arthritis starts before the onset of clinical symptoms of disease, as antibody formation and elevations of C-reactive protein have been observed even before the clinical onset of RA (Molenaar et al. 2001). This suggests that proinflammatory cytokines act before the onset of disease, thereby enabling immune activation and trafficking of immune cells to the target tissues.

However, the cytokine expression before the onset of arthritis is not thoroughly characterized even in animal models of human immune-mediated arthritis. Inhibition of TNF-α or IL-1, or both, significantly reduces but does not eliminate inflammation in rat models of adjuvant-induced arthritis (AIA) (Feige et al. 2000, Campagnuolo et al. 2002, Schett et al. 2005) and collagen-induced arthritis (CIA) (Stolina et al. 2005). Such residual disease might result from a submaximal blockade of the targeted cytokines, or alternatively might be a consequence of other proinflammatory cytokines, e.g. IL-6, IL-12, IL-15, IL-17, IL-18, transforming growth factor (TGF)-β (Gaffen 2004, McInnes & Gracie 2004), proinflammatory chemokines, e.g. CCL2 (formerly MCP-1) (Rollins 1996), CXCL8 (formerly IL-8) (Punzi et al. 2002) and pro-erosive entities (principally receptor activator of nuclear factor κB ligand (RANKL)) (Bolon et al. 2002).

Focal bone erosion in inflamed joints is a hallmark of immune-mediated arthritis and has been attributed to excessive RANKL-mediated osteoclast activity (Kong et al. 1999, Pettit et al. 2001, Crotti et al. 2002). In animal models, recombinant osteoprotegerin (OPG) showed anti-erosive effects in rats with clinical AIA or CIA (Kong et al. 1999, Romas et al. 2002) and inhibited inflammatory bone loss and erosions in TNF-transgenic mice (Redlich et al. 2002, Schett et al. 2003). Treatment of RA patients with denosumab, a fully human monoclonal antibody that binds and inhibits RANKL, increased bone mineral density and inhibited RA-related skeletal structural damage and erosions (Cohen et al. 2008).

We performed the current work in a standard rat CIA model to explore the evolution of the cytokine spectrum over time in both the local (joint and regional lymph nodes) and systemic (circulation and distant lymphoid tissues) compartments. We hypothesized that CIA might result from distinct cytokine signatures developing in inflamed joints (local sites) versus the circulation (systemic compartment). Our results suggest that proinflammatory molecules other than IL-1β and TNF-α emerge as potentially important biomarkers and/or mediators of the initiation and progression of arthritis.

## Materials and methods

These studies were conducted in accordance with federal animal care guidelines and were pre-approved by Amgen's Institutional Animal Care and Use Committee (IACUC).



### *Experimental design*

Rat CIA is a well-characterized system for exploring the pathogenesis of arthritis (Schett et al. 2005, Stolina et al. 2005). For this study, disease was investigated by taking clinical measurements as well as fluid (serum) and tissue (hind paws, femur, lymphoid organs) samples on 14 occasions: before onset ($-5$, $-3$, or $-1$ days), at onset (indicated by hind paw swelling and ambulatory difficulties) and during clinical arthritis progression ($+1, +2, +3, +4, +5, +7, +10, +14, +20$ and $+27$ days after onset).

### *Animals*

Female Lewis rats (7–8 weeks old; Charles River, Wilmington, MA, USA) were acclimated for 1 week and then randomly assigned to treatments (14 CIA groups with $n=8$ per time point and 28 non-arthritic controls, $n=2$ per time point). This cohort size was used because we have observed that interindividual variability is minimal in rats with CIA (unpublished data). Animals were given tap water and fed pelleted rodent chow (#8640, Harlan Teklad, Madison, WI, USA) *ad libitum*; calcium and phosphorus contents were 1.2% and 1.0%, respectively.

### *Induction of arthritis*

CIA was induced as described (Stolina et al. 2005) by intradermal injection of emulsified porcine type II collagen (1 mg per animal; Chondrex, Redmond, WA, USA) in incomplete Freund's adjuvant (Difco, Detroit, MI, USA) at 10 different sites over the back (100 μl/site). The day of disease induction was designated as study day 0.

### *Assessment of arthritis*

*Clinical evaluation.* Total body weights were taken to assess general health. Joint involvement was evaluated by measuring average hind paw volume using water plethysmography (Feige et al. 2000) and diameter via precision calipers (Stolina et al. 2005).

*Haematological assessment.* At necropsy, blood was collected by intracardiac puncture from rats anesthetized with $CO_2$. A complete blood count was acquired from EDTA-treated whole blood using an Advia 120 analyzer (Bayer Corporation, Tarrytown, NY, USA). Flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA, USA) to quantify leukocyte subpopulations was performed on whole blood and selected local (popliteal and inguinal lymph nodes) and systemic (mesenteric lymph node and spleen) lymphoid organs (dissociated in PBS) using antibody reagents (BD PharMingen, San Diego, CA, USA) directed against cell surface markers specific for B (CD45RA) and T (CD3, CD4, CD8) lymphocytes, antigen-presenting cells (CD11b/c), granulocytes (HIS48) and macrophages (HIS36).

*Protein extraction from joints.* One tibiotarsal region from one hind paw was flash-frozen, pulverized, and extracted using a 50 mM Tris buffer, pH 7.4, containing 0.1 M NaCl and 0.1% Triton X-100. Protein concentration in individual extracts was evaluated using a standard BCA Protein Assay (Pierce Co., Rockford, IL, USA).

*Biochemical assays for serum and joint extracts.* Separate aliquots of serum or paw protein extracts were used to quantify levels of various analytes. The major rat acute phase protein α1 acid glycoprotein (α1AGP) was measured by a precipitin ring assay (Ecos Institute, Furukawa, Miyagi, Japan). Multiple cytokines (IL-1α/β, TNF-α, TGF-β, IL-2, IL-4, IL-6, IL-10, IL-12, IL-17, IL-18, CCL2, KC/GRO, granulocyte/macrophage-colony stimulating factor (GM-CSF), interferon (IFN)-γ, RANKL) were assessed using multiplex rat-specific Luminex kits (Linco Research, St Charles, MO, USA) or single-plex mouse OPG-specific Luminex kits (Linco Research); the mouse OPG kit was 95–98% cross-reactive with rat OPG, based on a comparison of standard curves for recombinant mouse OPG (internal kit standard) versus recombinant rat OPG (Amgen Inc., Thousand Oaks, CA, USA). Prostaglandin $E_2$ ($PGE_2$) was evaluated using an enzyme immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, MI, USA). Total immunoglobulins were determined by enzyme-linked immunosorbent assay (Bethyl Laboratories, Montgomery, TX, USA). Concentrations of humoral immune modulators in paw protein extracts were evaluated as described above and normalized to the total protein concentration. Assays were performed according to the manufacturers' instructions.

*Histopathology.* The other hind paw was removed, fixed by immersion in zinc formalin, decalcified in eight serial changes of a 1:4 mixture of 8 N formic acid and 1 N sodium formate for approximately a week, trimmed and processed into paraffin. The extent of local disease was evaluated in the paw at all time points using standard criteria and a blinded analytical paradigm (Table I; Feige et al. 2000). Analyses were performed in sections stained with haematoxylin and eosin .

### *Statistical analysis*

For the CIA time-course study, two normal healthy control rats were killed at each time point. These rats were age and sex matched to the CIA group. None of the endpoints from these healthy rats showed any significant changes over the 32-day time course. We therefore pooled data from all 28 healthy control rats, and used these data for the non-arthritic control. Results are expressed as mean ± SEM. Student's *t*-test

Table I. Histopathology criteria for scoring joint lesions

| | |
|---|---|
| Inflammation | |
| 0 | Normal |
| 1 | Few inflammatory cells |
| 2 | Mild inflammation |
| 3 | Moderate inflammation |
| 4 | Marked inflammation (generally diffuse) |
| Erosion | |
| 0 | Normal |
| 1 | Minimal loss of cortical or trabecular bone at a few sites |
| 2 | Mild loss of cortical or trabecular bone at modest numbers of sites (generally tarsals) |
| 3 | Moderate loss of bone at many sites (usually the trabeculae of the tarsals, but sometimes the cortex of the distal tibia) |
| 4 | Marked loss of bone at many sites (usually as extensive destruction of trabeculae in the tarsals, but sometimes with partial loss of cortical bone in the distal tibia) |
| 5 | Marked loss of bone at many sites (with fragmenting of tarsal trabeculae AND full thickness penetration of cortical bone in the distal tibia) |

was used for the clinical data by comparing CIA versus non-arthritic control rats. Histopathology data were analyzed using the $\chi^2$ test. Statistical significance was delineated by a $p$-value $<0.05$.



## Results

### *Arthritis progression*

Clinical onset of CIA occurred between 9 and 11 days after induction, and was always observed first in hind paws. Hind paw volume peaked by onset + day 2, while hind paw diameter peaked at onset + day 3; these maximal measurements remained steady for the duration of the study (data not shown). At day + 14 post-onset the macroscopic signs of arthritis become evident for the fore paws. By disease onset and through the end of the study, total body weight in rats with CIA was significantly reduced compared with healthy control rats ($p<0.05$; data not shown).

Anatomical evidence of arthritis accompanied the clinical disease. Histological examination showed minimal but significant inflammation at day + 1 after onset, which peaked at onset + 7 days (Figure 1*A*, *C*). Significant skeletal erosions were evident beginning at onset + 2 days and reached near maximal values (erosion score = 4.2 out of 5) by day + 10 ($p<0.05$; Figure 1*B* (Stolina et al. 2005), 1*C*).

Based on these macroscopic and microscopic patterns, CIA progression was divided into three stages: (1) preclinical (from day −10 before onset to the day of clinically visible arthritis onset; designated day 0), where evidence of inflammation or bone erosion was lacking; (2) acute clinical (from day 0 to day + 14 post-onset), where hind paw swelling, body weight loss, inflammation and hind paw bone erosion (microscopic) were steadily progressing and macroscopic signs of CIA appeared on fore paws; and (3) chronic clinical (post-day + 14), where clinical (hind and fore paw swelling), and structural (inflammation and articular erosions in hind paws) evidence of joint involvement plateaued.

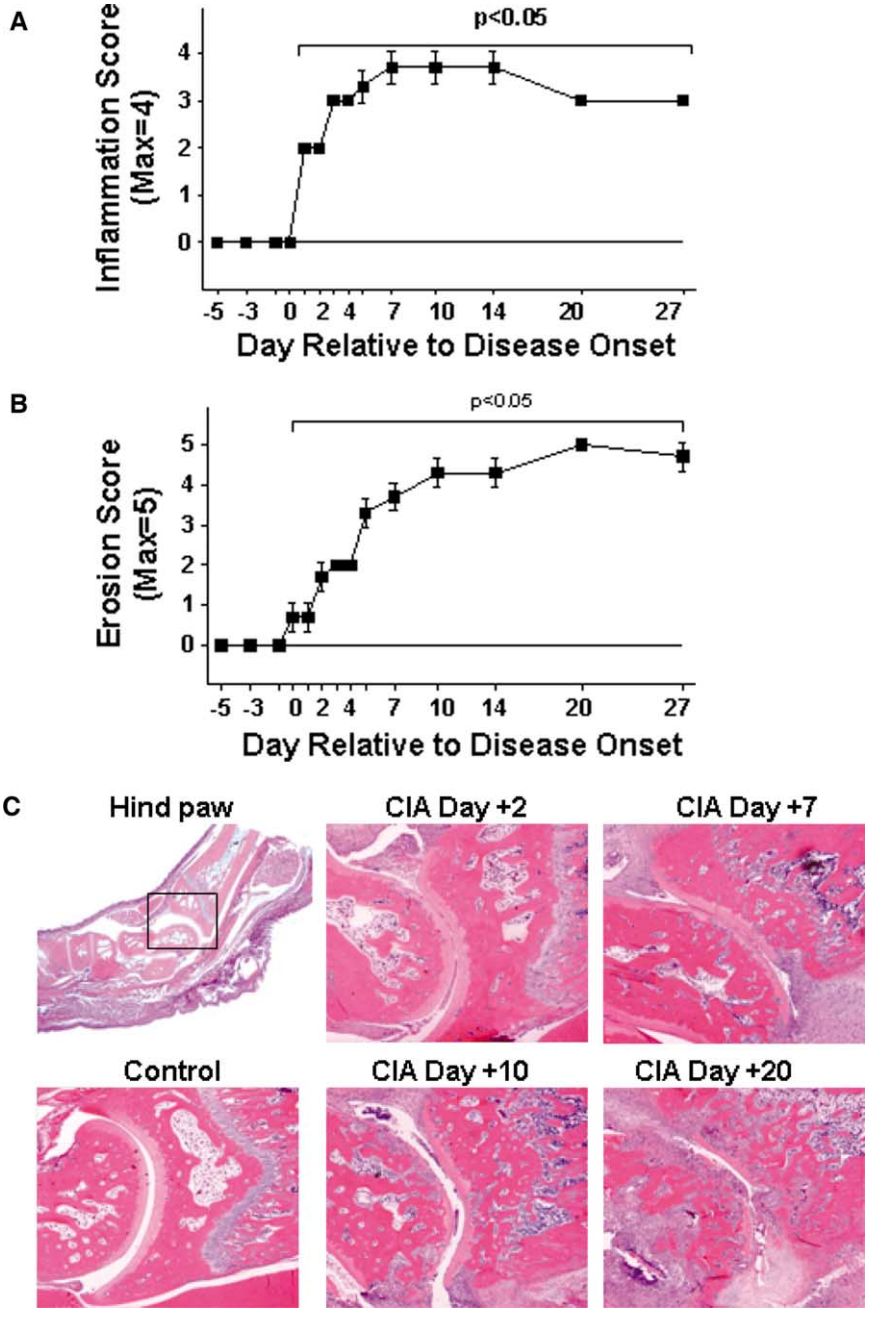


Figure 1. Progression of collagen-induced arthritis (CIA) in hind paws as indicated by histopathological scores for inflammation (A) and joint erosion (B), and representative images (C; original magnification 2x; haematoxylin and eosin stain; the principal site of CIA is denoted by solid square box on the whole paw image). Mean (±SEM) of arthritic groups (squares) defined by brackets were significantly different from values of non-CIA controls (straight line). Onset of clinical disease in the hind paw is designated as day 0 on the *x*-axis. The erosion panel (B) has been reproduced from Stolina et al. 2005 with permission of the American Society for Bone and Mineral Research.

*Haematological data*

Circulating blood cell populations in arthritic animals were altered at all time points (Figure 2*A*). Prior to onset, mean white blood cells (WBC), neutrophils and monocyte numbers were significantly increased ($p<0.01$ vs non-CIA control). Even though the populations of myeloid cells remained high throughout the study, the increased absolute numbers of WBC, neutrophils and monocytes demonstrated a biphasic shape, with the first peak coinciding with CIA onset (day 0), and the second with the first clinical evidence of arthritis in the fore paws (day+14 post-onset). In both the blood and spleen of CIA rats, B- and T-lymphocyte numbers were significantly reduced prior to disease onset and remained so through the clinical stages of CIA ($p<0.01$; Figure 2*A, B*). Conversely, the CD4+/CD8+ cell ratio within the T-lymphocyte population was significantly elevated in both blood and spleen throughout the preclinical and clinical stages of CIA progression ($p<0.05$ compared with controls).

Haematological and flow cytometric analyses of lymph node cell suspensions showed resident leukocyte populations in local lymph nodes (inguinal and popliteal LNs) were altered during the preclinical stage of AIA (Figure 3). The mean number of leukocytes residing in inguinal and popliteal LNs increased from $4.7\times10^6$ and $1.1\times10^6$ (basal levels in control rats, correspondingly) to $43.5\times10^6$ (inguinal LN) and $6.3\times10^6$ (popliteal LN) at day -5, ultimately reaching a maximum cellularity of $149.4\times10^6$ (inguinal LN, day+7) and $23.5\times10^6$ (popliteal LN, day+5) during acute CIA. The majority of resident leukocytes in draining LNs was represented by significantly increased numbers of B and T (both CD4+ and CD8+) lymphocytes ($p<0.05$). In contrast to what was observed in the blood and spleen, the CD4+/CD8+ T-cell ratio was not altered in local LNs of CIA rats compared with control rats (data not shown). The percentage of CD3+ T lymphocytes in local LNs of CIA rats was significantly reduced relative to that of control animals (58% to 75% *vs* 84%, respectively; $p<0.05$), whereas the percentage of CD45RA+ B lymphocytes was significantly elevated relative to that of non-CIA controls ($p<0.05$).

The absolute numbers of neutrophils and monocytes in inguinal LNs of CIA animals were significantly increased throughout all stages of arthritis progression ($p<0.05$, Figure 3), whereas significantly increased cellularity of popliteal LNs was observed at the beginning of acute clinical CIA (from day 0 to day+2) and after day +4. The percentage of myeloid cells in local LNs increased significantly from 3% at baseline to 7–12% at the peak of active disease (from day+7 to day+10, $p<0.05$). The cellularity within distant (mesenteric) LNs was not altered during disease progression (data not shown).

*Systemic immunoglobulin changes*

Serum levels of IgM (an efficient activator of the classical complement pathway) and IgG (the predominant antibody of the secondary immune response) were significantly enhanced during clinical CIA (Figure 4). Levels of both IgM and IgG were significantly elevated (1.5- to 6-fold, $p<0.05$) during acute CIA and moderately increased (1.5- to 3-fold, $p<0.05$) during chronic CIA. IgG1 and IgG2a (principal elements of Th1- or Th2-type immune responses, respectively (Kaplan et al. 2002)), IgG2b, and IgG2c (a co-marker for B-cell activation in the splenic marginal zone (Oliver et al. 1997)) followed the same pattern as total IgG. In contrast, IgE was

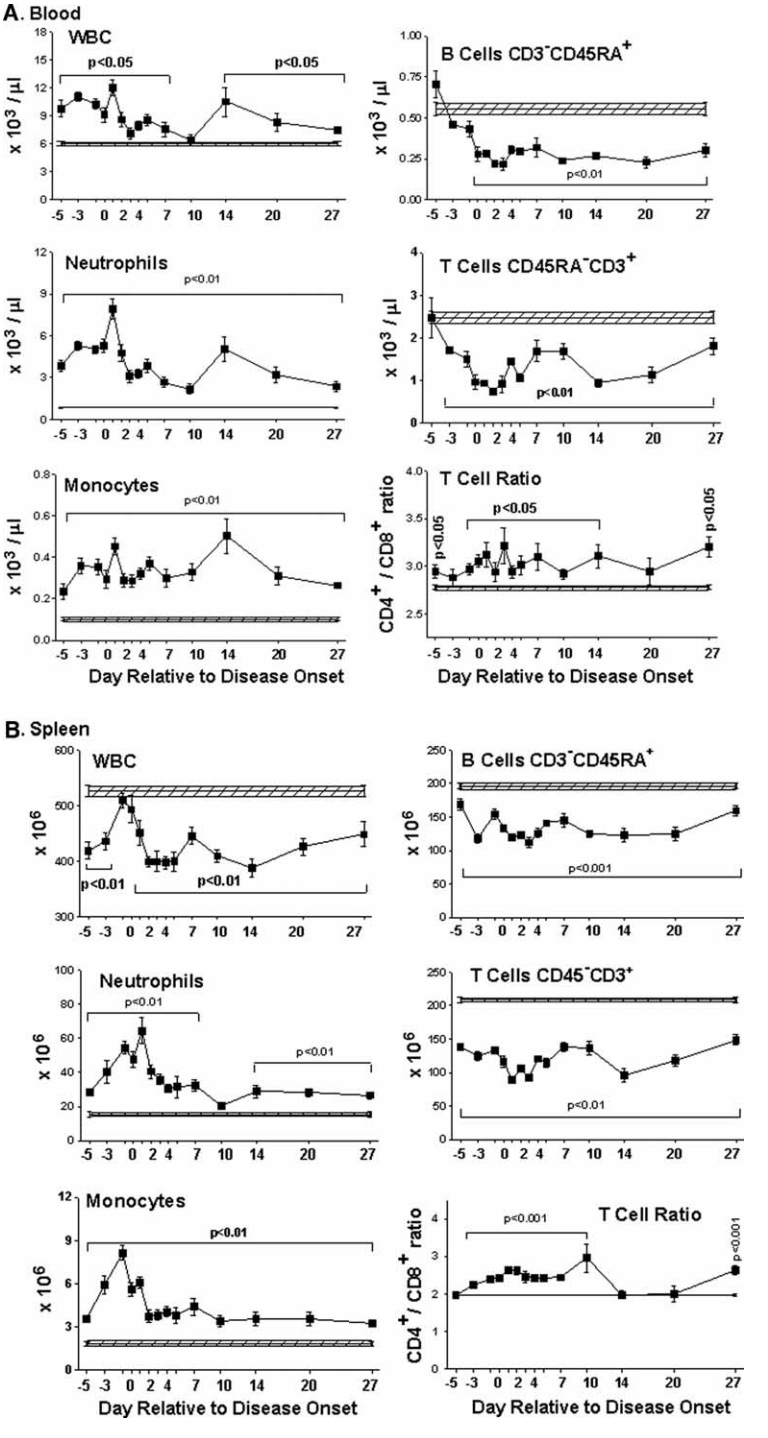


Figure 2. Systemic changes in leukocyte populations circulating in blood (A) or residing in spleen (B) during progression of collagen-induced arthritis. Mean (±SEM) of arthritic groups encompassed by the bracket were significantly different from non-CIA controls (horizontal hatched bar represents mean ± SEM of controls).

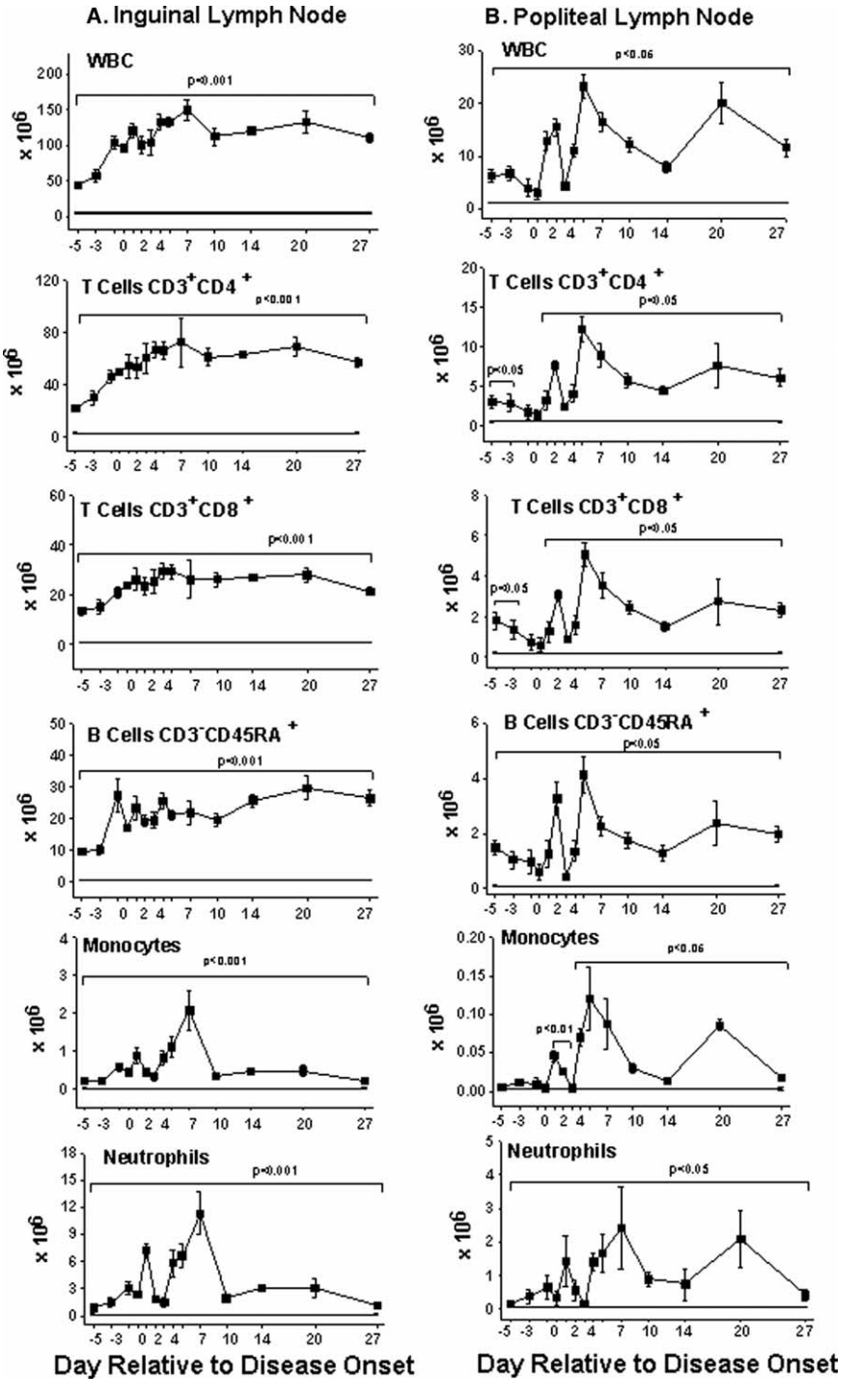


Figure 3. Local changes in leukocyte populations within draining inguinal lymph nodes (A) and popliteal lymph nodes (B) during progression of collagen-induced arthritis. Mean (±SEM) of arthritic groups encompassed by the bracket were significantly different from controls (horizontal line represents mean ±SEM of controls).

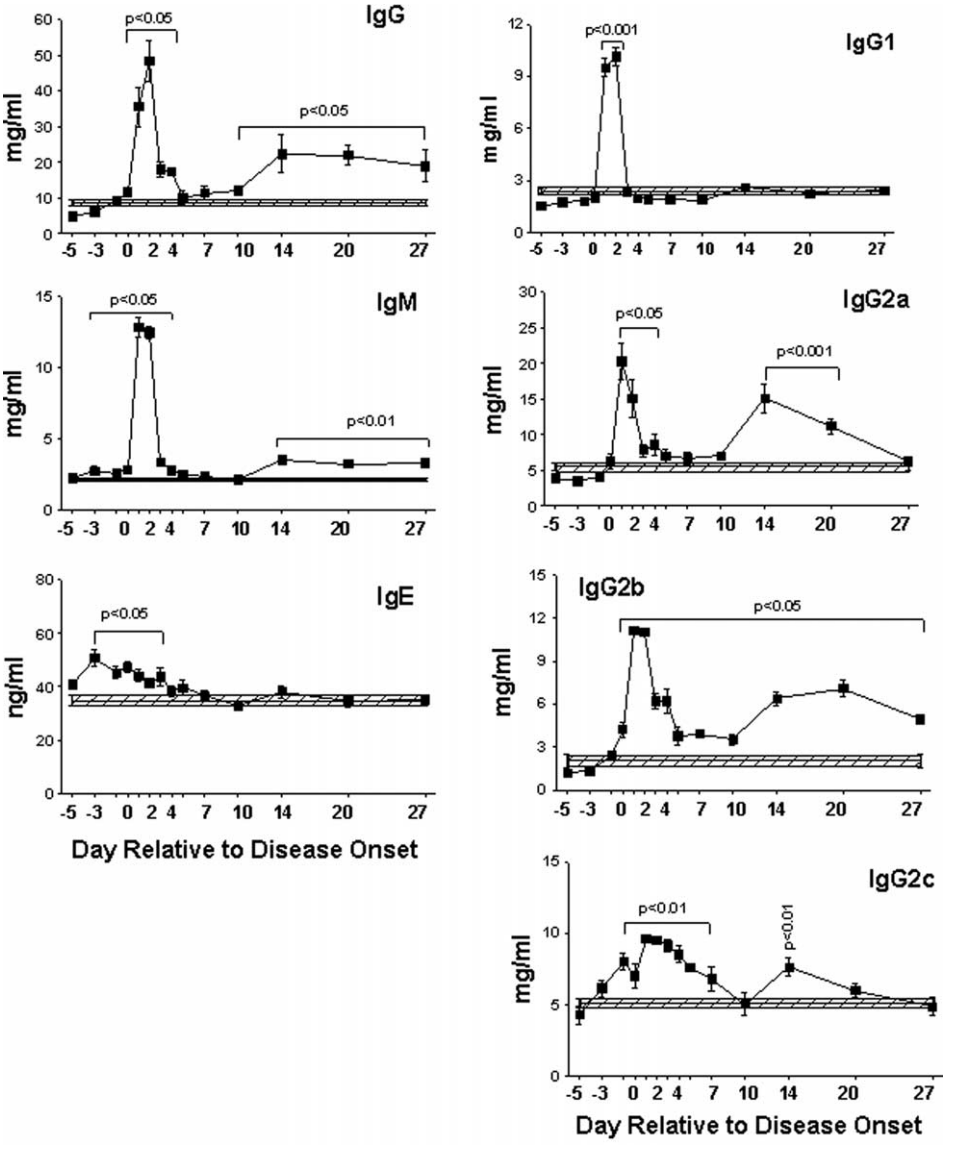


Figure 4. Systemic circulating immunoglobulin levels in serum during progression of collagen-induced arthritis. Mean (±SEM) of arthritic groups encompassed by the bracket were significantly different from non-CIA controls (horizontal hatched bar represents mean±SEM of controls).

significantly amplified during the preclinical and acute phase of disease ($p<0.05$ vs non-CIA controls).

*Systemic and local changes in biochemical markers of inflammation*

Multiple cytokines (IL-1α/β, TNF-α, TGF-β, IL-2, IL-4, IL-6, IL-10, IL-12, IL-17, IL-18, CCL2, KC/GRO, GM-CSF, IFN-γ, $PGE_2$ and α1AGP were evaluated in serum and hind paw protein extracts at all designated time points of CIA progression.

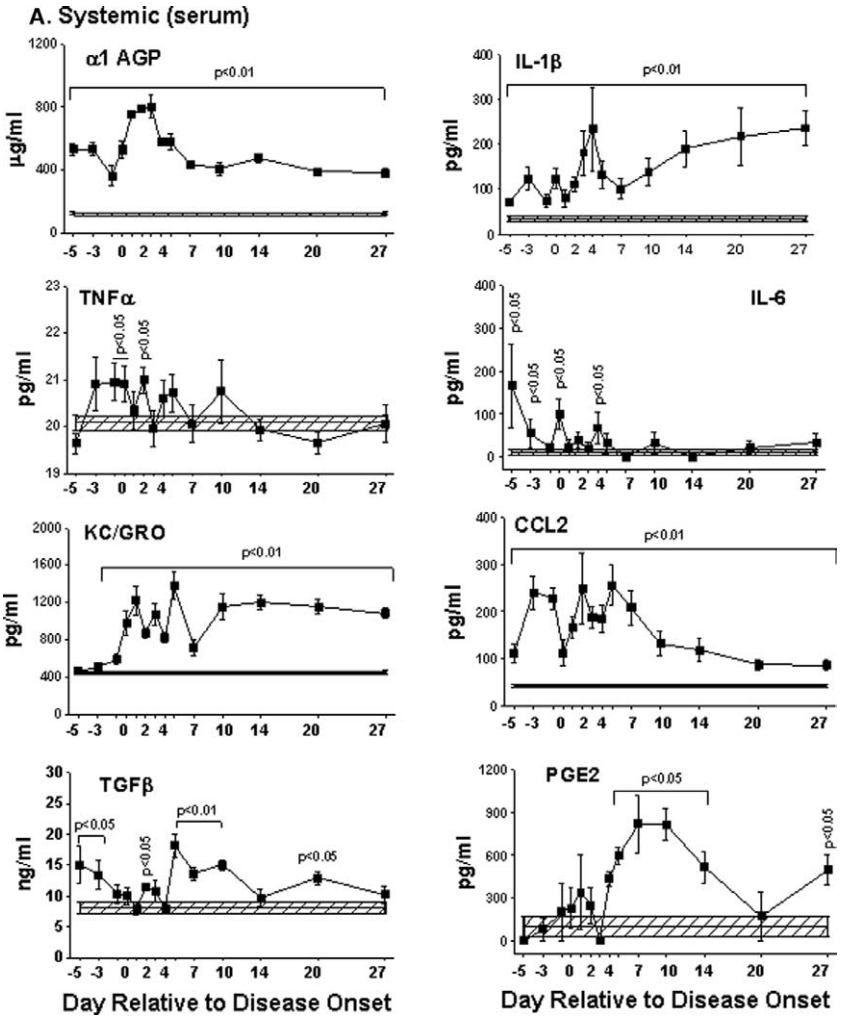


Figure 5. Systemic (serum, A) and local (hind paw protein extract, B) concentrations of immune modulators, cytokines and chemokines during progression of collagen-induced arthritis. Mean (±SEM) of arthritic groups encompassed by the bracket were significantly different from non-CIA controls (horizontal hatched bar represents mean±SEM of controls).

The systemic and local levels of IL-2, IL-4, IL-10, IL-12, IL-17 and GM-CSF were not different from those detected in non-CIA control samples at any time point examined (data not shown).

Changes in systemic (serum, Figure 5A) and/or local (hind paw tissue protein extracts, Figure 5B) concentrations of several immune modulators reflected the progression of arthritis through preclinical and clinical stages of CIA progression. Serum α1AGP, the dominant acute-phase protein in rats (Petersen et al. 2004), was significantly increased throughout CIA ($p<0.01$), while tissue α1AGP was significantly increased only during acute clinical disease.

Serum IL-1β was significantly elevated ($p<0.01$ compared with non-CIA control) throughout all stages of arthritis. Significant increases in tissue levels of IL-1β were

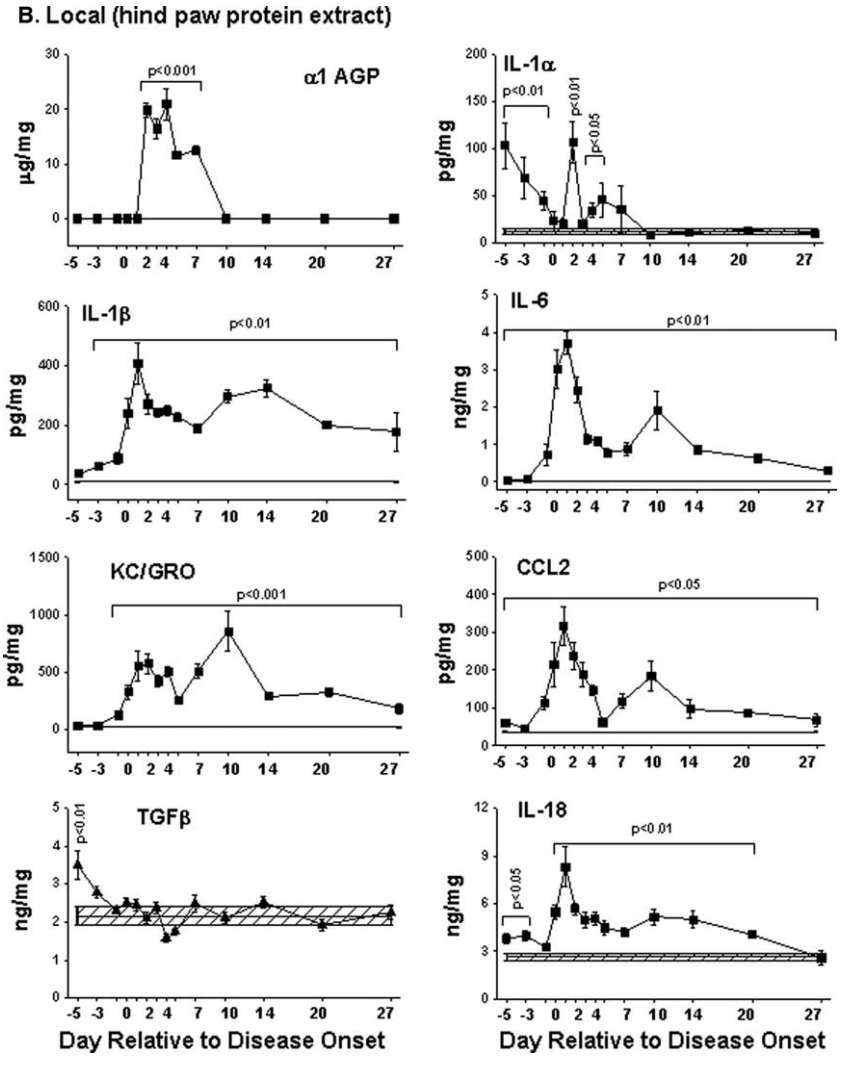


Figure 5 (Continued)

observed at day −3, and increased steeply to peak at day+1 (acute CIA) before gradually declining to a lower steady-state range (177–296 pg $mg^{-1}$ total protein).

IL-1$\alpha$ was not detected in serum; however, tissue IL-1$\alpha$ was significantly elevated 5 days before CIA onset (103 pg $mg^{-1}$ vs undetectable concentrations in non-CIA controls). A second significant, but transient, peak was observed during the acute stage of CIA.

Serum TNF-$\alpha$ was modestly elevated during preclinical CIA (day -3) and on the days immediately surrounding disease onset (day -1, day 0 and day+2), but did not exceed 21 pg $ml^{-1}$ (range 17–20 pg $ml^{-1}$ for non-CIA control rats). TNF-$\alpha$ protein was not detected in paw extracts of non-CIA control rats and, surprisingly, was also not detected in tissue extracts of arthritic animals. Using *in situ* hybridization, TNF-$\alpha$ expression was not detected in the non-CIA control rats, or during *pre*clinical stages of arthritis in rats with CIA. Low diffuse TNF-$\alpha$ signal was first detected in the inflamed

periarticular soft tissue of hind paws at disease onset. At later time points, occasional focal patches of moderate labelling were observed in marrow space areas adjacent to eroding bone (data not shown).

Serum IL-6 was increased significantly at day -5 before CIA onset (166 pg $ml^{-1}$, $p<0.05$), at onset (99 pg $ml^{-1}$, $p<0.05$), and day+4 (68 pg $ml^{-1}$, $p<0.05$). Concentrations of IL-6 in tissue extracts increased rapidly by day -1, peaked at day +2 (3.8 ng $mg^{-1}$ total protein), then rapidly declined to lower levels (0.7–1.9 ng $mg^{-1}$ total protein). These increases were significant on all days evaluated ($p<0.01$ vs non-CIA control).

KC/GRO was significantly elevated in both serum and tissue during all stages of disease ($p<0.05$ for both vs non-CIA controls). Serum levels of CCL2 were significantly elevated at day -5 and peaked at 208–240 pg $ml^{-1}$ (relative to non-CIA control levels of 43 pg $ml^{-1}$) during the acute phase of clinical CIA before gradually declining. In tissue extracts, CCL2 increased rapidly at day -1, peaked at day+1 (316 pg $mg^{-1}$ vs 36 pg $mg^{-1}$ in non-CIA controls), then declined. A second, but lower, spike in tissue levels of CCL2 occurred at day+10 (184 pg $mg^{-1}$ total protein).

TGF-β concentration in serum and tissue of CIA rats was significantly increased at the preclinical stage of CIA progression ($p<0.05$ vs non-CIA controls), and continued to be significantly higher in blood, but not tissue extracts, during clinical CIA.

Serum $PGE_2$ was significantly elevated on day+4 through day+14 ($p<0.05$ vs non-CIA control). However, no $PGE_2$ was detected in either CIA or non-CIA tissue extracts.

Serum concentrations of IL-18 were significantly elevated in arthritic rats at day +5 (207 pg $ml^{-1}$) and day+27 (276 pg $ml^{-1}$) relative to the concentrations in non-CIA control rats (67 pg $ml^{-1}$; $p<0.05$). In contrast, IL-18 concentrations in tissue extracts from CIA rats were significantly increased in preclinical CIA, peaked at day +1 (8.3 ng $mg^{-1}$ vs 2.6 ng $mg^{-1}$ in non-CIA controls), and remained significantly elevated through day+20.

### *Systemic and local changes in pro- and anti-erosive bone markers*

If levels of RANKL, a mediator of bone resorption, are inversely correlated with levels of OPG, an inhibitor of RANKL, bone destruction is favoured (Figure 6). As described previously (Stolina et al. 2005) and shown here for context, RANKL levels in CIA serum were significantly increased compared with levels in control serum starting at day+2, and remained elevated (range 88–110 pg $ml^{-1}$ vs 58 pg $ml^{-1}$ in control group; $p<0.05$) throughout CIA progression, except for a transient drop at day+3. The RANKL concentration in tissue extracts of arthritic animals was significantly increased at day+1 through day+27 ($p<0.05$ compared with non-CIA control rats). Intra-articular RANKL peaked at day+5. While, serum OPG concentrations in CIA rats were generally similar to those of control rats, tissue OPG levels began to decline during preclinical CIA (day -1). These level were significantly lower than control levels at disease onset (300 pg $mg^{-1}$ in CIA rats vs 1669 pg $mg^{-1}$ in non-CIA control; $p<0.001$) and at all subsequent time points evaluated. The RANKL:OPG ratio in arthritic rats was 1.5- to 2.4-fold above the ratio in non-CIA control rats on all days after clinical disease onset except for day+1 and day+3.

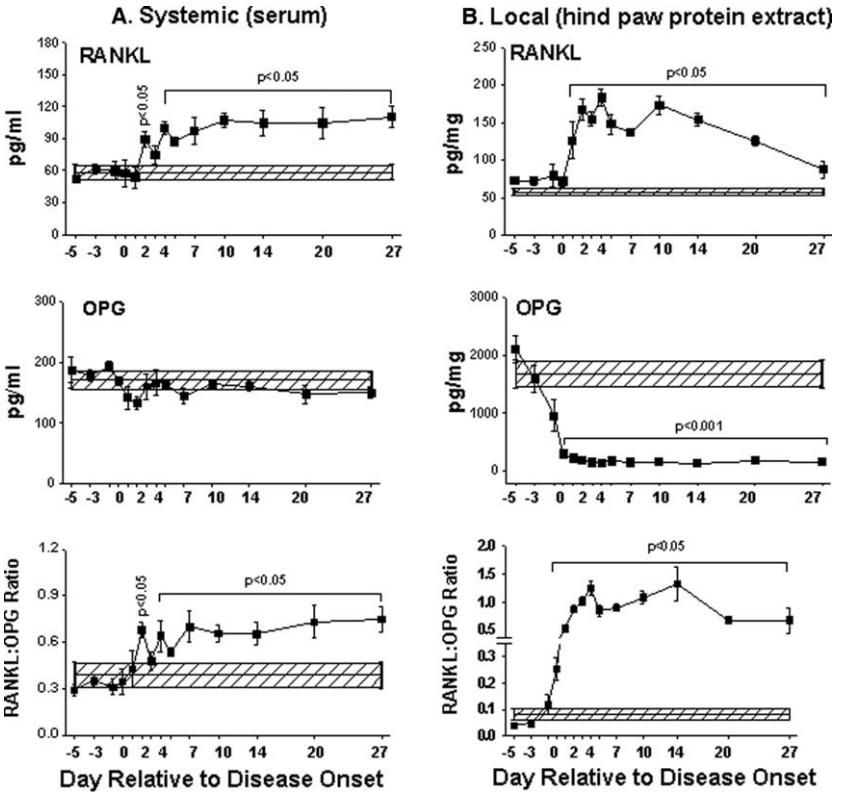


Figure 6. Systemic (serum, A) and local (hind paw protein extract, B) concentrations of bone turnover markers during progression of collagen-induced arthritis. Mean (±SEM) of arthritic groups encompassed by the bracket were significantly different from controls (horizontal hatched bar represents mean±SEM of non-CIA controls). The RANKL panels have been reproduced from Stolina et al. 2005 with permission of the American Society for Bone and Mineral Research.

Relative to the control group, the RANKL:OPG ratio in paw tissue from CIA rats was significantly higher starting at disease onset (2.5-fold; $p<0.05$) and reached a maximum (12.4-fold increase) by day+4. The local RANKL/OPG ratio was approximately 240% higher than the systemic RANKL:OPG ratio for days+4 through+27 ($p<0.05$).

## Discussion

To evaluate targets for therapeutic intervention, it is important to understand the spatial and temporal expression patterns of the myriad factors that have been implicated in RA progression. Peripheral blood provides an expedient source to assess systemic levels of these factors, while joint tissue provides important information on local changes (Arend 2001, Andreakos et al. 2002, Stolina et al. 2005). The majority of clinical studies focus on either systemic or local markers related to the specific stage of arthritis progression and frequently disconnects between local and systemic cytokines are revealed even within the disease state. However, very few studies also compare these differences with the normal healthy

state, an important comparison as whether or not a given cytokine appears higher in joints than in blood is only relevant if this relationship differs from the normal healthy state (Steiner et al. 1999, Arend 2001, Andreakos et al. 2002, Rosengren et al. 2003, Stolina et al. 2005).

During the past two decades TNF-α and IL-1 have been shown to serve as dominant mediators of immune-mediated arthritis in both humans (Arend & Dayer 1995, Bresnihan et al. 1998, Richard-Miceli & Dougados 2001) and animals (Kuiper et al. 1998, Joosten et al. 1999, Feige et al. 2000, Iwakura 2002). Nevertheless, the incomplete response in some patients (Kulmatycki & Jamali 2005, Koller 2006) and animals (Feige et al. 2000, Schett et al. 2005) with immune-mediated arthritis to TNF-α and IL-1 inhibitors suggests that other potential mediators exist. Indeed, several other cytokine inhibitors (Rollins 1996, Schulze-Koops & Kalden 2001, Punzi et al. 2002, Gaffen 2004, McInnes & Gracie 2004, Lubberts et al. 2005) have been shown to reduce disease severity in animal arthritis models.

We induced CIA in rats to probe the pathogenesis of progressive immune-mediated arthritis. Our two hypotheses were that local (joint and regional lymph nodes) and systemic (serum and distant lymphoid tissues) consequences might be driven by distinctive repertoires of signalling molecules, and that local and/or systemic increases in other cytokines besides IL-1 or TNF-α would emerge as potential mediators (and biomarkers) of arthritis.

Our first significant result was that regulation of local and systemic inflammation was a function of distinct mediator mixtures (Table II). This interpretation is supported by observations that while IL-1 was significantly increased in both CIA sera and inflamed joints, TNF-α protein was detected only in serum (Figure 5).



Table II. Comparison of systemic and local changes in biomarkers relative to the progression of collagen-induced arthritis (CIA).

| | CIA vs. Non-CIA Control (SERUM) | | | CIA vs. Non-CIA Control (PAW) | | |
|---|---|---|---|---|---|---|
| | | Clinical | | | Clinical | |
| Marker | Pre-clinical | Acute | Chronic | Pre-clinical | Acute | Chronic |
| α1AGP | ▲▲▲ | ▲▲▲ | ▲▲▲ | = | ▲▲▲ | = |
| TNFα | ▲ | ▲ | = | = | △ | △ |
| IL-1α | = | = | = | ▲▲▲ | ▲▲ | = |
| IL-1β | ▲▲ | ▲▲▲ | ▲▲▲ | ▲▲ | ▲▲▲ | ▲▲▲ |
| TGFβ | ▲ | ▲ | ▲ | ▲ | = | = |
| CCL2 | ▲▲▲ | ▲▲▲ | ▲ | ▲▲ | ▲▲▲ | ▲▲ |
| IL-6 | ▲ | ▲ | = | ▲▲ | ▲▲▲ | ▲▲▲ |
| IL-18 | = | = | = | ▲ | ▲▲ | ▲ |
| KC/GRO | ▲ | ▲▲ | ▲▲ | = | ▲▲▲ | ▲▲▲ |
| $PGE_2$ | = | ▲▲▲ | ▲▲ | = | = | = |
| RANKL | = | ▲ | ▲ | = | ▲▲▲ | ▲▲ |
| OPG | = | = | = | = | ▼▼▼ | ▼▼▼ |
| RANKL/OPG | = | ▲ | ▲ | = | ▲▲▲ | ▲▲▲ |
| TRACP-5B (Stolina et al. 2005) | = | = | = | = | ▲▲ | ▲▲▲ |

=, no change; one triangle, 1.3- to 2-fold change; two triangles, >2- to <3-fold change; three triangles, ≥3-fold change; black, detected by protein assay; white, detected by in situ hybridization; triangle apex up =increase, triangle apex down = decrease; tartrate-resistant acid phosphatase 5B (TRACP-5b; trends derived using data published in Stolina et al., 2005).

Furthermore, both IL-1 forms but not TNF-α were increased in paw protein extracts prior to CIA onset (Figure 5), and TNF-α mRNA expression in markedly inflamed hind paws was minimal and remained so over time in rats with CIA (*in situ* mRNA hybridization, data not shown). Our results are supported by a recently published report, showing that in arthritic joints TNF-α protein levels were quite low relative to levels of TNF-α mRNA expression, whereas for both IL-1β and IL-6 the levels of mRNA and protein correlated much more closely (Rioja et al. 2004). Systemic TNF-α augments progression of arthritis-related bone erosions by releasing osteogenic precursors from the bone marrow and by 'priming' them to respond to RANKL (Li et al. 2004, Ochi et al. 2007). This interpretation is further supported as a general principle for pro-arthritic signalling molecules by our prior demonstration that the different concentrations of RANKL (a TNF superfamily member that is an essential mediator of bone erosions) seen systemically and locally were strongly correlated with the induction of divergent degrees of systemic and local osteopenia in rats with CIA starting as early as the day of disease onset (Schett et al. 2005, Stolina et al. 2005). Taken together, these findings support the hypothesis that in rat CIA, IL-1 propels joint destruction locally while TNF-α drives systemic sequelae (van den Berg 2001).

Secondly, the nature of the inflammatory changes in CIA rats differed in regional lymph nodes (inguinal, popliteal) compared with more distant lymphoid tissues (mesenteric lymph nodes, spleen). Regional lymph nodes, but not spleen or distant lymph nodes, exhibited reactive hyperplasia (Figure 2B and 3). This anatomical distinction was reflected in absolute cell counts, as local nodes contained significantly more lymphocytes, neutrophils and monocytes (Figure 3), while more distant nodes did not (data not shown). Interestingly, the significant influx of B cells into local lymph nodes coincided with the peak of immunoglobulin levels in CIA serum (Figure 4), but was the reverse of the situation in spleen, where these two populations of lymphocytes were reduced relative to those in non-CIA control rats (Figure 2B). These unique biochemical and cellular signatures first began developing well before CIA onset (Figures 2, 3 and 5). Progression of CIA could be divided into three different stages: preclinical (before disease onset), acute clinical (from disease onset until 14 days after onset) and chronic clinical (more than 14 days after onset). The analysis of systemic and/or local biomarker profiles of CIA progression relative to the phase of arthritis development is represented inTable II. When partitioned in this fashion, some proinflammatory molecules were increased mainly during preclinical CIA (e.g. IL-1α and TGF-β in joints; TNF-α and IL-6 in serum), others were detected chiefly during clinical CIA (e.g. $PGE_2$ in serum, KC/GRO in paw extracts), and still others displayed as either sporadic spikes or consistent increases that spanned the whole course of disease (e.g. IL-1β and CCL2 in joints and serum; α1AGP and TGF- in serum; and IL-18 in joints). Intra-articular consequences are probably regulated by elevated tissue levels of proinflammatory mediators, while systemic effects are propelled by high circulating concentrations. This deduction implies that IL-1 (both forms), IL-6, IL-18, CCL2, and KC/GRO serve as the key regulators for inciting joint inflammation in CIA. Previous work implicating IL-18 (McInnes & Gracie 2004) and TNF-α (Maini et al. 1999) as possible mediators in RA suggests that these two molecules should receive further study as potential players in CIA.

In addition to proinflammatory mediators, our time-course data show marked dysregulation of mediators of bone turnover in inflamed joints. Local levels of RANKL – a primary mediator of osteoclast formation, function and survival and

hence a gauge for enhanced bone resorption – and its soluble decoy receptor OPG (an inhibitor of osteoclast formation, activation and survival) changed sharply in opposite directions (Figure 5, Table II) during the same time frame in which production of activated intra-articular osteoclasts is enhanced (Stolina et al. 2005) and the first joint erosions appear (Figure 1C). The RANKL:OPG ratio is considered to be an important determinant of bone resorption in arthritis (Geusens et al. 2006). Our newly validated methods for measuring OPG and RANKL in rats represent important technical advances, due to the widespread use of rats as models for investigating human bone disease. As a first application, we determined (Table II) that the local reduction in intra-articular OPG protein levels and the associated increase in the RANKL:OPG ratio coincided with significant local increases in intra-articular osteoclast numbers and levels of the osteoclast marker tartrate-resistant acid phosphatase 5B (TRACP-5b) (Stolina et al. 2005). Given the extreme proresorptive state associated with the local increase in RANKL:OPG ratio (Grimaud et al. 2003), we posit that RANKL is the primary driver of bone erosion in CIA. Other molecules capable of stimulating osteoclast differentiation (e.g. IL-1β, TGF-β, TNF-α) might enhance this function by recruiting preosteoclasts and promoting osteoclast differentiation (Lam et al. 2000, Cheon et al. 2002, Wei et al. 2005, Kindle et al. 2006) and/or by modulating RANKL and OPG levels (Hofbauer et al. 1999, Koenders et al. 2005). The dominance of RANKL in mediating bone erosions is further supported by the ability of OPG to cause greater suppression of bone erosions compared with inhibitors of TNF-α or IL-1 in these models, even when OPG treatment is undertaken in the presence of severe ongoing joint inflammation (Feige et al. 2000, Campagnuolo et al. 2002). The marked OPG reduction in inflamed joints (Figure 6), which was not apparent in peripheral blood, further highlights the value of evaluating local changes in arthritis.

Our study revealed the presence of several potential serum biomarkers that were expressed ahead of CIA onset. The immune system of rats injected with collagen was reacting vigorously long before clinical disease developed, as shown by altered leukocyte populations in local (inguinal lymph node, Figure 3) and distant (spleen,Figure 2B) lymphoid organs. These changes were accompanied by significant elevations in serum levels of the acute-phase protein α1AGP (4- to 7-fold greater) as well as the proinflammatory cytokines IL-1β (elevated by 2.5- to 7-fold) and CCL2 (3- to 6-fold higher). We propose that the best non-invasive method to indicate that an immune response has been provoked would be to measure two or more of these molecules in a single serum sample. The slight delay (until day+2) before serum RANKL concentrations started to rise suggests that this molecule will have little utility in evaluating the early stages of CIA. However, measurement of serum RANKL was clearly effective in following the disease course once skeletal damage was launched. As structural joint damage is already present in most human patients diagnosed with immune-mediated arthritis, quantification of serum RANKL is predicted to be another possible biomarker for following the progression of early disease. Additional work with patients with RA will be needed to define what other systemic markers of bone resorption would be suitable markers for following early disease (McInnes & Schett 2007).

Finally, the current CIA results considered in conjunction with data from a comparable study in Lewis rats with a mycobacteria-incited AIA model (Stolina et al. 2008) provides the foundation for a better molecular understanding of arthritis

syndromes. Rat models of immune-mediated joint diseases incited by collagen or mycobacteria arise from distinct immunopathogenic mechanisms (Cremer et al. 1983). Their divergent responses to anti-TNF-α and anti-IL-1 agents (Schett et al. 2005) suggest that differences in the cytokine milieu elaborated within the local and/or systemic compartments is a major factor in the unique progression of CIA and AIA; the dissimilar cytokine pattern and kinetics in our CIA (Table II) and AIA (Stolina et al. 2008) studies confirm this notion. One implication for patients with RA or spondyloarthropathy is that such conditions might represent a continuum of patient-specific syndromes rather than a single condition with one immutable pathogenesis. Further work will be required to evaluate which other cytokines have roles in regulating rheumatoid arthritis and other immune-mediated joint disease of sufficient impact to warrant the development of specific inhibitors directed against them.

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## References

Andreakos ET, Foxwell BM, Brennan FM, Maini RN, Feldmann M. 2002. Cytokines and anti-cytokine biologicals in autoimmunity: present and future. Cytokine and Growth Factor Reviews 13:299–313.

Arend WP. 2001. Physiology of cytokine pathways in rheumatoid arthritis. Arthritis and Rheumatism 45:101–106.

Arend WP, Dayer JM. 1990. Cytokines and cytokine inhibitors or antagonists in rheumatoid arthritis. Arthritis and Rheumatism 33:305–315.

Arend WP, Dayer JM. 1995. Inhibition of the production and effects of interleukin-1 and tumor necrosis factor alpha in rheumatoid arthritis. Arthritis and Rheumatism 38:151–160.

Bolon B, Shalhoub V, Kostenuik PJ, Campagnuolo G, Morony S, Boyle WJ, Zack D, Feige U. 2002. Osteoprotegerin, an endogenous antiosteoclast factor for protecting bone in rheumatoid arthritis. Arthritis and Rheumatism 46:3121–3135.

Breedveld FC. 1999. Future trends in the treatment of rheumatoid arthritis: cytokine targets. Rheumatology (Oxford) 38(Suppl. 2):11–13.

Brennan FM, Field M, Chu CQ, Feldmann M, Maini RN. 1991. Cytokine expression in rheumatoid arthritis. British Journal of Rheumatology 30(Suppl. 1):76–80.

Bresnihan B, Alvaro-Gracia JM, Cobby M, Doherty M, Domljan Z, Emery P, Nuki G, Pavelka K, Rau R, Rozman B, Watt I, Williams B, Aitchison R, McCabe D, Musikic P. 1998. Treatment of rheumatoid arthritis with recombinant human interleukin-1 receptor antagonist. Arthritis and Rheumatism 41: 2196–2204.

Calabrese LH. 2003. Molecular differences in anticytokine therapies. Clinical and Experimental Rheumatology 21:241–248.

Campagnuolo G, Bolon B, Feige U. 2002. Kinetics of bone protection by recombinant osteoprotegerin therapy in Lewis rats with adjuvant arthritis. Arthritis and Rheumatism 46:1926–1936.

Cheon H, Yu SJ, Yoo DH, Chae IJ, Song GG, Sohn J. 2002. Increased expression of pro-inflammatory cytokines and metalloproteinase-1 by TGF-beta1 in synovial fibroblasts from rheumatoid arthritis and normal individuals. Clinical and Experimental Immunology 127:547–552.

Cohen SB, Dore RK, Lane NE, Ory PA, Peterfy CG, Sharp JT, van der HD, Zhou L, Tsuji W, Newmark R. 2008. Denosumab treatment effects on structural damage, bone mineral density, and bone turnover in rheumatoid arthritis: a twelve-month, multicenter, randomized, double-blind, placebo-controlled, phase II clinical trial. Arthritis and Rheumatism 58:1299–1309.

Coxon A, Bolon B, Estrada J, Kaufman S, Scully S, Rattan A, Duryea D, Hu YL, Rex K, Pacheco E, Van G, Zack D, Feige U. 2002. Inhibition of interleukin-1 but not tumor necrosis factor suppresses neovascularization in rat models of corneal angiogenesis and adjuvant arthritis. Arthritis and Rheumatism 46:2604–2612.

Cremer MA, Hernandez AD, Townes AS, Stuart JM, Kang AH. 1983. Collagen-induced arthritis in rats: antigen-specific suppression of arthritis and immunity by intravenously injected native type II collagen. Journal of Immunology 131:2995–3000.

Crotti TN, Smith MD, Weedon H, Ahern MJ, Findlay DM, Kraan M, Tak PP, Haynes DR. 2002. Receptor activator NF-kappaB ligand (RANKL) expression in synovial tissue from patients with rheumatoid arthritis, spondyloarthropathy, osteoarthritis, and from normal patients: semiquantitative and quantitative analysis. Annals of the Rheumatic Diseases 61:1047–1054.

Feige U, Hu YL, Gasser J, Campagnuolo G, Munyakazi L, Bolon B. 2000. Anti-interleukin-1 and anti-tumor necrosis factor-alpha synergistically inhibit adjuvant arthritis in Lewis rats. Cellular and Molecular Life Sciences 57:1457–1470.

Gaffen SL. 2004. Biology of recently discovered cytokines: interleukin-17 – a unique inflammatory cytokine with roles in bone biology and arthritis. Arthritis Research and Therapy 6:240–247.

Geusens PP, Landewe R, Garnero P, Chen D, Dunstan CR, Lems WF, Stinissen P, van der Heijde D, van der Linden S, Boers M. 2006. The ratio of circulating osteoprotegerin to RANKL in early rheumatoid arthritis predicts later joint destruction. Arthritis and Rheumatism 54:1772–1777.

Grimaud E, Soubigou L, Couillaud S, Coipeau P, Moreau A, Passuti N, Gouin F, Redini F, Heymann D. 2003. Receptor activator of nuclear factor kappaB ligand (RANKL)/osteoprotegerin (OPG) ratio is increased in severe osteolysis. American Journal of Pathology 163:2021–2031.

Hofbauer LC, Lacey DL, Dunstan CR, Spelsberg TC, Riggs BL, Khosla S. 1999. Interleukin-1b and tumor necrosis factor-α, but not interleukin 6, stimulate osteoprotegerin ligand gene expression in human osteoblastic cells. Bone 25:255–259.

Iwakura Y. 2002. Roles of IL-1 in the development of rheumatoid arthritis: consideration from mouse models. Cytokine and Growth Factor Reviews 13:341–355.

Joosten LA, Helsen MM, Saxne T, van de Loo FA, Heinegard D, van den Berg WB. 1999. IL-1 alpha beta blockade prevents cartilage and bone destruction in murine type II collagen-induced arthritis, whereas TNF-alpha blockade only ameliorates joint inflammation. Journal of Immunology 163:5049–5055.

Kaplan C, Valdez JC, Chandrasekaran R, Eibel H, Mikecz K, Glant TT, Finnegan A. 2002. Th1 and Th2 cytokines regulate proteoglycan-specific autoantibody isotypes and arthritis. Arthritis Research 4:54–58.

Keller C, Webb A, Davis J. 2003. Cytokines in the seronegative spondyloarthropathies and their modification by TNF blockade: a brief report and literature review. Annals of the Rheumatic Diseases 62:1128–1132.

Kindle L, Rothe L, Kriss M, Osdoby P, Collin-Osdoby P. 2006. Human microvascular endothelial cell activation by IL-1 and TNF-alpha stimulates the adhesion and transendothelial migration of circulating human CD14+ monocytes that develop with RANKL into functional osteoclasts. Journal of Bone and Mineral Research 21:193–206.

Koenders MI, Lubberts E, Oppers-Walgreen B, van den Bersselaar L, Helsen MM, Di Padova F, Boots MH, Gram H, Joosten L, van den Berg WB. 2005. Blocking of interleukin-17 during reactivation of experimental arthritis prevents joint inflammation and bone erosion by decreasing RANKL and interleukin-1. American Journal of Pathology 167:141–149.

Koller MD. 2006. Targeted therapy in rheumatoid arthritis. Wiener Medizinische Wochenschrift 156: 53–60.

Kong YY, Feige U, Sarosi I, Bolon B, Tafuri A, Morony S, Capparelli C, Li J, Elliott R, McCabe S, Wong T, Campagnuolo G, Moran E, Bogoch ER, Van G, Nguyen LT, Ohashi PS, Lacey DL, Fish E, Boyle WJ, Penninger JM. 1999. Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. Nature 402:304–309.

Kourbeti IS, Boumpas DT. 2005. Biological therapies of autoimmune diseases. Current Drug Targets Inflammation and Allergy 4:41–46.

Kuiper S, Joosten LA, Bendele AM, Edwards CK, III, Arntz OJ, Helsen MM, van de Loo FA, van den Berg WB. 1998. Different roles of tumour necrosis factor alpha and interleukin 1 in murine streptococcal cell wall arthritis. Cytokine 10:690–702.

Kulmatycki KM, Jamali F. 2005. Drug disease interactions: role of inflammatory mediators in disease and variability in drug response. Journal of Pharmacy & Pharmaceutical Sciences 8:602–625.

Lam J, Takeshita S, Barker JE, Kanagawa O, Ross FP, Teitelbaum SL. 2000. TNF-α induces osteoclastogenesis by direct stimulation of macrophages exposed to permissive levels of RANK ligand. Journal of Clinical Investigation 106:1481–1488.

Li P, Schwarz EM, O'Keefe RJ, Ma L, Boyce BF, Xing L. 2004. RANK signaling is not required for TNFalpha-mediated increase in CD11(hi) osteoclast precursors but is essential for mature osteoclast formation in TNFalpha-mediated inflammatory arthritis. Journal of Bone and Mineral Research 19: 207–213.

Lubberts E, Koenders MI, van den Berg WB. 2005. The role of T-cell interleukin-17 in conducting destructive arthritis: lessons from animal models. Arthritis Research and Therapy 7:29–37.

Maini RN, Taylor PC, Paleolog E, Charles P, Ballara S, Brennan FM, Feldmann M. 1999. Anti-tumour necrosis factor specific antibody (infliximab) treatment provides insights into the pathophysiology of rheumatoid arthritis. Annals of the Rheumatic Diseases 58(Suppl. 1):I56–I60.

McInnes IB, Gracie JA. 2004. Targeting cytokines beyond tumor necrosis factor-alpha and interleukin-1 in rheumatoid arthritis. Current Rheumatology Reports 6:336–342.

McInnes IB, Schett G. 2007. Cytokines in the pathogenesis of rheumatoid arthritis. Nature Reviews Immunology 7:429–442.

Molenaar ET, Voskuyl AE, Familian A, van Mierlo GJ, Dijkmans BA, Hack CE. 2001. Complement activation in patients with rheumatoid arthritis mediated in part by C-reactive protein. Arthritis and Rheumatism 44:997–1002.

Ochi S, Shinohara M, Sato K, Gober HJ, Koga T, Kodama T, Takai T, Miyasaka N, Takayanagi H. 2007. Pathological role of osteoclast costimulation in arthritis-induced bone loss. Proceedings of the National Academy of Sciences of the USA 104:11394–11399.

Oliver AM, Martin F, Gartland GL, Carter RH, Kearney JF. 1997. Marginal zone B cells exhibit unique activation, proliferative and immunoglobulin secretory responses. European Journal of Immunology 27:2366–2374.

Petersen HH, Nielsen JP, Heegaard PM. 2004. Application of acute phase protein measurements in veterinary clinical chemistry. Veterinary Research 35:163–187.

Pettit AR, Ji H, von SD, Muller R, Goldring SR, Choi Y, Benoist C, Gravallese EM. 2001. TRANCE/RANKL knockout mice are protected from bone erosion in a serum transfer model of arthritis. American Journal of Pathology 159:1689–1699.

Punzi L, Calo L, Plebani M. 2002. Clinical significance of cytokine determination in synovial fluid. Critical Reviews in Clinical Laboratory Sciences 39:63–88.

Redlich K, Hayer S, Maier A, Dunstan CR, Tohidast-Akrad M, Lang S, Turk B, Pietschmann P, Woloszczuk W, Haralambous S, Kollias G, Steiner G, Smolen JS, Schett G. 2002. Tumor necrosis factor alpha-mediated joint destruction is inhibited by targeting osteoclasts with osteoprotegerin. Arthritis and Rheumatism 46:785–792.

Richard-Miceli C, Dougados M. 2001. Tumour necrosis factor-alpha blockers in rheumatoid arthritis: review of the clinical experience. BioDrugs 15:251–259.

Rioja I, Bush KA, Buckton JB, Dickson MC, Life PF. 2004. Joint cytokine quantification in two rodent arthritis models: kinetics of expression, correlation of mRNA and protein levels and response to prednisolone treatment. Clinical and Experimental Immunology 137:65–73.

Roberts S, Butler RC. 2005. Inflammatory mediators as potential therapeutic targets in the spine. Current Drug Targets Inflammation and Allergy 4:257–266.

Rollins BJ. 1996. Monocyte chemoattractant protein 1: a potential regulator of monocyte recruitment in inflammatory disease. Molecular Medicine Today 2:198–204.

Romas E, Sims NA, Hards DK, Lindsay M, Quinn JW, Ryan PF, Dunstan CR, Martin TJ, Gillespie MT. 2002. Osteoprotegerin reduces osteoclast numbers and prevents bone erosion in collagen-induced arthritis. American Journal of Pathology 161:1419–1427.

Rosengren S, Firestein GS, Boyle DL. 2003. Measurement of inflammatory biomarkers in synovial tissue extracts by enzyme-linked immunosorbent assay. Clinical and Diagnostic Laboratory Immunology 10:1002–1010.

Schett G, Middleton S, Bolon B, Stolina M, Brown H, Zhu L, Pretorius J, Zack DJ, Kostenuik P, Feige U. 2005. Additive bone-protective effects of anabolic treatment when used in conjunction with RANKL and tumor necrosis factor inhibition in two rat arthritis models. Arthritis and Rheumatism 52:1604–1611.

Schett G, Redlich K, Hayer S, Zwerina J, Bolon B, Dunstan C, Gortz B, Schulz A, Bergmeister H, Kollias G, Steiner G, Smolen JS. 2003. Osteoprotegerin protects against generalized bone loss in tumor necrosis factor-transgenic mice. Arthritis and Rheumatism 48:2042–2051.

Schulze-Koops H, Kalden JR. 2001. The balance of Th1/Th2 cytokines in rheumatoid arthritis. Best Practice & Research Clinical Rheumatology 15:677–691.

Steiner G, Tohidast-Akrad M, Witzmann G, Vesely M, Studnicka-Benke A, Gal A, Kunaver M, Zenz P, Smolen JS. 1999. Cytokine production by synovial T cells in rheumatoid arthritis. Rheumatology (Oxford) 38:202–213.

Stolina M, Adamu S, Ominsky M, Dwyer D, Asuncion F, Geng Z, Middleton S, Brown H, Pretorius J, Schett G, Bolon B, Feige U, Zack D, Kostenuik PJ. 2005. RANKL is a marker and mediator of local and systemic bone loss in two rat models of inflammatory arthritis. Journal of Bone and Mineral Research 20:1756–1765.

Stolina M, Bolon B, Middleton S, Dwyer D, Brown H, Duryea D, Zhu L, Rohner A, Pretorius J, Kostenuik P, Feige U, Zack D. 2008. The evolving systemic and local biomarker milieu at different stages of disease progression in rat adjuvant-induced arthritis. Journal of Clinical Immunology doi: 10.1007/s10875-008-9238-8.

Taylor PC. 2003. Anti-cytokines and cytokines in the treatment of rheumatoid arthritis. Current Pharmaceutical Design 9:1095–1106.

van den Berg W. 2001. Cytokines in bone and cartilage destruction and repair. Journal of Clinical Rheumatology 7:S8–S11.

Wei S, Kitaura H, Zhou P, Ross FP, Teitelbaum SL. 2005. IL-1 mediates TNF-induced osteoclastogenesis. Journal of Clinical Investigation 115:282–290.

Wooley PH, Dutcher J, Widmer MB, Gillis S. 1993a. Influence of a recombinant human soluble tumor necrosis factor receptor FC fusion protein on type II collagen-induced arthritis in mice. Journal of Immunology 151:6602–6607.

Wooley PH, Whalen JD, Chapman DL, Berger AE, Richard KA, Aspar DG, Staite ND. 1993b. The effect of an interleukin-1 receptor antagonist protein on type II collagen-induced arthritis and antigen-induced arthritis in mice. Arthritis and Rheumatism 36:1305–1314.