# EFFECTS OF AN ELR$^+$CXC CHEMOKINE ANTAGONIST IN A MODEL OF EXPERIMENTAL ARTHRITIS

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By

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**ABSTRACT**

Rheumatoid arthritis is an autoimmune disease that can cause chronic inflammation of the joints and other areas of the body. Neutrophils contribute to the pathogenesis of arthritis, and are recruited to the site of inflammation by chemokines. CXCL8/IL-8 is a member of a sub-family of chemokines ($ELR^+CXC$ chemokines) that activate and attract neutrophils through the CXCR1 and CXCR2 receptors. Our lab developed a high affinity human CXCR1/CXCR2 antagonist, called human $CXCL8_{(3-72)}$K11R/G31P (hG31P). This antagonist has been shown to be highly effective in blocking $ELR^+CXC$ chemokine-driven neutrophilic inflammation. In this study we looked at the therapeutic effect of blocking $ELR^+CXC$ chemokine receptors (CXCR1 and CXCR2) in an experimental model of arthritis. We induced type II collagen (CII)-induced arthritis (CIA) in mice and treated them with hG31P after the onset of disease. The parameters we looked at to assess disease severity were clinical scores (paws were graded on the severity of edema), clinical measurements (measuring inflammation by change in circumference of paw), serum levels of anti-CII antibodies, and inflammatory cytokines mRNA (IL-1β, TNF, KC, and MIP-2) and protein levels (IL-1β, IL-6, KC, and MIP-2) in paw tissue. Initially, when we analyzed all mice together, we were unable to see a change in clinical scores and measurement when CIA mice were treated with hG31P. All CIA mice did not develop arthritis simultaneously, but rather in a serendipitous fashion; therefore we subdivided our mice and analyzed data from mice that developed arthritis early versus those that developed it late. Treatment with hG31P in mice that developed arthritis early (within 5 weeks of initial CII injection) significantly reduced clinical scores ($p=0.02$) in one, but not both, of our experiments. When CIA mice were treated with hG31P we saw a significant reduction ($p<0.05$) in CII-specific IgG1 and MIP-2 protein levels in one of our experiments. Our results were variable and we did not see these changes in our other experiment. Treatment of CIA mice with G31P did not significantly affect inflammatory cytokine mRNA levels in the paws. During this study we found the production of anti-hG31P antibodies in our hG31P-treated mice. We used a $Ca^{2+}$ influx assay to determine if these hG31P antibodies were neutralizing. When

these antibodies were non-neutralizing we were able to see a significant reduction in the clinical scores ($p=0.02$) of our hG31P-treated CIA mice (that had developed early-onset arthritis) when compared to our saline-treated CIA mice. In the experiment in which we detected significant levels of neutralizing anti-hG31P antibodies, treatment with hG31P did not affect the clinical scores of our CIA mice. Although we cannot definitively say that hG31P has a therapeutic effect in CIA, we believe this line of research merits further investigation. Our research suggests to us that after some experimental refinement and reduction of the immune response mounted to hG31P, there could still be potential for hG31P to have a therapeutic effect in arthritis.

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**TABLE OF CONTENTS**

**LIST OF FIGURES**

## LIST OF ABBREVIATION

| | |
|---|---|
| AA | arachidonic acid |
| Ab | antibody |
| ABTS | 2-2'-azino di-[3-ethylbenzthiazoline sulphonic acid] |
| ADA | anti-drug antibodies |
| ANOVA | one-way analysis-of-variance |
| APC | antigen-presenting cell |
| BAFF | B-cell activating factor |
| BlyS | B lymphocyte stimulator |
| BPI | bacterial permeability-increasing protein |
| CII | type II collagen |
| CC | chemokine (C-C motif) |
| CD40 | TNF receptor superfamily member |
| C/EBP | CCAAT/enhanced-binding protein |
| CFA | Freund's complete adjuvant |
| CIA | type II collagen-induced arthritis |
| COX | cyclooxygenase |
| Csk | carboxy-terminal Srk kinase |
| CTAP-III | connective tissue activating protein-III |
| CTLA4 | cytotoxic T-lymphocyte antigen 4 |
| CXC | chemokine (C-X-C motif) |
| $CX_3C$ | chemokine (C-$X_3$-C) motif |
| DAG | diacylglycerol |
| DC | dendritic cell |
| DMARD | disease-modifying anti-rheumatic drug |
| ECM | extracellular matrix |
| ELISA | enzyme-linked immunosorbent assay |
| ELR | Glu-Leu-Arg |
| ENA-78 | epithelial-neutrophil activating protein-78 |
| ESAM | endothelial cell-selective adhesion molecule |

| | |
|---|---|
| FGF | fibroblast growth factor |
| fMLP | formyl-methionyl-leucyl-phenylalanine |
| G31P | $CXCL8_{(3-72)}$K11R/G31P (an $ELR^+$CXC chemokine antagonist) |
| G6PI | glucose-6-phosphate isomerase |
| GCP-2 | granulocyte chemotactic peptide-2 |
| G-CSF | granulocyte colony-stimulating factor |
| GM-CSF | granulocyte-macrophage colony-stimulating factor |
| GPCR | G protein-coupled receptors |
| GRO | growth-related oncogene |
| HCQ | hydroxychloroquine |
| hG31P | human $CXCL8_{(3-72)}$K11R/G31P (an $ELR^+$CXC chemokine antagonist) |
| HLA | human leukocyte antigen |
| HRP | horseradish peroxidase |
| iNOS | inducible nitric oxide synthase |
| ICAM-1 | intercellular adhesion molecule 1 |
| IFA | Freund's incomplete adjuvant |
| IFN | interferon |
| Ig | immunoglobulin |
| IL | interleukin |
| $IP_3$ | inositol 1,4,5-triphosphate |
| JAM | junctional adhesion molecules |
| KC | keratinocyte-derived cytokine |
| KIF | kinesin superfamily |
| LAF4 | lymphoid nuclear protein related to AF4 |
| LFA-1 | lymphocyte function-associated antigen-1 |
| LIF | leukemia inhibitory factor |
| LIX | lipopolysaccharide-induced CXC chemokine |
| LO | lipoxygenase |
| LPS | lipopolysaccharide |
| LT | leukotriene |
| MAC | membrane attack complex |

| | |
|---|---|
| MAC-1 | macrophage antigen-1 |
| MCP | monocyte chemoattractant protein |
| M-CSF | monocyte colony-stimulating factor |
| MDNCF | monocyte-derived neutrophil chemotactic factor |
| MHC | major histocompatability complex |
| MIF | macrophage migration inhibitory factor |
| MIP | macrophage inflammatory protein |
| MMEL1 | metallo-endopeptidase-like 1 |
| MMP | matrix metalloproteinase |
| MONAP | monocyte-derived neutrophil-activating factor |
| MPO | myeloperoxidase |
| MTX | methotrexate |
| NADPH | nicotinamide adenine dinucleotide phosphate |
| NAF | neutrophil-activating factor |
| NAP | neutrophil-activating peptide |
| NF-κB | nuclear factor-κB |
| NO | nitric oxide |
| NOS | nitric oxide synthase |
| NSAID | non-steroidal anti-inflammatory drug |
| OVA | ovalbumin |
| $PADI_4$ | peptidylardinine deaminase citrullinating enzyme 4 |
| PAF | platelet-activation factor |
| PAMP | pathogen-associated molecular pattern |
| PBS | phosphate-buffered saline |
| PDGF | platelet-derived growth factor |
| PECAM-1 | platelet endothelial cell adhesion molecule 1 |
| PG | prostaglandin |
| PKC | protein kinase C |
| $PLA_2$ | phospholipase $A_2$ |
| PLC | phospholipase C |
| PLD | phospholipase D |

| | |
|---|---|
| PMN | polymorphonuclear leukocyte (e.g., neutrophil) |
| PTPN22 | protein tyrosine phosphatase nonreceptor 22 |
| qRT-PCR | quantitative real time PCR |
| RA | rheumatoid arthritis |
| RANKL | receptor activator of NF-κB ligand |
| RANTES | regulated upon activation, normal T-cell expressed and secreted |
| RF | rheumatoid factor |
| ROI | reactive oxygen intermediates |
| ROS | reactive oxygen species |
| SDF-1 | stromal cell-derived factor-1 |
| SEM | standard error of the mean |
| SOD | superoxide dismutase |
| SSZ | sulfasalazine |
| STAT4 | signal transducer and activator of transcription 4 |
| TGF | transforming growth factor |
| TLR | toll-like receptors |
| TNF | tumor necrosis factor |
| u-PA | urokinase-type plasminogen activator |
| VCAM-1 | vascular cell adhesion molecule-1 |
| VEGF | vascular endothelial growth factor |
| VLA-4 | very late antigen-4 |

# 1. LITERATURE REVIEW

## 1.1 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disorder that involves damage to the synovial membrane, cartilage and bone resulting in joint destruction.(St. Claire 2004; McInnes 2007) The cause and full pathogenesis of RA have yet to be elucidated, but many factors that contribute to the disease progression have been identified. The following is a look at some of the inflammatory mediators and processes in RA.

### 1.1.1 Etiology

Rheumatoid arthritis is a chronic synovial inflammatory disease of unknown etiology that affects approximately 1% of the population (Sweeney 2004). The prevalence of RA is higher in women than in men (2:1 to 3:1 ratio in most studies) and the peak age of onset of RA occurs in individuals aged 40-60 (Alamanos 2005; Khurana 2005). Numerous risk factors have been identified as contributors to the progression of RA. These risk factors include combinations of both genetic and environmental factors.

#### 1.1.1.1 Genetics

Genetic factors contribute significantly to RA susceptibility (Seldin 1999). Using twin studies it has been estimated that the hereditability of RA is approximately 60% (MacGregor 2000). Several loci contribute to the genetic risk of RA, the most essential of these being a polymorphism in the human leukocyte antigen (HLA) DBR1 locus, which contributes about one third of the genetic susceptibility to RA (Deighton 1989; Buch 2002). The HLA-DBR1 gene encodes major histocompatability complex (MHC) class-II molecules which are involved in antigen processing and presentation. The HLA-DBR1 alleles associated with RA share a highly conserved amino acid sequence at position 70-74 in the third hyper variable region of their DRB1 chain. This group of

alleles is referred to as the shared epitope and is found within the peptide-binding groove of MHC class-II molecule (Gregersen 1987; Pratt 2009). The possible functional consequence of these genetic variants in the DBR1 locus, although not yet confirmed, could be a perturbation in the process of distinguishing self and foreign antigens, which could lead to autoimmune antibody (Ab) and T-cell responses (Gregersen 1987; Imboden 2009).

There are other genes associated with RA that have been identified outside of the MHC. The gene that has the second strongest association with RA is protein tyrosine phosphatase nonreceptor 22 (PTPN22) which codes for the intracellular tyrosine phosphatase Lyp. This protein inhibits T-cell receptor signaling through interaction with COOH-terminal Srk kinase (Csk) (Begovich 2004; Orozco 2010). The Arg620→Trp polymorphism causes a gain in PTPN22 activity which has been proposed to suppress T-cell receptor signaling during thymic selection, resulting in either a lack of negative selection of autoreactive T-cells or perhaps diminished activity of Tregs (Gregersen 2005; Vang 2005).

Another risk allele for RA that has been identified is STAT4 (signal transducer and activator of transcription 4) (Remmers 2007). STAT4 is a key transcription factor in the IL-12-mediated differentiation of Th-1 helper cells and the IL-23-mediated differentiation of Th-17 cells, both of which are involved in RA pathogenesis (Kaplan 2005; Orozco 2010).

Genome-wide association studies have led to the identification of additional loci that are associated with RA susceptibility, including tumor necrosis factor-receptor associated factor 1 and complement component 5 (TRAF1-C5) (Plenge 2007) and 6q23 (Plenge 2007; Thomson 2007). Other gene loci have been identified to be associated with RA risk, but with differing degrees of substantiation: peptidylarginine deaminase citrullinating enzyme 4 ($PADI_4$), chemokine (C-C motif) ligand 21 (CCL21), interleukin 2 receptor, beta (IL2RB), kinesin superfamily 5a (KIF5a), TNF receptor superfamily member 5 (CD40), metallo-endopeptidase-like 1 (MMEL1), lymphoid nuclear protein related to AF4 (LAF4), interleukin 2 and 21 (IL2-IL21) and cytotoxic T-lymphocyte antigen 4 (CTLA4) (Coenen 2009; Orozco 2010).

#### 1.1.1.2 Environmental Risk Factors

Smoking is a well-established environmental risk factor for RA (Symmons 1997; Harrison 2002). Other potential risk factors include silica dust exposure (Klockars 1987; Stolt 2005) and mineral oils (Sverdrup 2005). Different infectious agents have also been implicated in the cause of RA, including the Epstein-Barr virus, parvovirus, lentivirus, rubella, as well as the bacteria *Mycoplasma*, *Chlamydia*, and *Mycobacteria* species. However, as of yet there is no conclusive evidence for bacterial or viral involvement in RA (Buch 2002; Jenkins 2002).

### 1.1.2 Pathogenesis

The triggers for the disease have yet to be fully determined, but much has been learned of the contributing cells and their roles in arthritis. An inflamed synovium, the major characteristic of RA, is attributable to an influx of inflammatory cells and an increase in soluble inflammatory mediators (McInnes 2007). Normal synovial fluid is acellular, but RA synovial fluid is filled predominantly with neutrophils, as well as macrophages, T cells and B cells (Feldmann 1996; Ma 2005). The synovial membrane increases in cellularity from 1-2 cells to 6-8 cells in thickness and is made up of mostly activated macrophages, as well as fibroblast-like cells (synovial fibroblasts), T cells and B cells (Feldmann 1996; Ma 2005). The destruction of cartilage and bone, another characteristic of RA, is mediated mostly by neutrophils, chondrocytes, osteoclasts, and synovial fibroblasts (McInnes 2007).

#### 1.1.2.1 Cellular Mediators of Inflammation and Joint Damage

##### 1.1.2.1.1 Monocytes / Macrophages

Monocytes/macrophages are key participants in many inflammatory responses and in RA they are the primary cells in the synovium (Jenkins 2002). Monocytes migrate from the peripheral circulation and accumulate in large numbers in the thickening synovium and at the cartilage-pannus junction (Kinne 2000). Monocyte migration can be

induced by interleukin-17 (IL-17)(Shahrara 2009), interleukin-18 (IL-18)(Ruth 2010), Fractalkine ($CX_3CL1$)(Gevrey 2005), regulated upon activation, normal T-cell expressed and secreted (RANTES)(Volin 1998), macrophage inflammatory protein 1α (MIP-1α)(Szekanecz 2007), and monocyte chemotactic protein-1 (MCP-1)(Koch 1992). In the RA synovial membrane, recently immigrated monocytes differentiate to mature synovial tissue macrophages (Hogg 1985).

Macrophages can be activated by several factors expressed in arthritic joints. Immune complexes can activate macrophages by binding to their Fc receptor (St. Claire 2004). Numerous cytokines are also known to have stimulatory activity on macrophages. Macrophages can be activated by interferon-γ (IFN-γ), produced by T-cells, which stimulates macrophages to produce IL-1β, nitric oxide (NO), and increase expression of MHC class II antigens and adhesion molecules (Ma 2005). IL-17, produced by T-cells, strongly stimulates macrophages to produce interleukin-1β (IL-1β) and tumor necrosis factor alpha (TNFα) (Jovanovic 1998). Macrophages can also be activated by bacterial/viral components, which is relevant because of the possible infectious agent etiology of RA. For example, lipopolysaccharide (LPS) can bind and activate macrophages through the CD14 receptor, and Staphylococcal enterotoxin B is also a strong macrophage-activating factor (Kinne 2000). It has also been found that physiological concentrations of the hormone oestrogen can stimulate macrophages to produce IL-1 in RA, whereas high levels of oestrogen can inhibit IL-1 production, which correlates both with higher arthritis incidence in non-pregnant females as well as a reductions in arthritis symptoms during pregnancy (Cutolo 1997).

Macrophages can also be stimulated by contact with other inflammatory cells. T-cells reside adjacent to macrophages in the synovial membrane. Interleukin-15 (IL-15), produced by macrophages and other cells in the synovial lining, sustains T-cell/macrophage interactions to induce release of IL-1β, TNFα, interleukin-8 (IL-8) and MCP-1 by macrophages (Kinne 2000). Macrophages, which express MHC class II molecules, are also antigen-presenting cells that can activate T cells (Jenkins 2002). Macrophages and synovial fibroblasts also come into contact with one another in the synovial membrane. This interaction may occur through the interaction of CD97 on the macrophages and CD55 on the fibroblasts (Hamann 1999), which promotes the

production of interleukin-6 (IL-6), granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-8 by synoviocytes (Chomarat 1995). Monocytes and endothelial cells interact in RA, which is an essential to the continued influx of monocytes into the synovium. This monocyte-endothelial interaction relies on altered selectin/integrin expression on the cells (Szekanecz 2007).

Monocyte/macrophage function can be suppressed by cytokines produced by macrophages (e.g., IL-10), T cells (e.g., IL-4, IL-13) and stromal cells (e.g., IL-11) (Kinne 2007). Macrophages have many effector functions in RA. They are able to act as phagocytes to remove pathogens and debris from the joint, but they also secrete many proinflammatory mediators that promote joint damage in inflammation (Gierut 2010). Activated macrophages in RA synovial tissue produce high levels of cytokines and chemokines such as IL-1β, TNFα, IL-6, IL-8, GM-CSF, MIP-1α, MCP-1 (Deng 2006). Macrophages directly produce proteinases that are involved in tissue degradation, including matrix metalloproteinase (MMP)-1 (collagenase 1), MMP-2 (gelatinase A), and MMP-9 (gelatinase B)(Szekanecz 2007).

Macrophages also produce angiogenic factors such as vascular endothelial growth factor (VEGF) (Fava 1994), IL-8 (Koch 2001), growth-related oncogene alpha (GRO-α)(Szekanecz 2010), epithelial-neutrophil activating protein-78 (ENA-78) (Koch 1994; Koch 2001), and fractalkine (Ruth 2001; Volin 2001). These angiogenic mediators contribute to the production of new blood vessels in the synovium promoting entry of leucocytes that perpetuate inflammation (Maruotti 2006).

#### 1.1.2.1.2 T Cells

Another prominent cell in RA is the T cell, predominantly $CD4^+$ helper T cells with a bias towards a Th1 phenotype (Firestein 2003). Migration of T cells from the peripheral circulation into the synovium is regulated by adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells and very late antigen-4 (VLA-4) on T cells (van Dinther-Janssen 1991). Migration is also regulated by chemokines such as CCL5, CCL2, and CXCL12 (Shadidi 2003).

Activation of T cells in RA is initiated by T cell receptor (TCR) interaction with MHC-II-bound peptide on antigen-presenting cells (APCs) (Lee 2001). There are also several costimulatory molecules that are involved in T cell/APC interactions, the most prominent being the ligation of CD28 to CD80/CD86 (Lundy 2007). Dendritic cells (DCs) are strong APCs and can initiate Th1 immune responses through the production of IL-12 (Drakesmith 2000; Aarvak 2001). Monocytes/macrophages and B cells are also proficient APCs for activation of T cells in the synovium (Thomas 1998).

It is believed that autoantigen presentation by APCs and activation of autoreactive T cells is involved in the pathology of RA (Aarvak 2001). Although the initiating autoantigens have yet to be identified, there have been several potential candidates studied. Some of these include: type II collagen (CII) (Snowden 1997), cartilage proteoglycan (Goodacre 1992), cartilage glycoprotein-39 (Verheijden 1997), and glucose-6-phosphate isomerase (G6PI) (Kamradt 2005).

The effector functions of T cells in RA are realized in the synovial lining and intra-articular space of the joints (Lundy 2007). Activated T cells upregulate surface expression of CD40 ligand, which promotes B cell proliferation and Ab production (MacDonald 1997). Activated T cells produce a variety of proinflammatory cytokines, such as IFN-γ, IL-1, IL-2, IL-6, IL-17, TNF-α, and GM-CSF (Scrivo 2007). IL-2 acts as a survival factor for T cells, while IFN-γ activates monocytes/macrophages (Christodoulou 2006). IL-17 has effects on synovial fibroblasts, chondrocytes, and osteoclasts, including production of joint-damaging mediators, such as MMPs, prostaglandin $E_2$ ($PGE_2$), and NO (Miossec 2003). T cells also contribute to joint damage through the production of RANKL (receptor activator of nuclear factor κB (NF-κB) ligand), which can activate osteoclasts leading to bone erosion (Gravallese 2000; Firestein 2002).

Recent evidence suggests that a newly discovered subset of $CD4^+$ T helper cells, Th17 cells, are also involved the in the pathogenesis of RA. Th17 cells produce the inflammatory cytokine IL- 17 which can directly and indirectly increase inflammatory mediator production and joint destruction (Lundy 2007).

#### 1.1.2.1.3 B Cells

The inflammatory infiltrate in the rheumatoid synovium also consists of B cells and plasma cells. Accumulation and survival of B cells in the synovium is driven by B-cell activating factor (BAFF), CXCL13, and CXCL12, which can be produced by a variety of cells including synovial fibroblasts and dendritic cells (Mauri 2007). B cells can be activated through both T cell-dependent and -independent mechanisms. They can be activated by direct contact with T cells through CD40-CD40L ligation (Bugatti 2007), but also by Toll-like receptors (TLRs), which are one type of pathogen-associated molecular pattern (PAMP) receptor. TLRs are expressed by nearly every cell in the body; TLR-7 and TLR-9 are the main ones involved with B cell activation (Leadbetter 2002; Lau 2005).

B cells can play a number of roles in the pathogenesis of arthritis. Activated B cells can differentiate into plasma cells which produce the auto-Abs in arthritic joints (Mauri 2007). Rheumatoid factor (RF) is an auto-Ab that is specific for the constant region (Fc) of immunoglobulin G (IgG) (Kim 2000). RFs are found in more than 80% of RA patients and are involved in the formation of immune complexes (IC) and can fix and activate complement *in vitro* (classical pathway) (Vaughan 1975; Kim 2000). Downstream products of the complement cascade, such as small anaphylatoxins complement (C) 3a (C3a) and C5a, and membrane attack complex (MAC), contribute to inflammation in the joint. C5a can attract and activate neutrophils, macrophages and mast cells (Okroj 2007; Lee 2008). The C5b-C9 MAC can induce cell lysis, stimulate synovial fibroblasts, and can trigger the release of IL-1, oxygen radicals, eicosanoids, and collagen type IV (Jahn 1993). Another common auto-Ab detected in RA is anti-citrullinated proteins (e.g., anti-citrulline cyclic peptide, CCP) antibody (Goldbach-Mansky 2000; Goronzy 2005). Other auto-Abs produced in RA, although with a lower specificity, include Abs specific for CII and G6PI (Okroj 2007).

B cells can also function as antigen-presenting cells through their B cell receptor, thereby supporting the activation of autoreactive T cells (Silverman 2003). B cell depletion studies have shown that T cell activation in RA is B cell-dependent (Takemura 2001). B cells can also contribute to the pathogenesis of RA through the production of

inflammatory cytokines IL-6 and TNF-α. Both of these cytokines can activate macrophages and IL-6 is important to B cell growth (Dorner 2006).

#### 1.1.2.1.4 Fibroblasts

Rheumatoid arthritis synovial fibroblasts contribute to joint pathology through perpetuation of inflammation and joint destruction. RA synovial fibroblasts are also one of the cells responsible for synovial hyperplasia in RA (Mor 2005). RA synovial fibroblasts are both morphologically and functionally different from the fibroblasts found in non-arthritic joints. The precise events leading to RA synovial fibroblast growth and activation are still not fully understood, although it is believed that contributing factors include production of growth factors (e.g., fibroblast growth factor [FGF] and transforming growth factor [TGF]-β) , an up-regulation of transcriptional factors (e.g., NF-κβ) and proto-oncogenes (e.g., egr-1), a down-regulation of tumor suppressor genes (e.g., p53), and a resistance to apoptosis (Muller-Ladner 2007). TNF-α and IL-1 have also been found to be involved in the activation of RA synovial fibroblasts (Pap 2000).

The effector functions of RA synovial fibroblasts include the production of inflammatory mediators such as IL-1, -4, -6, -10, -12, -13, -15, -17, -18, and -21, TNF-α, TGF-β, IFN-γ, iNOS, cyclooxygenase-2 (COX-2) and prostaglandin $E_2$ ($PGE_2$)(Mor 2005). RA synovial fibroblasts contribute to the accumulation and survival of inflammatory cells, and most significantly interact with T cells, B cells, and monocytes/macrophages. They produce a variety of chemokines that are responsible for the attraction of these inflammatory cells into the joint, such as IL-8, RANTES, IP-10, ENA-78, MCP-1, MIP-1 and CXCL16 (Muller-Ladner 2007; Bartok 2010). RA synovial fibroblasts contribute to T-cell activation through direct cell-to-cell contact as well as through the production of a potent T-cell growth factor, IL-15 (Fox 2010). RA synovial fibroblasts have also been found to be responsible for a decrease in B-cell apoptosis through the increased production of the anti-apoptotic protein Bcl-$x_L$ (Hayashida 2000). RA synovial fibroblasts produce osteoclast differentiation factor (receptor activator of NF-κB ligand [RANKL]), which promotes differentiation of monocytes into osteoclasts (Shigeyama 2000).

Activated RA synovial fibroblasts have increased expression of adhesion molecules, such as integrins from the β1 subfamily, which allows for the attachment of RA synovial fibroblasts to the cartilage (Rinaldi 1997). Regular fibroblasts are involved in both assembly and removal of extracellular matrix molecules, whereas RA synovial fibroblasts are primarily involved in matrix degradation through the increased expression of MMPs and cysteine proteases (cathepsins) (Muller-Ladner 2007).

MMP expression is regulated by a variety of factors. The inflammatory cytokines IL-1 and TNF-α are among the important inducers of MMPs in RA synovial fibroblasts (Niedermeier 2010). RA synovial fibroblasts produce a number of MMPs, including MMP-1, MMP-3 (stromelysin-1), MMP-10 (stromelysin-2), MMP-13 (collagenase 3), MMP-14 (membrane-type metalloproteinase-1 [MT-I-MMP]), and MMP-16 (MT-3-MMP) (Lindy 1997; Pap 2001; Muller-Ladner 2002; Tolboom 2002). Cathepsins, which are involved in the breakdown of extracellular matrix components, are also induced by the inflammatory cytokines IL-1 and TNF-α (Huet 1993). The main cathepsins produced by RA synovial fibroblasts involved in joint destruction are cathepsins B, L and K (Lemaire 1997; Hou 2001).

#### 1.1.2.1.5 Neutrophils

Neutrophils are the predominant cell infiltrating the synovial fluid, and they can also be found at the cartilage-pannus interface (Mohr 1981; Jenkins 2002). Chemoattractants involved in the accumulation of neutrophils in the synovial fluid include IL-8, GRO-α, TGF-β, complement component C5a, leukotriene $B_4$ ($LTB_4$), platelet-activation factor (PAF), and ENA-78 (Sewell 1993; Koch 1994; Koch 1995; Edwards 1997). An increase in neutrophil adherence and transmigration through the endothelium in RA is due to an IL-1-induced increase in expression of E-selectin and intercellular adhesion molecule-1 (ICAM-1) (Pillinger 1995).

Neutrophils can be activated through binding of immune complexes (e.g., RF) which engage Fcγ receptors on the cells, leading to their degranulation (Wright 2010). Immune complexes can be found in the synovial fluid or bound to the cartilage surface where they can act as opsonins leading to 'frustrated phagocytosis', resulting in the

release of toxic agents that damage surrounding tissue (Jasin 2000). The attachment between the neutrophil and the cartilage surface protects the neutrophils' cartilage-damaging enzymes from proteolytic inhibitors (e.g., $\alpha_1$-proteinase) found in the synovial fluid (Brown 1988).

Activated neutrophils can release a variety of enzymes and reactive oxygen intermediates (ROIs) that are major contributors to joint damage. Proteases released by neutrophils that can damage the cartilage include MMP-3, MMP-8 (collagenase 2), MMP-9, cathepsins G, and lactase (Janusz 1991; Kakimoto 1995; Arner 1997; Grillet 1997; Nerusu 2007). Neutrophils can use oxygen ($O_2^-$) to produce ROIs, such as hydrogen peroxide ($H_2O_2$), hypochlorite ion ($OCl^-$), hydroxyl radical ($^{\cdot}OH$), and super oxide anion ($O_2^{\cdot -}$) (Henrotin 2005). ROS are capable of degrading cartilage directly or indirectly by activating latent neutrophil collagenases and by reducing matrix component synthesis (Henrotin 2003). Oxygen-derived free radicals released from neutrophils can degrade hyaluronic acid, the lubricating component of synovial fluid, which in turn decreases the viscosity of synovial fluid (Greenwald 1986). Hyaluronic acid inhibits neutrophil phagocytosis and chemotaxis and can inhibit proteoglycan release from cartilage; therefore its degradation can contribute to inflammation and cartilage degradation (Tamoto 1993; Villar-Suarez 2005).

Activated neutrophils are also able to release a variety of inflammatory cytokines such as IL-1, TNF-α, IL-6, TGF-β and MIP-1α (Hatano 1999; Wipke 2001). There are multiple products of the arachidonic acid (AA) cascade that are released by activated neutrophils (e.g., prostaglandins and leukotrienes) and these contribute to inflammation in the joint (Kitsis 1991). Mediators secreted by neutrophils that contribute to the chemotaxis of other neutrophils include IL-8, $LTB_4$, GRO-α, and TGF-β (Edwards 1997). Neutrophil apoptosis is delayed under the low oxygen conditions (hypoxia) in arthritic joints, which may lead to increased tissue damage and inflammation (Cross 2006).

There have been neutrophil depletion studies done in experimental models of arthritis that show disease amelioration, including studies in K/BxN serum-induced arthritis and anti-type II collagen-induced arthritis (Wipke 2001; Nandakumar 2003).

#### 1.1.2.1.6 Chondrocytes

Chondrocytes, the only resident cell in normal articular cartilage, are responsible for cartilage remodeling. The cartilage matrix, synthesized by chondrocytes, is composed primarily of types II, IX, and XI collagens and the aggregating proteoglycan aggrecans (Goldring 2009).

Chondrocytes activated by IL-1 and TNF-α produce cartilage-destroying MMPs, such as MMP-1 MMP-2, MMP-8, MMP-10, MMP-13 and MMP-14, and NO (Goldring 2003; Ainola 2005; Murphy 2005; Barksby 2006). Other enzymatic factors, including cathepsins B, D, and L, are produced by chondrocytes and contribute to the degradation of the cartilage matrix (Nagase 2002). IL-1 and TNF-α can also induce chondrocytes to produce IL-6, leukemia inhibitory factor (LIF), IL-17, and IL-18, all of which can contribute to cartilage loss (Otero 2007). Activated chondrocytes are also involved in the chemoattraction of other inflammatory cells through their expression of chemokines, including IL-8, MCP-1, MCP-4, MIP-1α, MIP-1β, RANTES, and GROα (Otero 2007).

#### 1.1.2.1.7 Osteoclasts

Bone erosion in arthritis is mediated by osteoclasts. Bone resorption by osteoclasts is mediated by the parallel action of a proton pump and chloride channel that reduce the pH at the border between osteoclast and bone surface dissolving the bone mineral which exposes the organic matrix to the matrix-degrading enzymes secreted by osteoclasts such as MMPs and cathepsins (Schlesinger 1997; Schett 2007). Osteoclasts are hematopoietic cells that differentiate from mesenchymal cells (monocytes/ macrophages). They are found in abundance in synovial inflammatory tissue (Schett 2006). Synovial fibroblasts and T cells express two key molecules, monocyte colony-stimulating factor (M-CSF) and RANKL, which are required for osteoclast differentiation and function (Seitz 1994; Gravallese 2000). T cells are also able to trigger osteoclastogenesis through the production of IL-17, which supports osteoclast formation (Udagawa 2003). IL-17 can stimulate production of RANKL by synovial fibroblasts and can also stimulate macrophages to produce inflammatory cytokines IL-1, TNF-α and IL-6 (Nakashima 2009). IL-1, TNF-α, IL-6 and MCP-1 are all also able to increase osteoclast

differentiation and function, predominantly through the induction of RANKL expression (Walsh 2005; Novack 2008).

#### 1.1.2.1.8 Endothelial Cells

The vascular endothelium in RA is involved in inflammatory cell migration and angiogenesis, both of which are key processes in the pathogenesis of RA. Leukocyte adhesion and migration into the synovium is mediated by a number of adhesion molecules that are involved in the four steps of leukocyte emigration; rolling, activation, firm adhesion, and diapedesis (Luster 2005). Rolling is mediated by selectins, of which E- and P-selectins have been found on endothelial cells in RA (Tak 1995; Tedder 1995). Endothelial cells have also been found to express VCAM-1 and ICAM-1, which are ligands for integrins found on leukocytes and are involved in firm adhesion of leukocytes to endothelial cells in RA (Koch 1991; Albelda 1994). Platelet endothelial cell adhesion molecule 1 (PECAM-1) is also found on RA endothelial cells, and is involved in diapedesis (Middleton 2004). Inflammatory cytokines IL-1, TNF-α, and IFN-γ have been found to upregulate expression of several of these adhesion molecules, thereby stimulating leukocyte-endothelial cell adhesion (Szekanecz 2000).

Endothelial cells also play an important role in the formation of new blood vessels (angiogenesis) in RA. Endothelial cells can be activated by inflammatory cytokines IL-1 and TNF-α (Pober 2007). Endothelial cell migration and proliferation is induced by VEGF, which can be expressed by fibroblasts in response to IL-1 and TNF-α stimulation (Koch 2003). Endothelial cells can secrete proteases, such as MMP-8, MMP-1, and cathepsin B, which degrade basement membrane and extracellular matrix to make room for new blood vessel growth (Hanemaaijer 1997; Middleton 2004). Endothelial cells can then migrate to the site of new blood vessel formation in response to the $ELR^{+}CXC$ chemokines IL-8, GROα, ENA-78, and granulocyte chemotactic peptide-2 (GCP-2) (Speyer 2011). Endothelial cells are also able to express angiogenesis-promoting growth factors such as FGF (Paleolog 2002).

#### 1.1.2.2 Soluble Mediators of Inflammation and Disease

##### 1.1.2.2.1 Cytokines / Chemokines

Cytokines are regulatory proteins that mediate communication between immune cells and contribute to the pathogenesis of RA (Lee 2001). Many cytokines have been found in the synovial fluid and tissue of RA patients, including, but not limited to, TNF-α, IL-1, IL-6, IL-15, IL-18, and IL-17.

Both TNF-α and IL-1 have primary roles in the pathogenesis of RA. TNF-α is primarily produced by monocytes and macrophages, but also by T cell, B cells, and fibroblasts (Christodoulou 2006). TNF-α is able to induce the production of cytokines such as IL-1, IL-6, IL-8, and GM-CSF (Larche 2005). IL-1 is also produced by monocytes and macrophages, but also by endothelial cells, T cells, and B cells (Christodoulou 2006). IL-1 and TNF activate a variety of cells found in RA, including macrophages, fibroblasts, chondrocytes, and osteoclasts (Henrotin 1996; Pap 2000; Walsh 2005; McInnes 2007). IL-1 and TNF-α can each induce the expression of the other and can work synergistically together (Nawroth 1986; Lee 2001).

IL-1 and TNF-α can contribute to the joint damage found in RA in many ways. Both upregulate expression of adhesion molecules (VCAM-1, ICAM-1, and E-selectin) on fibroblasts and endothelial cells, and these can contribute to the increase in inflammatory cells in the joint (Proudman 1999). They can also induce production of a variety of inflammatory mediators that contribute to joint destruction. IL-1 stimulates chondrocytes to produce NO and $PGE_2$ (Abramson 2002). IL-1 and TNF-α can both stimulate fibroblasts and macrophages to produce $PGE_2$ (Badolato 1996). IL-1 and TNF-α can increase bone resorption by upregulating RANKL expression on osteoblasts which leads to increased osteoclast differentiation and stimulation (Roux 2000). They are also both involved in the stimulation of chondrocytes, fibroblasts, and endothelial cells to release degradative enzymes (e.g., MMPs) that contribute to cartilage destruction (Shingu 1993).

IL-6 is produced by synovial fibroblasts, macrophages, chondrocytes, neutrophils, and endothelial cells (Shingu 1993). IL-1 and TNF-α are the primary inducers of IL-6

from fibroblasts (Guerne 1989). IL-6 is known to work synergistically with IL-1 and TNF-α to increase MMP production (Badolato 1996). IL-6 induces the differentiation of B cells into antibody-producing plasma cells and naïve T cells into Th17 cells (Choy 2001; Kimura 2010).

Other inflammatory cytokines include IL-15, which is produced by T cells, macrophages, fibroblasts, and endothelial cells. It is capable of activating monocytes, T cells, and neutrophils to release of TNF-α and IFN-γ (Christodoulou 2006). IL-18 is produced by monocytes and endothelial cells (McInnes 2007). It can activate macrophages and induce IFN-γ production as well as Th1 differentiation of T cells (Sweeney 2004). IL-12 is secreted by macrophages and is also able to induce Th1 differentiation of T cells and IFN-γ production (Agarwal 2005; McInnes 2007). IL-17 is produced by T cells and can work with IL-1 and TNF-α to activate fibroblasts and stimulate bone degradation (Sweeney 2004).

Chemoattractant cytokines, called chemokines, have also been found in the synovium of RA patients and have been found to play an important role in leukocyte recruitment and activation (Lee 2001). Chemokines can be divided into 4 different families on the basis of the positioning of their amino sub-terminal cysteine residues. These include the CXC, CC, $CX_3C$, and C subfamilies (Luster 1998). Chemokines found to be important in regulating pathogenesis in RA include IL-8, GRO-α, ENA78, connective tissue activating protein-III (CTAP-III), stromal cell-derived factor-1 (SDF-1), RANTES, MIP-1α, MIP-3α, and MCP-1 (Feldmann 1996; Szekanecz 2003).

IL-8 (CXCL8), GRO-α (CXCL1), ENA-78 (CXCL5), and NAP-2 (CXCL7) are all $ELR^+CXC$ chemokines that induce neutrophil chemotaxis (Loetscher 2005). IL-8 is detected in large quantities in RA and is predominantly expressed by macrophages, fibroblasts, neutrophils and chondrocytes (Edwards 1997; Deng 2006; Otero 2007; Bartok 2010). ENA-78 is expressed by macrophages, fibroblasts and endothelial cells (Koch 1994). GRO-α is produced by neutrophils, mononuclear cells, fibroblasts, and chondrocytes (Hosaka 1994; Koch 1995). IL-8, GRO-α and ENA-78 can be induced by stimulation with IL-1 and TNF-α (Badolato 1996; Szekanecz 2003; Koch 2005). CTAP-III is a human platelet-derived growth factor that can stimulate the synthesis of proteoglycan by fibroblasts (Szekanecz 2003). All 4 of these $ELR^+CXC$ chemokines are

angiogenic (Belperio 2000; Szekanecz 2005). IL-8 is able to induce adhesion of neutrophils expressing CXCR1 and CXCR2, which upregulates firm adhesion of rolling cells (Haringman 2004). IL-8 is not only chemotactic for neutrophil, but it can also stimulate neutrophil degranulation resulting in cartilage damage (Elford 1991). SDF-1 (CXCL12) is a CXC chemokine (lacking the ELR motif) that is expressed by synovial fibroblasts and localizes on endothelial cells, where it promotes angiogenesis and inflammatory cell infiltration (Pablos 2003). IL-17 and IL-18 can induce the production of SDF-1 by fibroblasts (Gorman 2008). In RA SDF-1 is also able to increase expression of IL-8 by synovial fibroblasts (Loetscher 2005).

MCP-1 (CCL2), MIP-1α (CCL3), MIP-3α (CCL20), and RANTES (CCL5) are all CC chemokines that are involved primarily in the recruitment of monocytes and lymphocytes (Loetscher 2005). MCP-1, MIP-1α, MIP-3α, and RANTES are expressed by synovial fibroblasts and synovial macrophages (Koch 1992; Hogan 1994; Koch 1994; Szekanecz 2003), and are also upregulated by IL-1 and TNF-α (Hosaka 1994; Badolato 1996; Szekanecz 2003). MCP-1 and RANTES have also been found to increase expression of IL-8 by synovial fibroblasts in RA (Loetscher 2005).

Chemokines regulate their activity through interactions with the 7-transmembrane G protein-coupled receptors (GPCRs) (Borish 2003). There have been 18 chemokine receptors identified that interact with approximately 50 chemokines (Moser 2001). These receptors are named according to the family of chemokine they bind (e.g., CXCR, CXCR, $CX_3CR$, or CR) (Moser 2004). While the receptors are always family-specific, most receptors are able to bind to multiple chemokines, although some are specific for only one chemokine (Zlotnik 2006). In RA CXCR1 and CXCR2 expression on synovial macrophages and neutrophils have been implicated in disease progression (Patterson 2002; Coelho 2008). CXCR1 is predominantly a receptor for IL-8, while CXCR2 is a receptor for IL-8, GRO-α, ENA-78, and CTAP-III (Koch 2005). CXCR4, which is expressed on endothelial cells, is a receptor for SDF-1 (Vergunst 2005). CCR1 and CCR2 are expressed on synovial T lymphocytes and monocytes (Loetscher 2005). CCR1 and CCR5 are receptors for MIP-1α and RANTES, while CCR2 is a receptor for MCP-1 (Koch 2005). In RA CCR5 has been found on synovial fibroblasts and T lymphocytes.

CCR6, a receptor for MIP-3α, has been found on infiltrating white blood cells in the synovial tissue (Koch 2005).

There are also mitogenic cytokines that have been identified in the RA synovium. These include platelet-derived growth factor (PDGF), FGF, VEGF, and GM-CSF (Feldmann 1996). PDGF is produced by platelets and monocytes/macrophages and functions as a growth factor for fibroblasts (Remmers 1991; Rosengren 2010). FGF is expressed by fibroblasts and its main function is to stimulate fibroblasts and angiogenesis (Bucala 1991; Sano 1993). VEGF is expressed by macrophages and fibroblasts and its main function is stimulating endothelial cell proliferation, an important component of angiogenesis (Fava 1994; Koch 2003). GM-CSF expression by fibroblasts and macrophages is stimulated by IL-1 and TNF-α. This growth factor can stimulate and increase effector functions of neutrophils and macrophages (Alvaro-Gracia 1991; Cornish 2009).

**1.1.2.2.2 Enzymatic Factors**

Matrix metalloproteinases are involved in the degradation of cartilage and bone in RA. The majority of MMPs found in the RA synovium include collagenases 1, 2, and 3 (MMP-1, -8, and -13), gelatinases A and B (MMP-2, and -9), stromelysin-1 (MMP-3), and membrane type I MMP (MT1-MMP; MMP-14) (Otero 2007). The collagenases degrade fibrillar forms of collagen (I, II, and III). In RA they mainly degrade type II collagen, the primary component of cartilage, although MMP-1 is more effective against type III collagen, MMP-8 has a higher affinity for type I collagen, and MMP-13 has greatest activity against type II collagen (Duffy 2000; Vincenti 2002). MMP-13 is predominantly expressed by chondrocytes, MMP-8 by neutrophils, and MMP-1 by fibroblasts and chondrocytes (Burrage 2006). MMP-3 can directly degrade proteoglycan, laminin, fibronectin and collagen IV, but it is also involved in the activation of proMMPs, such as MMP-1 (So 1999). MMPs are secreted as inactive proenzymes (proMMPs), which require extracellular activation by other proteases to become active (Murphy 1999). MMP-3 is mainly expressed by rheumatoid synovial fibroblasts and chondrocytes (Tetlow 1993; Cawston 1996). MMP-2 and MMP-9 complete collagen

degradation by digesting gelatins produced by the action of collagenases. They can also degrade articular substances, such as aggrecans and type I collagen (Yamanaka 2000; Itoh 2002). MMP-9 is expressed by macrophages and neutrophils, while MMP-2 is produced by a variety of cells, including chondrocytes (Cawston 1996; Itoh 2002). MMP-14 is involved in the activation of MMP-2 and MMP-13, and also contributes to the degradation of the extracellular matrix (Yamanaka 2000; Pap 2001). Macrophages and fibroblasts have been found to express MMP-14 (Pap 2001). MMP-1, MMP-3, MMP-9 and MMP-13 are upregulated by IL-1β and TNF-α, while MMP-2 and MMP-14 are constitutively expressed in RA (Cunnane 2001; Burrage 2006).

The cysteine cathepsins are proteolytic enzymes that also contribute to cartilage and bone damage in RA. Synovial fibroblasts, macrophages and osteoclasts can be stimulated to express cathepsin K by IL-1β and TNF-α in RA (Hou 2002). Cathepsin K is a key enzyme involved in osteoclastic bone resorption and can contribute to cartilage destruction by degrading native fibrillar collagen (Salminen-Mankonen 2007). Cathepsin B contributes to damage in RA by activating MMP-1 and degrading MMP inhibitors (Hashimoto 2001). High levels of cathepsins B and L have been found in RA synovial fluid and these enzymes can hydrolyze proteoglycans and collagens (types II, IX, and XI) and have been implicated in participating in the destruction of bone (Lemaire 1997). Cathepsins B and L can be produced by macrophages, fibroblasts, chondrocytes and osteoclasts (Cunnane 1999).

#### 1.1.2.2.3 Reactive Oxygen Intermediates

Reactive oxygen species (ROS) are associated with the inflammatory response and contribute to the tissue-damaging effects of inflammatory reactions (Afonso 2007). ROS are reactive molecules derived from the metabolism of oxygen. The ones most commonly involved in inflammation are superoxide ($O_2^-$), hydrogen peroxide ($H_2O_2$), hydroxyl radical ($OH^\cdot$), hypochlorous acid (HOCl), and nitric oxide ($NO^\cdot$).

Activated phagocytic (neutrophils, macrophages) cells undergo oxidative bursts leading to the production of toxic ROS. This oxidative burst is mediated by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which reduces oxygen ($O_2$) to $O_2^-$

(Fialkow 2007). NADPH oxidase can be induced by inflammatory cytokines IL-1β, TNF-α, and IFN-γ (Filippin 2008). Superoxide can then be converted to $H_2O_2$ by superoxide dismutase (SOD), and $H_2O_2$ can then be further reduced to the most active oxygen radical, $OH^{\cdot}$, by its interaction with ferrous iron ($Fe^{2+}$). In the presence of myeloperoxidase (MPO), $H_2O_2$ can oxidize $Cl^-$ to from HOCl, a very powerful oxidant (Fialkow 2007). These ROS can contribute to the pathogenesis of RA by damaging the cartilage and bone. $O_2^-$ can inhibit type II collagen and proteoglycan synthesis and $OH^{\cdot}$ can degrade collagen (Henrotin 2003; Hitchon 2004). $H_2O_2$ is capable of inhibiting cartilage proteoglycan synthesis and can damage chondrocytes (Halliwell 1995). $H_2O_2$ and $O_2^-$ are involved in osteoclast activation associated with bone resorption (Garrett 1990; Hall 1995). HOCl generated by neutrophils can activate latent collagenase which mediates tissue damage in RA (Weiss 1985). ROS can activate the transcription factor NF-κB, which up-regulates cartilage-degrading MMP genes (Henrotin 2005).

The second major oxidant-generating pathway involves $NO^{\cdot}$ synthesis by nitric oxide synthase (NOS), which oxidizes L-arginine to L-citrulline (Folkerts 2001). Inducible NOS (iNOS) can be upregulated in inflammation and is found in chondrocytes, neutrophils, synovial fibroblasts and macrophages (Palmer 1993; Santos 1997; Cedergren 2002). The inflammatory mediators IL-1β, TNF-α, and IL-6 can induce iNOS to release high levels of $NO^{\cdot}$, which has been found to increase MMP activity in articular cartilage and to suppress proteoglycan synthesis (Taskiran 1994; Murrell 1995). Chondrocytes are the major source of $NO^{\cdot}$ in the joints (Rediske 1994). Increased levels of the $NO^{\cdot}$ product nitrate ($NO_3^-$) can be found in the synovial fluid of RA patients (Farrell 1992). The interaction of $NO^{\cdot}$ and superoxide anion can lead to the production of highly reactive peroxynitrite ($ONOO^-$). This radical is involved in the activation of COX to produce prostaglandins, and the increased expression of MMP-3 and MMP-13 (Landino 1996; Afonso 2007). $NO^{\cdot}$ also affects the migration of lymphocytes into the joint by downregulating the expression of P and E selectins, ICAM-1, and VCAM-1 (Veihelmann

2002). Another mechanism by which $NO^{\cdot}$ can be toxic in RA is by $NO^{\cdot}$-mediated chondrocyte apoptosis (Jang 1998).

#### 1.1.2.2.4 Eicosanoids

There are numerous inflammatory mediators that are derived from phospholipids. Arachidonic acid (AA) is released from phospholipid cell membranes by the action of phospholipase $A_2$ ($PLA_2$). AA can be metabolized into the eicosanoids, such as prostaglandins (PGs) or leukotrienes (LTs) by the enzymes cyclooxygenase (COX) and lipoxygenase (LO), respectively (Heller 1998). Eicosanoids are not stored in cells and must be newly synthesized in response to inflammatory stimuli (Marcus 1984).

Production of eicosanoids from AA is dependent on $PLA_2$ (Park 2006). IL-1, IL-6, TNF-α, and macrophage migration inhibitory factor (MIF) induce the expression of $PLA_2$ in various cells (Andreani 2000; Sampey 2001). 5-lipoxygenase (5-LO) is required for the synthesis of leukotriene $B_4$ ($LTB_4$), the LT most relevant in RA. 5-LO, mostly expressed in macrophages and neutrophils in the RA synovium (Gheorghe 2009), can be induced by formyl-methionyl-leucyl-phenylalanine (fMLP), C5a, IL-8, and $LTB_4$ (Werz 2002). Elevated levels of $LTB_4$ are found in arthritic joints (Kim 2006). The major source of $LTB_4$ is neutrophils (Chen 2006). The main function of $LTB_4$ is the stimulation of neutrophil chemotaxis, degranulation, adhesion and survival (Mathis 2007), but LTB4 can also work with prostaglandin $E_2$ ($PGE_2$) to increase vascular permeability (Davidson 1983).

COX-2 is a key enzyme involved in the synthesis of $PGE_2$, which is the PG most relevant in RA. COX-2 is rapidly induced following pro-inflammatory stimulation, such as with IL-1β, TNF-α and MIF (Crofford 1994; Berenbaum 1996; Sampey 2001). COX-2 is expressed by mononuclear cells, endothelial cells, chondrocytes and fibroblasts in RA (Martel-Pelletier 2003). The major sources of $PGE_2$ are endothelial cells and mononuclear phagocytes (Amin 1999). $PGE_2$ is involved in angiogenesis, but also in cartilage and bone degradation in arthritis. $PGE_2$ activates synovial fibroblasts leading to upregulation of VEGF, an angiogenic factor (Hla 1999). $PGE_2$ can also increase MMP production by chondrocytes and fibroblasts (Martel-Pelletier 2003). Bone resorption in

RA is also stimulated by $PGE_2$, which can upregulate RANKL production by osteoblasts (Robinson 1975; Li 2000). $PGE_2$ also delays neutrophil apoptosis (Wright 2010). Both $LTB_4$ and $PGE_2$ exert their effects on cells by binding to GPCRs (Hikiji 2008).

### 1.1.3 Treatment of Disease

There have been many developments in the treatment of RA in recent years. Before 1999 the treatment of RA initially involved non-steroidal anti-inflammatory drugs (NSAIDs) followed by disease-modifying anti-rheumatic drugs (DMARDs)(Lee 2001). NSAIDs, such as aspirin and celecoxib, provide symptomatic relief of RA by decreasing pain and inflammation. These drugs inhibit COX, which are key enzymes in prostaglandin synthesis (Doan 2005). DMARDs include drugs from many classes, such as methotrexate (MTX), leflunomide, sulfasalazine (SSZ), and hydroxychloroquine (HCQ), and can improve inflammatory symptoms or slow disease progression in RA (Klareskog 2009). The mechanism of action of most DMARDs is incompletely understood (Furst 1996). MTX, the most commonly used DMARD, blocks purine synthesis and works well in combination with other DMARDs and biologic therapies (Borchers 2004). Leflunomide inhibits pyrimidine synthesis, blocks TNF-mediated activation of NF-κB, suppresses T cell/monocyte contact activation, and inhibits MMP in RA (Breedveld 2000). SSZ weakly inhibits the AA cascade and has been shown to downregulate neutrophil chemotaxis and degranulation (Feely 2009). HCQ is an antimalarial agent believed to inhibit the antigen processing ability of macrophages, leading to subsequent decreased activation of T cells and downregulation of autoimmune responses (Fox 1993).

New drugs used in RA include biologic therapies, which have shown disease control associated with prevention of joint destruction (Combe 2009). Biologics are usually directed towards a specific cytokine or cell surface molecule. There are several TNF-α inhibitors, including infliximab, etanercept, and adalimumab, which have all shown significant clinical improvements in RA through reduction of synovial inflammation, bone resorption and cartilage degradation (van Vollenhoven 2009). Infliximab is a chimeric monoclonal anti-TNF-α Ab (Perdriger 2009). Etanercept is a

recombinant human TNF receptor p75 Fc fusion protein that inhibits TNF-α from binding to its receptors (Moreland 1999). Adalimumab is a recombinant fully humanized anti-TNF-α IgG1 monoclonal Ab (Voulgari 2006). Anti-TNF agents used in combination with MTX have more significant effect in RA than anti-TNF agents used alone (van Vollenhoven 2009). Anakinra, a recombinant IL-1 receptor antagonist, has been found effective in treatment of RA and has been found to suppress collagenase production, $PGE_2$ secretion and proteoglycan degradation (Furst 2004). Another biologic agent used in treatment of RA is Rituximab, a chimeric monoclonal Ab to CD20 (a cell marker only expressed on mature B cells and pre-B cells), which selectively depletes B cells (Shaw 2003). Abatacept is a fusion protein consisting of CTLA-4 and the Fc portion of IgG1, which binds to CD80/CD86 on APCs and thereby prevents T cell activation (Buch 2008). A final biologic agent worth mentioning in RA is tocilizumab, which is a recombinant monoclonal humanized Ab directed at the IL-6 receptor that leads to significant improvement in RA (Smolen 2006).

Although these therapies have provided benefits to numerous patients, DMARDs and biologic therapies still do not work for all patients and can both have adverse side effects (Doan 2005; Voll 2006). Additional drawbacks of biologic therapies are that they are often quite costly and required parenteral administration (Sweeney 2004). Biologics have also been associated with an increase risk of infection (Listing 2005). More specifically, the immunosuppressive effects of TNF-α antagonists were associated with an increased incidence of tuberculosis (CDC 2004) and the possible increased risk of lymphoma (Brown 2002). Consequently, RA is sub optimally treated with the currently available drugs, such that there is still a need for alternate therapies in the treatment of RA.

A growing concern with biologic therapies is the development of immunogenicity, such as anti-drug Abs (ADAs), that could interfere with the efficacy and safety of these biologics (Sethu 2012). ADAs can be neutralizing or non-neutralizing Abs. Non-neutralizing Abs bind to a biologic without disturbing the interaction with its target, whereas neutralizing Abs bind to sites that are critical for drug-target interaction, thereby blocking the biologics activity, making it therapeutically ineffective (Sathish 2013). Most often neutralizing Abs are responsible for adverse immune responses and

are connected with reduced efficacy, decreased drug survival, and increased occurrence of dose escalation (Alawadhi 2012). Different biologics have different immunogenicities and therefore different efficacies. TNF-α inhibitors infliximab and adalimumab have been linked to reduced clinical efficacy in RA and nearly half of RA patients treated with these biologics develop neutralizing ADAs (Wolbink 2006; Kosmac 2011; van Schouwenburg 2013). Whereas, very few RA patients treated with etanercept produce etanercept-specific ADAs (~6%), and all were found to be non-neutralizing (Dore 2007). The mechanism for generation of Ab to biologics similar to their endogenous counterparts is often due to the breaking of immune tolerance that generally exists to self-antigen (Sethu 2012). Possible ways to reduce immunogenicity found in biologics include humanization, induction of tolerance, and glycosylation (Sethu 2012). So, although biologics have drastically changed the way we treat RA, there are still problems that occur and the development of immunogenicity often decreases the efficacy of these biologic.

## 1.2 Neutrophils

### 1.2.1 General Aspects of Neutrophils

Neutrophils are phagocytes that play an important role in the primary defense against invading microorganisms (e.g. bacteria, fungi, and viruses) (Aratani 1999). They are also a central component in the inflammatory response and are the first cells to arrive at the site of injury (Theilgaard-Monch 2006). Neutrophils can be activated by bacterial products, cytokines (e.g. TNF), chemokines (e.g. IL-8), eicosanoids (e.g. $LTB_4$), and complement factors (e.g. C5a). The such activated neutrophils migrate to sites of injury along chemotactic gradients where they eliminate the invading pathogen through release of ROS products generated via the respiratory burst and cytotoxic proteins and hydrolytic enzymes released from granules (Pettersen 2002; Wright 2010). Inappropriate regulation of neutrophil activation can cause tissue damage, which is associated with inflammatory diseases (Clemens 2004) such as ischemia-reperfusion injury (Jordan 1999), chronic

obstructive pulmonary disease (COPD), cystic fibrosis (CF) (Gernez 2010), Crohn's disease (Biagioni 2006), and rheumatoid arthritis (Edwards 1997).

### 1.2.2 Neutrophil Development

Neutrophils develop from hematopoietic stem cells in the bone marrow. Their development starts from myeloid stem cells and proceeds through the following stages: promyelocyte, myelocyte, metamyelocyte, band cell, and mature neutrophil (Bainton 1971). Neutrophil differentiation can be stimulated by a specific set of cytokines and growth factors, the most common being granulocyte colony-stimulating factor (G-CSF) and GM-CSF (St. Claire 2004). The process of neutrophil differentiation involves the development of granules, which is a trait of granulocytes, a grouping that also includes basophils and eosinophils (Berliner 1998). The neutrophil primary granules develop in the promeyelocyte stage, while the secondary granules appear in the myelocyte stage (Berliner 1998). In humans there are approximately $10^{11}$ neutrophils produced daily (Furze 2008). They are the most abundant granulocyte subtype in the blood, and they have a short half-life in both the circulation and tissue (8-12 hours and 1-2 days, respectively) (Luo 2008).

### 1.2.3 Neutrophil Biology

Neutrophils have distinctive multilobular (3 to 5 lobes) nuclei and are often referred to as polymorphonuclear leukocytes (PMNs) (St. Claire 2004). The neutrophil has four distinct intracellular granules: the primary (azurophilic), secondary (specific), tertiary (gelatinase), and secretory granules (Theilgaard-Monch 2006). These granules contain a variety of antimicrobial enzymes, adhesion molecule receptors, extracellular matrix proteins, anti-bacterial products, and soluble mediators of inflammation (Faurschou 2003).

The primary granules contain acid hydrolases and antimicrobial proteins, and contribute mainly to the killing and degradation of phagocytosed microorganisms (Faurschou 2003). Some of the enzymes and anti-microbial peptides found in primary granules are MPO, defensins, bacterial permeability-increasing protein (BPI), lysozyme,

and serine proteases (e.g. elastase, cathepsins, and proteinase-3) (Borregaard 1993). MPO is an enzyme that reacts with $H_2O_2$ to form HOCl, a potent microbial ROI that can attack surface membranes of microorganisms or can lead to tissue damage (Kumar 2002). Defensins display antimicrobial activities against many bacteria, fungi, and some viruses and some are able to induce chemotaxis of monocytes and T cells (Faurschou 2003). BPI is a potent antibacterial agent that plays a role in killing gram-negative bacteria by both intracellular and opsonic methods (Iovine 1997). Lysozyme is an enzyme that can degrade gram-positive bacterial cell wall peptidoglycans, leading to their death (Nash 2006). The serine proteases have proteolytic activity against extracellular matrix (ECM) components such as elastin, fibronectin, laminin, and type IV collagen, and proteoglycans (Owen 1999). These serine proteases are also able to modify cytokine activity. Proteinase-3 is able to process membrane-bound pro-TNF-α, pro-IL-1β, and IL-18 into their active forms (Wiedow 2005). Also, N-terminal truncation of some chemokines (IL-8 and ENA-78) by proteases (proteinase-3 and cathepsin G, respectively) causes these chemokines to have a higher affinity for their receptors (Pham 2006).

The secondary granules products also participate in the antimicrobial activities of the neutrophil. They contain enzymes and proteins such as lysozyme, lactoferrin, collagenase, and $PLA_2$ (Rubin 2009). Lactoferrin can bind iron, a requisite molecule for bacterial growth, such that it impairs bacterial growth ultimately leading to their death (Levay 1995). It can also enhance the production of OH by neutrophils (Ambruso 1981). Neutrophil collagenase (MMP-8) is involved in the degradation of collagens (I-III) (Owen 1999). $PLA_2$ is able to cleave membrane phospholipids, which leads to the release of AA and subsequent eicosanoid production (Balsinde 1988).

Tertiary granules contain lysozyme, gelatinase, and urokinase-type plasminogen activator (u-PA) (Faurschou 2003). Gelatinase is an MMP that is able to degrade gelatin (previously degraded collagens), collagen types IV, V, VII, and X, elastin, and basement membrane components, which plays an important role in neutrophil transendothelial migration (Owen 1999). u-PA cleaves plasminogen to form plasmin which can degrade a number of matrix components (fibrin, fibronectin, and laminin) and can activate procollagenases (Saksela 1988; Mauch 1998).

The membranes of secretory vesicles in neutrophils contain many membrane-associated receptors required for neutrophils' responses to inflammation, including the $\beta_2$-integin CD11/CD18, the complement receptor 1 (CR1), FcγIII receptor (CD16), receptors for formylated bacterial peptides (fMLP receptors), and the LPS receptor (CD14) (Faurschou 2003). All these receptors can be integrated into the neutrophils cellular membrane and increase neutrophil function, such as adhesion to endothelium and binding opsonins to aid in phagocytosis (Borregaard 2007).

### 1.2.4 Neutrophil Recruitment and Activation

In order for neutrophils to respond to extravascular bacterial and inflammatory signals they must exit the bloodstream and migrate across tissue to reach the site of injury (Borregaard 2010). Neutrophil migration into the inflamed tissue is important for protection against invading microbes and tissue injury (Williams 1990). This migration is a multistep process that includes neutrophil tethering, rolling, activation, firm adhesion, transmigration, and chemotaxis into the tissue toward the site of injury (Ley 2007).

The interactions between neutrophils and endothelial cells are mediated by three major families of proteins: selectins, integrins, and adhesion receptors of the immunoglobulin (Ig) superfamily. Selectins are the molecules that are involved in tethering, the initial interaction of neutrophils and endothelial cells, and in rolling, the movement of neutrophils along the vessel wall (Kelly 2007). P-selectin and E-selectin are expressed on endothelial cells and L-selectin is expressed on neutrophils. P-selectin glycoprotein ligand 1 (PSGL1) is a ligand for all three of these selectins and is found on both cells (Ley 2007). E-selectin can be upregulated on endothelial cells by inflammatory mediators such as LPS, TNF-α, and IL-1β (Ward 1989). The endothelium can also be stimulated to upregulate expression of P-selectin by inflammatory mediators such as complement products, oxygen free radicals, or various cytokines (Wagner 2000).

Neutrophils are activated by chemokines (IL-8, MIP-2) or other chemoattractants (C5a, $LTB_4$, PAF) binding to GCPR (Witko-Sarsat 2000; Borregaard 2010; Wright 2010). This activation mediates the binding of integrins to Ig superfamily proteins (Ley 2007). Integrins such as VLA-4, lymphocyte function-associated antigen-1 (LFA-1) and

macrophage antigen-1 (MAC-1) on neutrophils can interact with adhesion molecules (Ig superfamily proteins) on endothelial cells, such as ICAM-1 and -2, and VCAM-1, to mediate firm adhesion (Wright 2010). Neutrophils stimulated by IL-8 or LPS produce integrin-modulating factor-1, which significantly increases CD18 (a component of LFA-1 and MAC-1) binding affinity (Wagner 2000). Chemokines and chemoattractants are able to drive integrin-mediated adhesion by increasing the integins' avidity (Ley 2007). MAC-1 activation on neutrophils can be induced by chemoattractants (PAF, IL-8, fMLP, C5a), cytokines and growth factors (TNF-α, GM-CSF), and bacterial products (LPS) (Witko-Sarsat 2000). VCAM-1, ICAM-1 and -2 can be upregulated by cytokines such as TNF-α and IL-1 (Kelly 2007).

Neutrophils then migrate into the tissues through the intercellular junctions of endothelial cells (paracellular migration) (Wright 2010). Transendothelial migration of neutrophils is facilitated by cell adhesion molecules of the Ig-superfamily such as PECAM-1 and junctional adhesion molecules (JAM)-A, -B, and -C (Muller 2003). Other molecules that may be involved in paracellular transmigration of neutrophils include the non-immunoglobulin molecule CD99 and endothelial cell-selective adhesion molecule (ESAM) (Ley 2007). PECAM-1, which can bind to other PECAM-1 molecules, is found evenly distributed over the surface of neutrophils but predominantly at junctions between neighboring endothelial cells (Wagner 2000). JAMs are also able to react with other JAMs, but they are also able to interact with integrins. Activated endothelial cells can rearrange their junctional molecules to favor neutrophil transendothelial migration. PECAM-1 and JAM can mobilize to the surface of the endothelial cells and create an adhesive gradient that guides neutrophils to the junctions (Ley 2007).

After neutrophils have left the circulation they migrate toward the inflamed tissue along a chemoattractant gradient. Neutrophils chemoattractants include soluble bacterial product fMLP, $LTB_4$, C5a, PAF and $ELR^+CXC$ chemokines (Rollins 1997; Wagner 2000). The binding of these chemoattractants to GCPRs generates intracellular signals that lead to the alterations in the cytoskeleton resulting in neutrophil motility (Downey 1994). During the migration of the neutrophil through the tissue, primary and secondary granules undergo partial exocytosis, releasing collagenase and serine proteases which aid in neutrophil migration by degrading the surrounding ECM (Faurschou 2003). At the site

of injury, opsonized targets (bacteria) are ingested and phagocytosed by neutrophils (Wright 2010). Phagocytosis of C3b-opsonized targets is complement receptor 3 (CR3)-dependent, while phagocytosis of Ig-opsonized targets is FcγII receptor (FcγRII)-dependent (Witko-Sarsat 2000). These activated neutrophils can undergo a respiratory burst and degranulation, releasing ROS and hydrolytic enzymes that kill the phagocytosed pathogens (Dale 2008). Inappropriately activated neutrophils can lead to surrounding tissue damage through the release of these toxic molecules (Clemens 2004).

Both oxygen-dependent and -independent mechanisms are used by neutrophils to digest and kill phagocytosed pathogens. Oxygen-independent methods include the release of granular antimicrobial proteins and enzymes such as BPI, defensins and serine proteases into the phagosome, as previously described (Levy 2004). Oxygen-dependent killing is mediated by the release of ROS from the neutrophil, including superoxide ($O_2^-$), hydrogen peroxide ($H_2O_2$), hydroxyl radical ($OH^{\cdot}$), and hypochlorous acid (HOCl) (Robinson 2008). During phagocytosis there is a large increase in oxygen consumption by the cell termed the respiratory burst, which leads to the generation of these ROS (Dale 2008). The respiratory burst is triggered by enzymes found in the membrane of the phagosome, called NADPH oxidase, which is activated during phagocytosis (Kobayashi 2003). NADPH oxidase uses NADPH as an electron donor to form $O_2^-$ from molecular oxygen, which is then rapidly reduced (spontaneously or enzymatically by SOD) to $H_2O_2$ (Smith 1994). $H_2O_2$ can be converted into $OH^{\cdot}$ in the presence of metal catalysts like $Fe^{3+}$, or it can be converted into HOCl in the presence of a halide ($Cl^-$) and MPO (Splettstoesser 2002). $O_2^-$ is only weakly bactericidal, although it is still quite important as it acts as a precursor to more potent ROS, and $H_2O_2$ is only bactericidal at high concentrations (Smith 1994; Hampton 1998). HOCl is a potent oxidant and is 100-fold more powerful as an antimicrobial agent than $H_2O_2$ (Ochoa 1997). HOCl can attack the double bonds of unsaturated fatty acids and cholesterols in lipids which can lead to chlorohydrin formation or peroxidation, both of which contribute to bacterial death (Pennathur 2010). ROS are also able to trigger expression of adhesion molecules by endothelial cells, which promotes the tethering of neutrophils (Smith 1994). ROS ( such

as HOCl) not only kill invading pathogens, but they can also contribute to tissue injury found in inflammation (Dahlgren 1999; Pullar 2000).

### 1.2.5 Neutrophil Inflammatory Mediators

It has been shown that neutrophils can participate in inflammatory responses by producing tissue-damaging mediators, including ROS (e.g. HOCl, $OH^{\cdot}$) and hydrolytic enzymes (e.g. collagenase, gelatinase). Neutrophils can also participate in and amplify inflammation by synthesizing soluble mediators that that can recruit and activate more cells to the site of inflammation (Nathan 2006). Activated neutrophils can synthesize a variety of inflammatory cytokines, such as IL-1, TNF-α, IL-6, TGF-β, oncostatin M, and B lymphocyte stimulator (BLyS) (Wright 2010). Neutrophils are also able to secrete a variety of chemokines, such as IL-8, GRO-α, MIP-1α, MIP-1β, IP-10, and MIG, some of which are neutrophil chemoattractants, while others are chemoattractant for other inflammatory leukocytes (Scapini 2000). Other mediators of the inflammatory response produced by neutrophils include oxidation products of the AA cascade. AA is released from lipid membranes by $PLA_2$ (released from specific granules) and then serves as a precursor for prostaglandin and leukotriene production, of which $PGE_2$ and $LTB_4$ are the most important in inflammation (Pillinger 1995). The regulation of many inflammatory mediator, such as cytokines and chemokines, in neutrophils is mediated by the transcriptional activation of the corresponding genes by the NF-κB and C/EBP (CCAAT/enhanced-binding protein) transcription factors (Cloutier 2007; Cloutier 2009).

As previously mentioned, pathogenic effects develop if the neutrophils' response is disproportionately large, resulting in the inappropriate release of hydrolytic enzymes and reactive oxygen intermediates, which damage surrounding tissue (Li 2002). So, although neutrophils are essential to host defense, they have been implicated in the pathology of many inflammatory diseases. For instance, in RA, proteases released from neutrophils are able to degrade collagen and proteoglycan, while oxygen radicals are able to activate latent collagenases and can release proteoglycan from the cartilage matrix, all which result in damage to the articular cartilage (Kitsis 1991). $ELR^{+}CXC$ chemokines are important to the neutrophil influx that causes pathology in these inflammatory

conditions (Gordon 2005). This suggests that blocking the ELR$^+$CXC chemokines could potentially interrupt a critical check-point in the inflammation cascade by inhibiting neutrophil chemotaxis and activation.

## 1.3 ELR$^+$CXC Chemokines

### 1.3.1 Human and Mouse ELR$^+$CXC Chemokines

A subfamily of the CXC chemokines, all of which attract and activate neutrophils, are the ELR$^+$CXC chemokines. The ELR$^+$CXC chemokines are characterized by four highly conserved cysteine residues, where a non-conserved amino acid residue separates the first two cysteines, and also by a Glu-Leu-Arg (ELR) tripeptide motif which is located on the $NH_2$ terminus adjacent to the conserved CXC region (Strieter 2004). In humans, the ELR$^+$CXC chemokines include CXCL1 (GRO-α), CXCL2 (GRO-β), CXCL3 (GRO-γ), CXCL5 (ENA-78), CXCL6 (GCP-2), CXCL7 (neutrophil-activating peptide-2; NAP-2), and CXCL8 (IL-8) (Laing 2004). Mice do not possess a CXCL8 gene, but alternate rodent ELR$^+$CXC chemokines have been found, including MIP-2, keratinocyte-derived chemokine (KC), lipopolysaccharide-induced CXC chemokine (LIX), and lungkine (CINC; CXCL15). KC and MIP-2 are related to the three human GRO chemokines and LIX is most closely related to human ENA-78 and GCP-2 (Rovai 1998; Laing 2004).

In humans ELR$^+$CXC chemokines bind to their target cell via two receptors, the CXCR1 and CXCR2 (Laing 2004). Human neutrophils express both of these receptors (Feniger-Barish 1999). The CXCR1 binds only CXCL8 with high affinity and CXCL7 with low affinity, whereas the CXCR2 binds CXCL8 with high affinity and all other ELR$^+$CXC chemokines with low affinity (Baggiolini 1995; Busch-Petersen 2006). The rodent homolog of the CXCR2 (mCXCR2) binds MIP-2 and KC with high affinity (Luan 2001). It was believed that CXCR2 was the only ELR$^+$CXC chemokine receptor found in mice, and until recently no function homolog of CXCR1 has been identified. However, a possible mouse CXCR1 (mCXCR1) homolog has been identified and characterized and it has been found to share 84% , 64%, 57%, and 57% homology with

rat CXCR1, hCXCR1, hCXCR2, and mCXCR2, respectively (Moepps 2006). mCXCR1 has been found to bind mouse GCP-2 (LIX) (Fan 2007).

### 1.3.2 CXCL8

CXCL8 is an inflammatory mediator that promotes the recruitment and activation of neutrophils. In the past it has had other names, such as neutrophil-activating factor (NAF), monocyte-derived neutrophil chemotactic factor (MDNCF), monocyte-derived neutrophil-activating peptide (MONAP), and NAP-1 (Matsushima 1988; Baggiolini 1989; Lindy 1997). Human CXCL8 occurs as a functional 72-amino acid protein that is derived from cleavage of a 99-residue precursor protein (Lindley 1988). CXCL8 exists as both a monomer and a dimer, although the monomer predominates at the functional concentration range. It is the monomer that binds and activates receptors and is therefore believed to be the functional form of CXCL8. Each monomer has a $NH_2$-terminal region, a loop region, three antiparallel β–strands, and a COOH-terminal α–helix (Clark-Lewis 1995). The chemokine structure is stabilized by two disulfide bonds ($C^7$-$C^{34}$ and $C^9$-$C^{50}$) and by hydrophobic interactions between a portion of the β–sheet and one side of the COOH-terminal α–helix (Clark-Lewis 1991; Fernandez 2002). The ELR (4-6) motif in the N-terminus is the most critical region for receptor activity (Leong 1997), while receptor binding specificity is determined by the sequences found in the $NH_2$-terminal loop region. Greater specificity for CXCR1 is determined by the residues YSKPK (13-17) in CXCL8 (Fernandez 2002). Another region that is involved in CXCL8 recognition of CXCR1 and CXCR2 is the a hydrophobic pocket comprised of $F^{17}$, $F^{21}$, $I^{22}$, and $L^{43}$ (Williams 1996). The $G^{31}$ and $P^{32}$ motif in the 30-35 turn region is essential in maintaining structure, and substitutions in this motif can influence the conformation of the ELR motif (Clark-Lewis 1995).

### 1.3.3 CXCR1 & CXCR2

In humans CXCL8 binds to its target cells via two receptors, the CXCR1 and CXCR2. They are both seven-transmembrane GPCR (Laing 2004). GPCR have their N-terminus and three extracellular loops on the outside of the cell, while their C-terminus

and three intracellular loops face the cytoplasm inside the cell (Allen 2007). CXCR1 and CXCR2 have a sequence homology of 80% (Mukaida 2003), but have different $ELR^{+}CXC$ chemokine specificities, as noted above. CXCR1 and CXCR2 differ significantly in their N-terminal sequences, and it is this region that is the major determinant of ligand binding affinity and specificity. For example, the first extracellular loop in CXCR2 is critical for binding of CXCL8, CXCL1 and CXCL7 and it binds each of these via different contact points (Katancik 1997).

Specific regions of the chemokine receptors are involved in chemokine interaction. The sites that are involved in chemokine recognition and activation are different (Fernandez 2002). Activation of CXCR1 and CXCR2 by CXCL8 is dependent on their interaction at two sites. The first site involves the interaction of CXCL8 N-terminus (11-17) loop and/or surface hydrophobic pocket with the receptors' N-terminal domain. The second site involves interactions between the ELR motif of the chemokine and the receptors' extracellular/transmembrane residues (Hammond 1996; Joseph 2010).

Signal transduction of GPCR involves the heterotrimeric G proteins that are bound to the intracellular loops. When a ligand binds a GPCR it activates the heterotrimeric G protein resulting in exchange of GDP for GTP in the α subunit of the G protein which leads to the dissociation of Gα from Gβγ, both of which are able to activate downstream effectors (Allen 2007). Gβγ activates phospholipase C (PLC) leading to an increase in inositol 1,4,5-triphosphate ($IP_3$) and diacylglycerol (DAG) which results in $Ca^{2+}$ mobilization and activation of protein kinase C (PKC) (Luster 2001). Activated PKC and increased levels of cytosolic $Ca^{2+}$ can induce PLC and phospholipase D (PLD) to produce DG and phosphatidic acid, resulting in a positive feedback loop. After this a variety of downstream effectors are phosphorylated and activated leading to several distinct signaling pathways resulting in a variety of cellular responses, such as chemotaxis, degranulation, and respiratory burst (Ben-Baruch 1995).

When GPCR have been activated the C-terminus becomes phosphorylated, which leads to receptor internalization and homologous and/or heterologous desensitization (Feniger-Barish 1999). Internalization of activated CXCR2 occurs much faster than that of CXCR1 (~90% vs ~10% after 5 min) (Nasser 2007). Homologous desensitization occurs when the activation of a receptor by an agonist results in the diminished

responsiveness to that same agonist. Heterologous desensitization occurs when the activation of a receptor by one agonist results in the diminished responsiveness to different agonists (Ben-Baruch 1995). Although CXCL8 can activate both CXCR1 and CXCR2, only CXCR1 in involved in heterologous desensitization (Nasser 2005).

Other important neutrophil chemoattractants besides CXCL8 include fMLP, C5a, $LTB_4$, and PAF, which also induce their effector functions by binding the GPCRs fMLPR, BLTR, and PAFR, respectively (Luster 2001). Each of these chemoattractant can initiate homologous desensitization, but it is these specific chemoattractants that are involved in heterologous desensitization of GPCR. In neutrophils CXCL8, C5a and fMLP are involved in heterologous desensitization of one another as well as to PAF and $LTB_4$ (as determined using calcium mobilization assays). However, PAF and $LTB_4$ are not able to desensitize the receptors for fMLP, C5a or CXCL8 (Tomhave 1994; Richardson 1996; Richardson 1998). Heterologous desensitization by chemoattractant has been seen to have somewhat different downstream signaling effects, and they do not desensitize each other's receptors to the same degree. For example, fMLP and C5a can cross-desensitize both chemotaxis and AA release stimulated by each other, while CXCL8 can desensitize chemotaxis by fMLP and C5a, but is less efficient in blocking AA release (Ali 1999). Thus, chemoattractant receptors have a hierarchy in their abilities to desensitize other chemoattractants, wherein fMLP is the most potent, followed by C5a, and then CXCL8 (Kitayama 1997).

In response to CXCL8 both CXCR1 and CXCR2 can induce chemotactic responses, neutrophil intracellular $Ca^{2+}$ flux, and degranulation, but activation of the respiratory burst and phospholipase D has been found to be CXCR1-dependent (Jones 1996). It is believed that CXCR2 is more involved in the initiation of IL-8-induced neutrophil migration (not at the site of inflammation), where IL-8 has a lower concentration, whereas CXCR1 is involved in mediating neutrophil chemotaxis at the site of inflammation, where IL-8 has a higher concentration (Feniger-Barish 1999).

### 1.3.4 ELR$^+$CXC Chemokine Expression and Regulation

CXCL8 can be expressed by a wide variety of cells, including monocytes, macrophages, T cells, neutrophils, fibroblasts, endothelial cells, and epithelial cells (Baggiolini 1989; Baggiolini 1995; Williams 1996). Expression of CXCL8 can be induced by inflammatory stimuli such as LPS, IL-1, and TNF-α (Hebert 1991). CXCL8s' effects on neutrophils include induction of neutrophil shape change, chemotaxis, respiratory burst, and the release of granules that contain degradative enzymes (Lindley 1988). CXCL8 also induces a transient rise in the concentration of intracellular free $Ca^{2+}$ in neutrophils (Holmes 1991). CXCL8 production is regulated through NF-κB and AP-1, which are both transcription factors involved in the regulation of inflammation (Mukaida 2003). NF-κB can be induced by inflammatory mediators such as IL-1, TNF-α, LPS and ROS (Ali 2004).

ELR$^+$CXC chemokines play an important part in inflammatory diseases such as RA, which are characterized by neutrophilic inflammation. CXCL8 is found at high levels in the synovial fluids and tissues of RA patients, where it is responsible for neutrophil recruitment in to the joints. It is here that the cells release mediators, such as ROS and proteases, that can damage the joint tissues (Szekanecz 2003; Cascao 2010). Other ELR$^+$CXC chemokines found in the RA synovium include GRO-α, GCP-2 and ENA-78 (Erdem 2005). These chemokines are also able to stimulate neovascularization in inflammation and play a central role in angiogenesis in RA (Szekanecz 2003). Since the ELR$^+$CXC chemokines are expressed redundantly in inflammation (including RA) it would seem that a strategy to antagonize both CXCR1 and CXCR2 is required to effectively block ELR$^+$CXC chemokine-induced neutrophil chemotaxis and activation.

### 1.3.5 ELR$^+$CXC Chemokine Antagonism

Development of a therapeutic agent that can block inflammation through antagonizing ELR$^+$CXC chemokines has become a major clinical priority. ELR$^+$CXC chemokines and their receptors are attractive therapeutic targets because of their roles in migration and activation of neutrophils in inflammatory diseases (Bizzarri 2006).

A number of Abs to different ELR$^+$CXC chemokines have been generated in the search of a good antagonist. A monoclonal anti-CXCL8 Ab (WS-4) was found to protect against acute lung injury in a rabbit model of necrotizing pancreatitis, as seen by a decrease in serum IL-8 and TNF-α and a significant reduction in neutrophil infiltration into the lungs (Osman 1998). WS-4 was also able to prevent neutrophil infiltration and tissue injury in a rabbit model of lung reperfusion injury (Sekido 1993). WS-4 was found to reduce neutrophil infiltration and neutrophil-dependent synovial membrane damage in a LPS/IL-1-induced arthritis model (Harada 1994). A neutralizing anti-CINC Ab was found to offer protection against pancreatitis-associated lung injury in rats, which was demonstrated by a reduction in MPO activity and vascular permeability (Bhatia 2000). But, we know that ELR$^+$CXC chemokines play a redundant role in inflammation, so being able to block all ELR$^+$CXC chemokines might be predicted to be a better approach than blocking just one (Le 2004).

There have also been viral chemokine homologues reported. Cytomegalovirus encodes a gene UL146, whose product has sequence similarity to CXC chemokines and has been designated vCXC-1. vCXC1 is a glycoprotein that has been found to induce neutrophil chemotaxis, $Ca^{2+}$ mobilization, and degranulation It is almost as potent as CXCL8, although it only activates the CXCR2 and not the CXCR1 (Penfold 1999). The murine γ–herpesvirus secretes a protein, hvCKBP, which had broad chemokine-binding capabilities. It interacts with the receptor-binding domains of chemokines and it has a strong affinity for CXCL8 and moderate affinity for CXCL1, but also interacts with chemokines from the CC subfamily (Parry 2000).

Chemokine receptor antagonists are also a common therapeutic target and a few will be discussed here. A small nonpeptide receptor antagonist SB 225002 [N-(2-hydroxy-4nitrophenyl)-N'-(2-bromophenyl)urea] is able to inhibit ELR+CXC chemokine-receptor interactions and is selective for CXCR2 (>150 fold over CXCR1). SB225002 was able to inhibit rabbit and human neutrophil chemotaxis by IL-8 and GRO-α *in vitro*, and was able to block CXCL8-meidated neutrophil margination in rabbits *in vivo* (White 1998). SB225002 has also been found to ameliorate acute experimental colitis in mice through the reduction in neutrophil influx, MPO activity, cytokine levels (IL-1β, MIP-2, KC, and VEGF), iNOS, and COX-2 (Bento 2008). Another potent

CXCR2 antagonist is Sch527123, which has been found to inhibit neutrophil chemotaxis and MPO release in response to CXCL1 and CXCL8, but was not able to antagonize the effects of C5a or fMLP (Gonsiorek 2007). Treatment with Sch527123 was able to block CXCR2-mediated neutrophil accumulation and goblet cell hyperplasia in the lungs in a mouse model of LPS-induced pulmonary inflammation (Chapman 2007). Even though Sch527123 can bind at both CXCR1 (nanomolar affinity) and CXCR2 (picomolar affinity), in effect it is CXCR2-selective as Sch527123 has a very slow dissociation rate from the CXCR2 and a very rapid dissociation from the CXCR1 (Gonsiorek 2007).

So far the receptor antagonists have been focused on CXCR2, but we know that CXCR1 is also involved in pathogenesis of inflammatory diseases through the release of reactive oxygen species, therefore being able to antagonize both CXCR1 and CXCR2 would be preferred (Jones 1996). Repertaxin (R(-)-2-(4-isobutylphenyl)propionyl methanesulfonamide) is a non-competitive allosteric inhibitor of CXCR1/CXCR2. Repertaxin binds to CXCR1 and locks it in an inactive conformation, preventing ligand binding and signaling. It inhibits chemotaxis of neutrophils *in vivo* and can protect organs against reperfusion injury in a rat model of liver ischemia-reperfusion injury, as seen by a reduction in neutrophil recruitment, and reductions in liver damage (Bertini 2004). Repertaxin was also found to reduce neutrophil infiltration into the lung in a LPS-induced model of acute lung injury (Zarbock 2008). Another non-competitive allosteric inhibitor of CXCR1/2 is called DF 2162 (4-[(1R)-2-amino-1-methyl-2-oxoethyl]phenyl trifluoromethane sulphonate) and it is an orally active compound. DF2162 was found to inhibit neutrophil chemotaxis mediated by CXCL8 and CXCL1, but was not able to affect neutrophil chemotaxis mediated by C5a or fMLP. It was also found to have no effect on CXCL8 binding to neutrophils. DF2162 was found to diminish neutrophil influx and inflammatory hypernociception in mice and was able to ameliorate collagen-induced arthritis in mice, as seen by a reduction in paw edema, disease scores, and neutrophil infiltration into the paws (Cunha 2008). DF2162 was also found to ameliorate adjuvant-arthritis in rats, although when rats received therapeutic, rather than prophylactic treatments there was not as great of an effect (Barsante 2008).

#### 1.3.5.1 $CXCL8_{(3–72)}$K11R/G31P

In the Gordon lab the strategy in making a bovine $ELR^{+}CXC$ chemokine antagonist was to first develop a high affinity analogue of CXCL8, and then to delete this agonists' ability to activate the CXCR1 and CXCR2. It had been found that amino-terminal truncation of CXCL8 increased its receptor affinity (Clark-Lewis 1994). Truncation of the 5' terminal end of bovine CXCL8, combined with a Lys to Arg substitution at amino acid 11 ($CXCL8_{(3-73)}$K11R) created a high affinity CXCR1 and CXCR2 agonist. $CXCL8_{(3-73)}$K11R had increased activity for CXCR1 and CXCR2 relative to wild-type CXCL8, which lead to an increased ability to chemoattract neutrophils and induce degranulation (Li 2001). The receptor signaling function of this agonist was then targeted and a Gly to Pro substitution at amino acid 31 ($CXCL8_{(3-73)}$K11R/G31P; bG31P) created a very high affinity CXCL8 antagonist for neutrophils. bG31P had no discernible neutrophil agonist activity, was able to bind to both the CXCR1 and CXCR2 with higher affinity than CXCL8, and was able to block CXCL8-mediated chemotactic responses (Li 2002).

Bovine G31P was found to have a higher affinity for neutrophils than CXCL8 by 2-3 orders of magnitude, such that 0.5 nM bG31P blocked 50% of the neutrophil chemotactic response to 129 nM CXCL8. It was also found to antagonize the chemotactic activities of other $ELR^{+}CXC$ chemokines, such as GRO-α and ENA-78. In wash fluids from bacterial pneumonia or experimental endotoxin-induced mastitis lesions of cattle bG31P was able to inhibit neutrophil chemotactic responses by 93-97%. *In vivo*, bG31P was able to reduce neutrophilic infiltration into intradermal LPS-challenge sites in cattle by 98% for up to 2-3 days (Li 2002). Treatment with bG31P in a piglet airway endotoxemia model resulted in an 86% reduction in airway neutrophil influx and a 70% and 83% reduction in airway TNF and IL-1 levels, respectively (Gordon 2009). In an airway endotoxemia model in guinea pigs, treatment with bG31P after the onset of fever, when delivered 3 or 6 h after endotoxin challenge, was able to reduce pulmonary neutrophilia by up to 85%, and resulted in a 50-85% reduction of lung hemorrhagic responses. In this model, G31P was also able to reduce pulmonary IL-1 and TNF responses by 75-95% (Gordon 2005).

The next step that was taken was the humanization of bG31P. A series of bovine-human chimeric proteins were generated, one of which combined the amino terminal half of bG31P with the carboxy terminal half of human CXCL8 (bCXCL8$_{(3-44)}$K11R/G31P-hCXCL8$_{(45-72)}$; bhG31P). This bhG31P retained the ELR$^+$CXC chemokine antagonist activity of bG31P and it was able to block the chemotactic or ROI release responses of human neutrophils to hCXCL8. It proved to be an effective antagonist *in vivo* in a guinea pig model of airway endotoxemia (Zhao 2007).

A fully human antagonist was then generated from a commercially synthesized full-length human CXCL8 cDNA with Lys$^{11}$ to Arg and Gly$^{31}$ to Pro substitutions. This fully human CXCL8$_{(3-72)}$K11R/G31P (hG31P) was able to inhibit chemoattractant responses and intracellular $Ca^{2+}$ flux in neutrophils. hG31P was also found to desensitize heterologous GPCR on neutrophils better than CXCL8. It could desensitize neutrophils responses to C5a, fMLP, and $LTB_4$, as seen by 52-86% reductions in their chemotactic responses and intracellular $Ca^{2+}$ flux. In a guinea pig model of airway endotoxemia hG31P had effects comparable to bG31P, as it was also able to block neutrophil recruitment (>95%) and activation in the airways. Therefore, human G31P is also a high affinity antagonist and has potent anti-inflammatory effects (Zhao 2009).

Human G31P was also tested in a rat model of intestinal ischemia reperfusion-induced injury. It increased survival levels from 50% to 80% and was able to significantly reduce neutrophil infiltration and pathology scores in the intestine and lung (Zhao 2010). Treatment with hG31P in a guinea pig model of aspiration pneumonia leads to 86% reductions in airway neutrophilic infiltration. In this model it was also seen that treatment with hG31P did not predispose to bacterial outgrowth in the airways (Zhao 2010).

In summary, hG31P is a potent ELR$^+$CXC chemokine antagonist that binds with high affinity to both the CXCR1 and CXCR2 receptors and has negligible agonist activity. It has proven to be successful in blocking neutrophil infiltration in numerous models of acute inflammation, including airway endotoxemia (Zhao 2009), and aspiration pneumonia (Zhao 2010), and it was able to block neutrophil infiltration and suppress pathology in intestinal ischemia reperfusion-induced injury (Zhao 2010). hG31P is able to reduce the response of inflammatory cytokines IL-1 and TNF and is also able to

antagonize the chemotactic activity of other $ELR^+CXC$ chemokines (GRO-α and ENA-78). It is able to cross-desensitize heterologous GPCR for other neutrophil chemoattractants, such as fMLP, C5a and $LTB_4$. G31P was also found to not have a significant effect on bacterial clearance in an aspiration pneumonia model, which indicates that neutrophil blockade by G31P did not interfere with the innate anti-bacterial responses. This suggested that G31P could be successful in treatment of other neutrophil and $ELR^+CXC$ chemokine-mediated inflammatory diseases, such as RA.

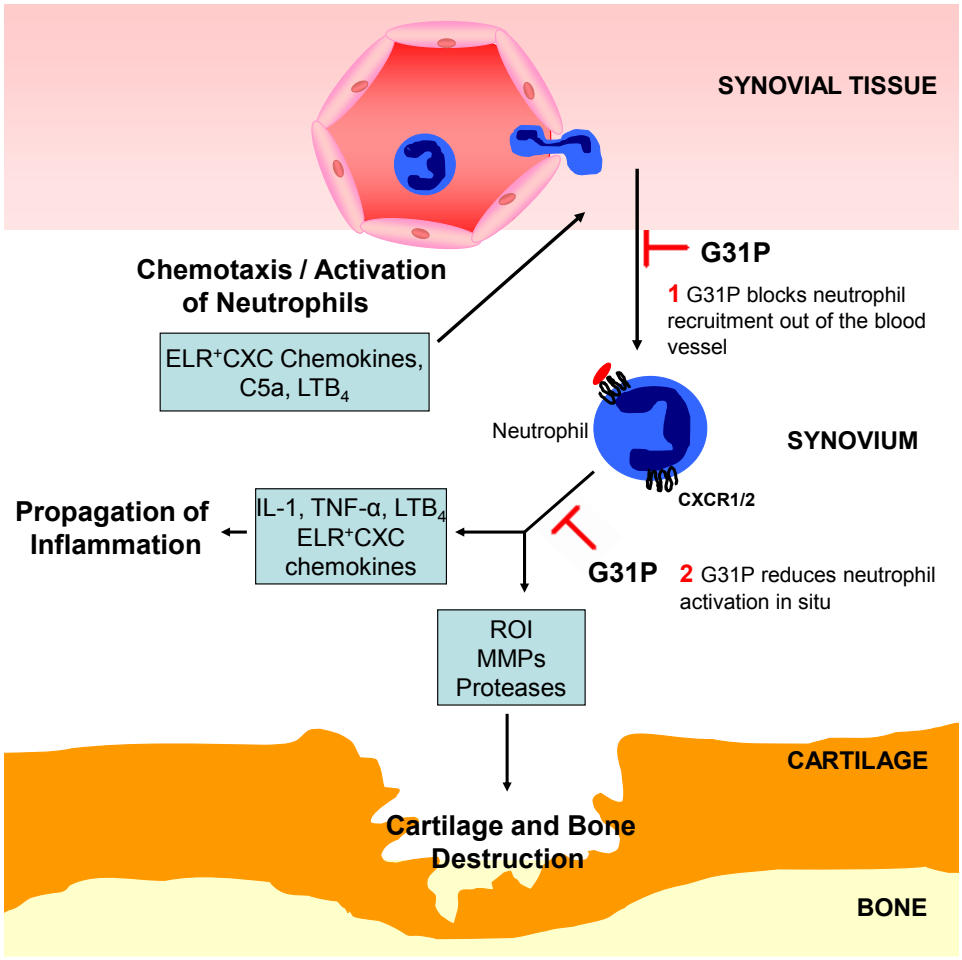



**Figure 4.1 Proposed action of hG31P in CIA.** Neutrophils are recruited to the synovial tissue by ELR$^+$CXC chemokines (e.g. MIP-2, KC) and other chemoattractants (e.g. C5a, $LTB_4$). Once activated, neutrophils release a variety of pro-inflammatory mediators (e.g. IL-1, TNF-α, $LTB_4$, ELR$^+$CXC chemokines), that in turn can propagate the inflammatory response. Neutrophils also release a variety of mediators that contribute to cartilage and bone destruction (e.g. ROIs, proteases, MMPs). hG31P is able to bind neutrophils through CXCR1/CXCR2 and partially block activities of ELR$^+$CXC chemokines as well as cross-desensitize heterologous GPCR for other neutrophil chemoattractants (e.g. fMLP, C5a, $LTB_4$). hG31P has been shown to 1 – block neutrophil chemotaxis, and 2 – reduce neutrophil activation. If hG31P is able to do so in arthritis it can greatly reduce inflammation and cartilage and bone destruction cause by neutrophils.

## 2. HYPOTHESIS AND OBJECTIVES

My hypothesis is that:

Blockade of $ELR^+$CXC chemokine receptors (CXCR1 and CXCR2) will have significant therapeutic effects in an experimental model of arthritis.

My objectives were to:

1) Establish and characterize a model of collagen-induced arthritis (CIA) in mice .
2) Assess the ability of $ELR^+$CXC chemokine blockade to reverse inflammation in established disease.

# 3. MATERIALS AND METHODS

## 3.1 Reagents and Supplies

The following materials were purchased commercially: bovine type II collagen (Sigma-Aldrich Co., Mississauga, ON); acetic acid, aracel A, heavy mineral oil and dextran (Sigma-Aldrich Co., St. Louis, MO); *Mycobacterium tuberculosis* H37RA (Difco Laboratories, Detroit, MI); Immulon-4 ELISA plates (Dynatech Laboratories Inc., Chantilly, VA); rat anti-mouse biotinylated anti-IgG1, IgG2a and IgG2b antibodies (BD Bioscience, Rockville, MD); streptavidin horseradish peroxidase (Vector Laboratories, Inc., Burlingame, CA); ABTS-peroxidase substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD); RNAlater, QIA shredder, and RNeasy Mini Kits (QIAGEN Inc, Mississauga, ON); qRT-PCR Master Mix Kit (Stratagene Cloning Systems, La Jolla, CA); primers (Invitrogen, Burlington, ON); Bio-plex mouse cytokine assays and Bio-plex filter plates (Bio-Rad Laboratories, Hercules, CA); paired anti-MIP-2 capture and detection antibodies and recombinant MIP-2 (R&D Systems, Minneapolis, MN); lymphocyte separation media (MP Biomedical, Aurora, ON); fluo-4 AM (Invitrogen Inc., Burlington, ON). Male DBA/1 mice (6-10 weeks old) were purchased from Taconic Farms Inc (Hudson ,NY); all experiments were carried out according to the guidelines established by the Canada Council on Animal Care and were approved by our university's animal ethics panel.

## 3.2 Induction of Collagen-Induced Arthritis

Type II collagen-induced arthritis (CIA) was induced using established methods (modified from (Brand 2007)) in 6-10 week-old male DBA/1 mice. Mice were immunized intradermally (i.d.) 1.5 cm distal to the base of the tail with 100 μg bovine type II collagen (CII) (dissolved in 0.01 M acetic acid) emulsified with an equal volume of Freund's complete adjuvant (CFA) containing *Mycobacterium tuberculosis* H37RA (final concentration 2 mg/ml). The mice were boosted on day 21 by an intraperitoneal (i.p.) injection of 100 μg of CII in Freund's incomplete adjuvant (IFA: 15% Aracel A and

85% heavy mineral oil). Arthritic mice were treated with 500 μg/kg hG31P subcutaneously (s.c.) three times per week once they exhibited clinical signs of arthritis (score ≥ 1), while control mice were given equal volumes of saline.

### 3.3 Clinical Assessment of CIA

Arthritis development was monitored three times per week and inflammation of the four paws was graded, as determined by signs of edema. The severity of the disease was graded using an arthritis index for each foot, on a scale of 0 to 4, resulting in a maximum possible score of 16 per animal (modified from (Nasu 2008)). The clinical score criteria for each paw was as follows: 0, normal; 1, mild swelling of the ankle or swelling of two digits, activity levels unaffected; 2, moderate swelling of the ankle or swelling of three or more digits, decreased activity but not limping; 3, severe swelling of the ankle, and swelling of four or more digits, evidence of lameness, favouring the affected paw; 4, severe swelling encompassing the ankle, foot and digits, lack of mobility. Once there were clinical signs of arthritis we also quantified the swelling of the paws 3 times per week by measuring the thickness of the ankle on the front paws and the thickness of the hind paws by measuring their circumference (Simoes 2003). Circumference was measured by wrapping a string around the paw, marking it, and then measuring it on a ruler. All scoring was done in a blinded fashion.

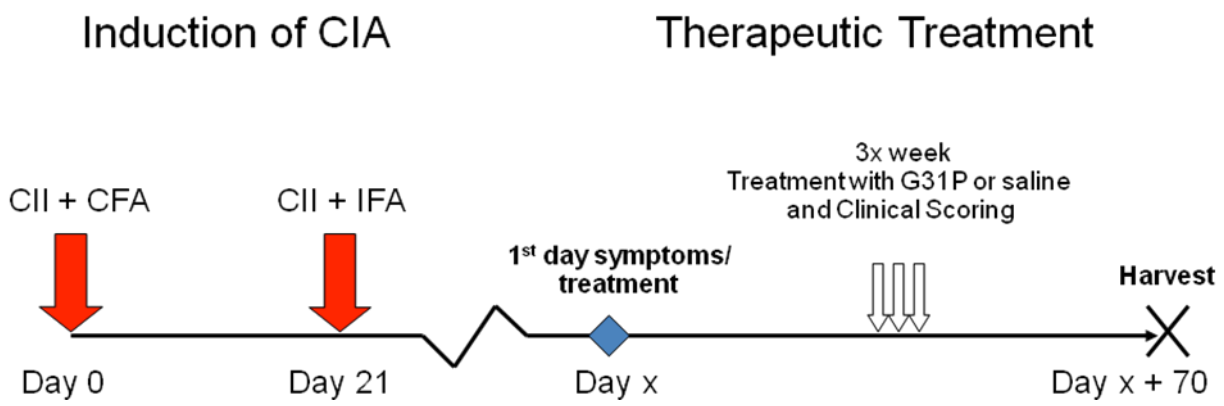



**Figure 4.2 Experimental design.** CIA is induced by i.d. injection of CII emulsified with CFA on day 0. A booster injection of CII emulsified in IFA is given i.p. on day 21. For therapeutic treatment we injected (500 µg/kg hG31P or saline) the mice s.c. three times a week, beginning when their clinical score had reached ≥1. Mice were harvested on day 70 after first exhibiting signs of arthritis. Experiment 1: Saline-treated (n=10), G31P-treated (n=9), non-arthritic (n=5). Experiment 2: Saline-treated (n=12), G31P-treated (n=13), non-arthritic (n=5).

### 3.4 ELISA of anti-CII Antibody

Serum samples were collected at the indicated time points (pre-mortem from the tail artery, post-mortem by cardiac puncture) and examined for anti-CII Ab by ELISA as previously described (Mukherjee 2003). Immulon-4 ELISA 96-well plates were coated with coating buffer (0.1M $NaCO_3$ pH 8.2) containing 3 µg of bovine CII and were incubated at 4°C overnight. After being washed with PBS-0.05% Tween 20 (PBST) 3 times, non-specific binding was blocked with 5% skim milk in PBS for 2 hours at room temperature. After washing 4 times with PBST, mouse serum samples were added (dilutions: $10^{-2}$, $10^{-3}$, $10^{-4}$) and incubated overnight at 4°C. After washing 5 times with PBST, biotinylated rat anti-mouse IgG1 or IgG2a Ab was added at 100 pg and incubated at room temperature for 2 hours. After washing 6 times with PBST, a 1:1000 dilution of streptavidin-HRP was added and incubated at room temperature for 2 hours. Plates were washed 8 times with PBST, followed by development of the reaction with ABTS-peroxidase substrate. The assays were read at 405 nm on the Novostar plate reader (BMG Labtech Ltd., Durham, NC). These results are presented in optical density ($OD_{405}$) units.

### 3.5 Isolation of Paw Tissue RNA

Paw tissues were harvested and stored in 500 µl RNAlater at -20°C. Paw tissues were homogenized using a Polytron homogenizer (Brinkman Instruments, Mississauga, ON) and then the RNA was extracted using RNeasy Mini Kits according to the protocol from the manufacturer. The total RNA was quantified using a Nanodrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE) and stored at -80°C.

### 3.6 Quantitative Real Time PCR (qRT-PCR)

For qRT-PCR a commercial one-step master mix kit was used with the thermocycling profile: first segment, 50°C for 30 min, and 95°C for 10 min (1 cycle); second segment, 95°C for 30 sec, 63°C for 30 sec, 72°C for 30 sec (40 cycles); and third segment 95°C for 1 min, 55°C for 30 sec (data read at this point), 95°C for 30 sec (1 cycle). The reverse transcription and then amplification were performed in a Mx3005P

thermocyler (Stratagene Cloning Systems, La Jolla, CA). As an internal control, we used primers for mouse β-actin (sense: 5′ AGA GGG AAA TCG TGC GTG AC 3′; antisense: 5′ CAA TAG TGA TGA CCT GGC CGT 3′). The sequences of the mouse gene-specific primers were: IL-1β (sense: 5' TGC CAC CTT TTG ACA GTG ATG AG 3'; antisense: 5'-TGA TGT GCT GCT GCG AGA TTT-3'); TNF (sense: 5' CAG CCG ATT TGC TAT CTC ATA CC 3'; antisense: 5' GTA CTT GGG CAG ATT GAC CTC AG 3'); KC (sense: 5' GAT TCA CCT CAA GAA CAT CCA GA 3'; antisense: 5' GGA CAC CTT TTA GCA TCT TTT GG 3') and MIP-2 (sense: 5' AAC ATC CAG AGC TTG AGT GTG AC 3'; antisense: 5' GCC TTG CCT TTG TTC AGT ATC TT 3'). Our primers were designed in-house using OligoPerfect Software (Invitrogen, Burlington, ON). Relative gene expression was determined using a comparative (ΔΔ) ct method with β–actin as an internal control (Schmittgen 2008). Results were analyzed using MxPro Software (Stratagene Cloning Systems, La Jolla, CA)

### 3.7 Multiplex Assay of Cytokines

A multiplex magnetic bead-based assay, containing individual colour-coded fluorescent beads conjugated to monoclonal Abs specific for a target protein, was used for cytokine measurement according to the manufacturer's instructions. The cytokines measured were IL-1β, IL-6, MIP-2, and KC. Briefly, at the time of harvest mouse paw tissues were snap frozen in liquid nitrogen and stored at -80°C. The tissues were ground with a mortar and pestle, the powdered tissue resuspended in PBS, and the supernatants thereof used for multiplex analysis. Then, 50 µl of tissue supernatant was incubated on a shaker with 1x antibody-coupled beads for 30 min (room temperature) in a 96-well filter plate. After washing 3 times with sheath fluid, the plate was incubated on a shaker with 1x biotinylated detections Ab (2 µg/ml of MIP-2 detection Ab) for 30 min. After washing 3 times, the plate was incubated on shaker with 1x streptavidin-PE for 10 min. After washing 3 times, assay buffer was added and then the plates were read on a multiplex array reader (Bio-Rad Laboratories, Hercules, CA). The data is presented as pg/ml, based on a range of recombinant cytokines used to establish standard curves. The concentrations were calculated using Bio-Plex Manager Software (Bio-Rad Laboratories,

Hercules, CA). All reagents, except recombinant MIP-2 and anti- MIP-2 paired capture and detection Ab, were provided in Bio-Plex cytokine assay kits. Assay sensitivities are between 0.2 and 9.4 pg/ml.

### 3.8 ELISA Assay of anti-G31P Antibody

Serum samples were examined for anti-G31P Ab by ELISA. Immulon-4 ELISA 96-well plates were coated with coating buffer (0.1M $NaCO_3$, pH 8.2) containing 10 μg/ml of hG31P and were incubated at 4°C overnight. After being washed 3 times with PBS-0.05% Tween 20 (PBST), non-specific binding was blocked with 5% skim milk in PBS for 2 hours at room temperature. Plates were washed 4 times with PBST, and then mouse serum samples were added (dilutions: $10^{-2}$, $10^{-3}$, $10^{-4}$) and incubated overnight at 4°C. After washing plates 5 times with PBST, biotinylated rat anti-mouse IgG1, IgG2a and IgG2b Ab was added at 1 μg/ml and incubated at room temperature for 2 hours. Plates were washed 6 times with PBST, and then a 1:1000 dilution of streptavidin-HRP was added and incubated at room temperature for 2 hours. Plates were washed 8 times with PBST, followed by development of the reaction with ABTS-peroxidase substrate. The assays were read at 405 nm on the Novostar plate reader (BMG Labtech Ltd., Durham, NC). These results are presented in optical density ($OD_{405}$) units.

### 3.9 Assay of Intracellular $Ca^{2+}$ Flux

This assay was used to test the neutralizing ability of the anti-hG31P Ab found in our hG31P-treated arthritic mouse serum. Protocols have been adapted from those previously described (Zhao 2009). To generate neutrophils, heparin-anticoagulated human peripheral blood was mixed at a 1:1 ratio with 4.5% Dextran T500 in PBS, and then the red blood cells were allowed to sediment. The white blood cell layer was fractioned on a standard density gradient, and then cleared of contaminating red blood cells by hypotonic lysis. Purified neutrophils ($5x10^6$ cells/ml in $Ca^{2+}$-free $PBS^+$) were incubated for 30 min at 37°C with 2 μM Fluo-4. The cells were washed twice with $Ca^{2+}$-free $PBS^+$ and then resuspended to $4x10^6$ cells/ml in $PBS^+$ (0.5 nM $CaCl_2$). For the assay, 50 μl of cells were incubated with 50 μl media or antagonist (hG31P) +/- mouse

serum (1:250 dilution), then challenged with 50 µl of medium or agonist (GRO-α) and then intracellular $Ca^{2+}$ flux was determined using a Novostar spectrofluorometer (emission wavelength 520 nm; excitation wavelength 488 nm). The data is presented as the area under the curve. In preliminary experiments we determined that optimal experimental parameters for our PMN intracellular $Ca^{2+}$ mobilization assay could be induced with 1:250 serum dilution (from healthy mice treated with G31P), 100 ng/ml GRO-α and 10 ng/ml hG31P.

### 3.10 Statistical Analyses

All data was expressed as the mean ± SEM. Clinical scores and measurements were analyzed using repeated measure analysis-of-variance (ANOVA). Assumption of normal distribution not violated, as determined by statistical normality test (Kolmogorov-Smirnov test). Neutralizing hG31P data (Fig 4.13) was not normally distributed, and therefore statistical differences among the experimental groups were explored by ranking the data and then performing ANOVA. Tukey's test was then used to compare the means of the ranks. All other data was analyzed by ANOVA and post-hoc Bonferroni testing. Differences were considered significant when P-values were less than 0.05.

# 4. RESULTS

## 4.1 Establishment and Characterization of a Mouse Model of Collagen-Induced Arthritis

Our ultimate goal was to test the effectiveness of the CXCR1/CXCR2 antagonist, hG31P, in a CIA model of experimental arthritis. In order to do this, the CIA model had to first be established in our lab and its pathology understood. We decided to use the CIA model because it is one of the most widely used models for RA and it shares many pathological features with RA, such as inflammation of synovial joints, pannus formation, destruction of cartilage and bone erosion (Hegen 2008). CIA also shares immunological features with RA, including high levels of Ab to CII, which is a major protein in cartilage (Cho 2007). The susceptibility to both RA and CIA are linked to the expression of specific MHC class II molecules (Myers 1997). There are several inflammatory cytokines found in RA that are also expressed in the joints of CIA mice, including TNF-α, IL-1β, IL-6, IL-12, IL-10, and TGF-β (Malfait 1998; Williams 2007).

To establish the concentration of CII needed to induce patent CIA, several concentrations of CII were used, including 2, 1.5, 1, 0.75, 0.5, 0.25, or 0.1 mg/ml CII and a negative control group of mice that received saline alone. CIA was induced in 6-10 week-old male DBA/1 mice (n=5). Mice were immunized intradermally 1.5 cm distal to the base of the tail with varying concentrations of bovine CII (dissolved in 0.01 M acetic acid) emulsified with an equal volume of CFA. The mice were boosted on day 21 by an intraperitoneal injection of varying concentrations of CII in Freund's incomplete adjuvant. This pilot experiment was only done once due to the difficulties in consistently inducing CIA (see Discussion).

### 4.1.1 Clinical Assessment of Disease

One of the parameters assessed when determining the optimal concentration of CII required in the induction of CIA was that of clinical symptoms.

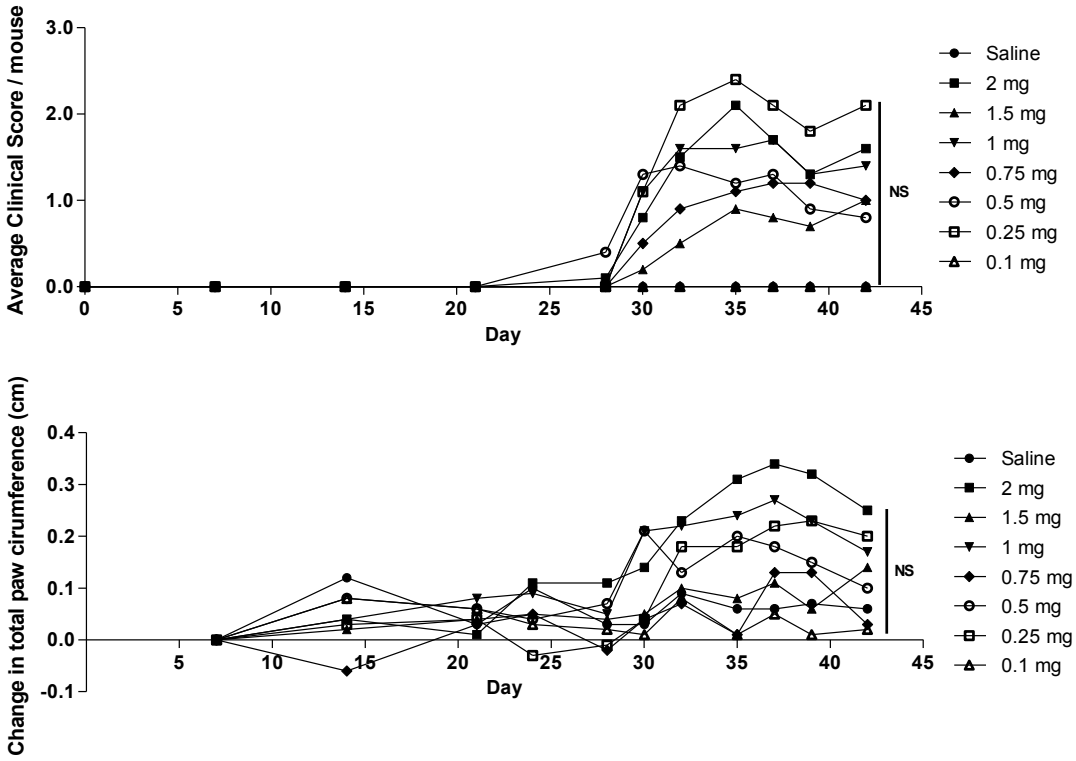



**Figure 4.3 Clinical assessment of CIA**. Different concentrations of bovine CII (2, 1.5, 1, 0.75, 0.5, 0.25, and 0.1 mg/ml) were used in the induction of CIA in DBA/1 mice as described in section 3.2 of Materials and Methods. Mice were monitored and the severity of diseases was graded using an arthritis index for each foot, on a scale of 0 to 4, resulting in a maximum possible score of 16 per animal. The clinical score criteria are as follows: 0, normal; 1, mild swelling of the ankle or swelling of two digits, activity levels unaffected; 2, moderate swelling of the ankle or swelling of three or more digits, decreased activity but not limping; 3, severe swelling of the ankle, and swelling of four or more digits, evidence of lameness, favouring the affected paw; 4, severe swelling encompassing the ankle, foot and digits, lack of mobility. Once there were clinical signs of arthritis the swelling of the paws was quantified by measuring paw circumference (the thickness of the ankle on the front paws and the thickness of the paw on the hind paw). All scoring was done in a blinded fashion. Values are mean (n=5). All P values were determined by repeated measures ANOVA.

There were no significant differences found between the different concentrations of CII ($P>0.05$) when looking at both the clinical scores and clinical measurements. So, in order to choose an optimal concentration, we looked for the greatest mean measurement in multiple parameters, without the SEM. When looking at the clinical scores (Fig 4.1), the mice induced with 0.25 mg/ml had the greatest scores followed by the mice induced with 2 mg/ml. When looking at clinical measurements of paw circumference the mice induced with 2 mg/ml had the greatest change in paw circumference followed by the mice induced with 1 mg/ml or 0.25 mg/ml. Based on these results, two concentrations of CII that could be associated with a strong induction of CIA were 2 mg/ml or 0.25 mg/ml.

### 4.1.2 Serum Levels of anti-CII Antibody

Arthritis severity was also assessed by measuring the serum levels of anti-CII Abs. It has been reported that the levels of CII-specific Ab correlate with the presence or absence of arthritis (Brand 2007). In our CIA mice (as compared to normal mice) we expected to see an increase in total CII-specific IgG2a because arthritis is reportedly attributed to a T helper type 1 (Th1) response. The anti-CII Abs found in CIA have been reported to be mainly of the IgG2 subclass and to be essential for pathogenesis (Watson 1985; Cho 2007). Nevertheless, IgG1, a Th2-associated Ab, can also be found in the serum of arthritic mice (Luross 2001). There were significantly higher levels of CII-specific IgG1 ($P<0.05$) when CIA was induced with 2 mg/ml of CII as opposed to 0.1 mg/ml of CII (Fig 4.2). When we looked at the levels of CII-specific IgG2a (Fig 4.2) there were significantly higher levels associated with 2 mg/ml of CII than with the 0.75 mg/ml ($P<0.05$) or 1 mg/ml ($P<0.05$) doses. A CII concentration of 2 mg/ml appeared to provide the most significant increase in both IgG1 and IgG2a anti-CII Abs.

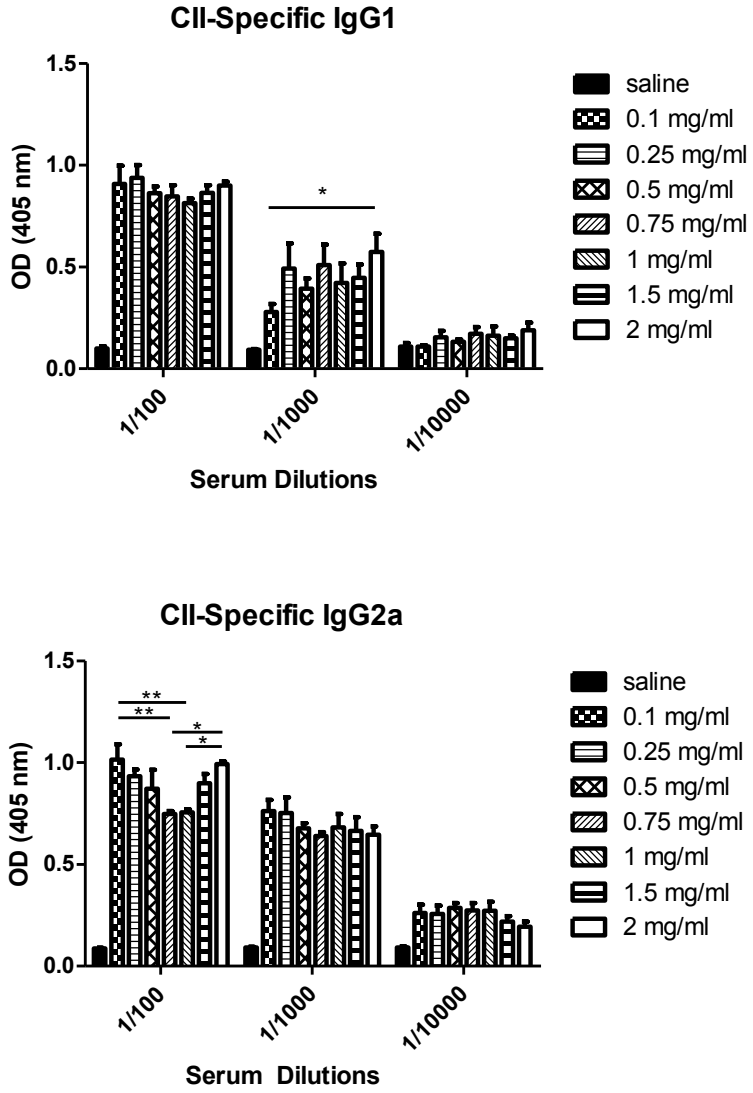
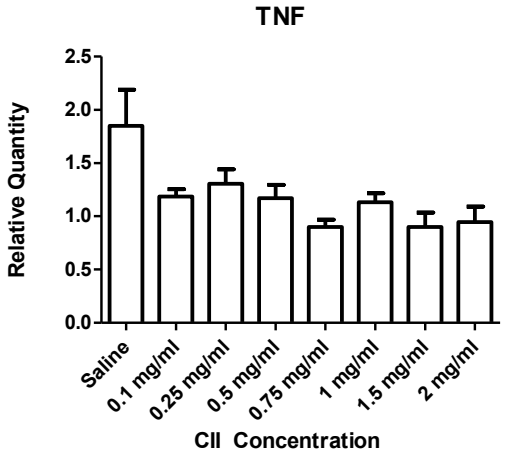


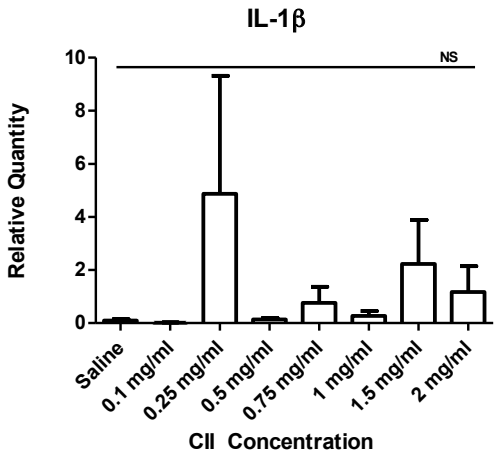

**Figure 4.4 Serum levels of anti-CII antibody**. Different concentrations of bovine CII (2, 1.5, 1, 0.75, 0.5, 0.25, and 0.1 mg/ml) were used in the induction of CIA in DBA/1 mice as described in section 3.2 of Materials and Methods. Serum was collected at time of harvest. To examine changes in the production of anti-CII Abs, the levels of CII-specific IgG1 and IgG2a in the serum were measured by ELISA as described previously. This pilot experiment was done once. Values are mean ± SEM (n=5). All P values were determined by ANOVA.*P<0.05, **P<0.01.

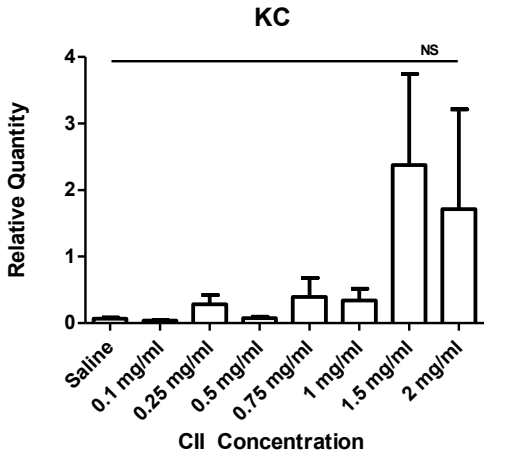
### 4.1.3 Inflammatory Cytokine Expression Levels

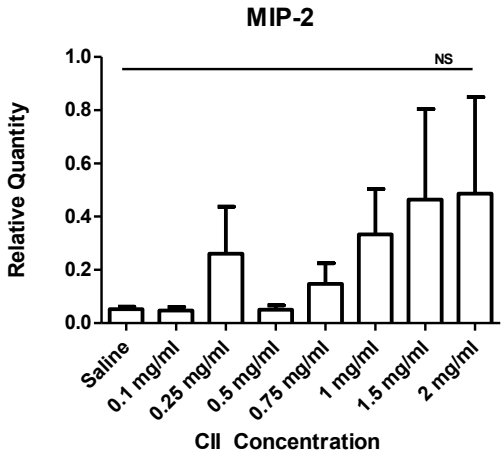
Inflammatory cytokines produced by resident cells or cells infiltrating the synovium can contribute to the inflammatory response in arthritis. Some of the inflammatory cytokines expressed in CIA mice include IL-1β, IL-6, and TNF, and the $ELR^{+}CXC$ chemokines KC and MIP-2, which are involved in neutrophil recruitment (Onodera 2004; Williams 2007). Quantitative real-time PCR (qRT-PCR) was used to assess the expression of the inflammatory cytokines IL-1β, TNF, KC and MIP-2 in the paws of CIA mice induced with varying concentrations of CII (Fig 4.3). There were no statistically significant differences in inflammatory cytokine levels ($P>0.05$) between the different concentrations of CII. The ultimate goal of these experiments was to determine the optimal concentration of CII in arthritis induction; therefore the possible elevated levels of $ELR^{+}CXC$ chemokine KC and MIP-2 in the 1.5 and 2 mg/ml groups could implicate these as candidates for the optimal CII concentration. Moreover, this correlates with the clinical score and serum anti-CII Ab data, where 2mg/ml was the CII concentration found to be associated in inducing patent CIA.

**Figure 4.5 Inflammatory cytokine expression levels**. Different concentrations of bovine CII (2, 1.5, 1, 0.75, 0.5, 0.25, and 0.1 mg/ml) were used in the induction of CIA in DBA/1 mice as described in section 3.2 of Materials and Methods. For determination of mRNA expression of inflammatory mediators IL-1β, TNF, KC, and MIP-2 RNA from each paw was purified and gene expression was evaluated using qRT- PCR. The reaction conditions used were: first segment, 50°C for 30 min, and 95°C for 10 min (1 cycle); second segment, 95°C for 30 sec, 63°C for 30 sec, 72°C for 30 sec (40 cycles); and third segment 95°C for 1 min, 55°C for 30 sec (data read at this point), 95°C for 30 sec (1 cycle). Relative gene expression was determined using comparative (ΔΔ) ct method using β–actin as an internal control. Results were calculated as relative to calibrators, where the calibrator samples were assigned a quantity of 1 and all the samples are expressed as fold changes relative to calibrators. Each paw was processed individually and only paws that exhibited arthritic symptoms were included. This pilot experiment was done once. Values are mean ± SEM (n=5). All P values were determined by ANOVA.


TNF
Relative Quantity
2.5
2.0
1.5
1.0
0.5
0.0
Saline
0.1 mg/ml
0.25 mg/ml
0.5 mg/ml
0.75 mg/ml
1 mg/ml
1.5 mg/ml
2 mg/ml
CII Concentration


IL-1β
NS
Relative Quantity
10
8
6
4
2
0
Saline
0.1 mg/ml
0.25 mg/ml
0.5 mg/ml
0.75 mg/ml
1 mg/ml
1.5 mg/ml
2 mg/ml
CII Concentration


KC
NS
Relative Quantity
4
3
2
1
0
Saline
0.1 mg/ml
0.25 mg/ml
0.5 mg/ml
0.75 mg/ml
1 mg/ml
1.5 mg/ml
2 mg/ml
CII Concentration


MIP-2
NS
Relative Quantity
1.0
0.8
0.6
0.4
0.2
0.0
Saline
0.1 mg/ml
0.25 mg/ml
0.5 mg/ml
0.75 mg/ml
1 mg/ml
1.5 mg/ml
2 mg/ml
CII Concentration

When looking at all of our experimental parameters, the concentration of CII that created the greatest difference in all parameters was 2 mg/ml. At this concentration we saw the greatest clinical manifestation of CIA, and increases in both IgG1 and IgG2a anti-CII Abs as well as the mean values for KC and MIP-2. We recognize that the differences observed in mean values for these parameters were not statistically significantly different, but in the face of not having a more discriminating signal we chose to employ 2mg/ml CII as out preferred concentration for induction of disease.

## 4.2 Assessing the Ability of ELR$^+$CXC Chemokine Blockade to Reverse Inflammation in Established Disease

The aim of our research was to test whether hG31P could be used to effectively treat arthritic mice. ELR$^+$CXC chemokines have been implicated in the pathophysiology of arthritis, which suggests that blocking their receptors, CXCR1/CXCR2, could be beneficial in the treatment of arthritis.

The induction of CIA in DBA/1 mice was done as described in Materials and Methods. Arthritic mice were treated with 500 μg/kg hG31P subcutaneously three times per week once they exhibited clinical signs of arthritis (score $\geq$ 1), while control mice were given equal volumes of saline. All mice were harvested 70 days after their first day of treatment. A side effect of selecting the tail as the site of disease induction was the development of sores on the tails of the mice, to the point that we were unable to take blood from the tail during the course of the trial. This experiment was done twice; in the 1$^{st}$ experiment the incidence of arthritis was 95% and 9 mice received hG31P and 10 mice received saline, while in the 2$^{nd}$ experiment the incidence of arthritis was 87% and 13 mice received hG31P and 12 mice received saline.

### 4.2.1 Clinical Assessment of Disease

To evaluate the effects of CXCR1/2 blockade on arthritis we assessed the severity of disease by measuring the clinical scores and paw circumference of our CIA mice, as described in Materials and Methods. As shown in Fig. 4.4, which depicts the mean scores for all mice in each group, in both experiments there were no significant

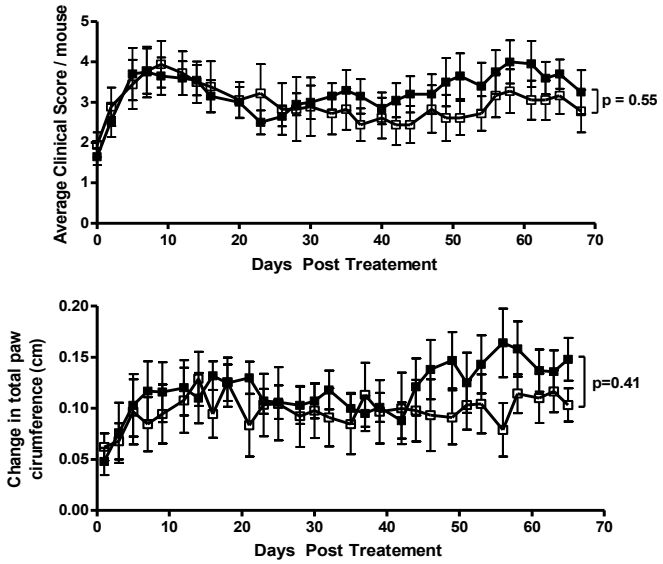
differences (P>0.05) between the hG31P-treated and saline-treated arthritic mice in both clinical scores and measurements. Data was found to be normally distributed and was assessed as stated in materials and methods.

In the CIA model the mice did not develop initial signs of arthritis simultaneously. Therefore we decided to divide the mice up into groups based on their time of CIA onset and look at their clinical scores. The time of onset varied between 4-9 weeks after first exposure to CII. Group 1 consisted of mice that developed arthritis within 5 weeks of initial CII injection, while group 2 consisted of mice that developed arthritis later, 7 weeks after initial CII injection. As shown in Fig 4.5, in the first experiment we found that the hG31P-treated mice who developed arthritis early were seen to have a significant reduction in clinical scores (p=0.02), whereas hG31P-treated mice that developed arthritis later did not show a significant change in clinical scores relative to saline-treated controls. In the second experiment we found that both mice that developed arthritis earlier and later both still showed no significant differences between the hG31P-treated and saline-treated arthritic mice when arthritis. Once again, we assessed normality of distribution, as stated in materials and methods, and found that data was normally distributed.

### 4.2.2 Serum Levels of anti-CII Antibody

To investigate the influence of hG31P-treatment on the production of CII-specific Abs IgG1 and IgG2a in CIA, we collected serum at the time of animal sacrifice (10 wk) and measured the levels of these Abs by ELISA. The effects of CXCR1/2 blockade on anti-CII Ab levels in CIA can be seen in Fig. 4.6. In the second experiment hG31P treatment resulted in a statistically significant decrease in CII-specific IgG1 when compared to saline-treated CIA (P<0.05). We did not see similar results in our first experiment, and in both experiments treatment of CIA mice with hG31P did not result in a significant reduction in the production of CII-specific IgG2a at any of the serum dilutions.

**A. 1st Experiment**




**B. 2nd Experiment**

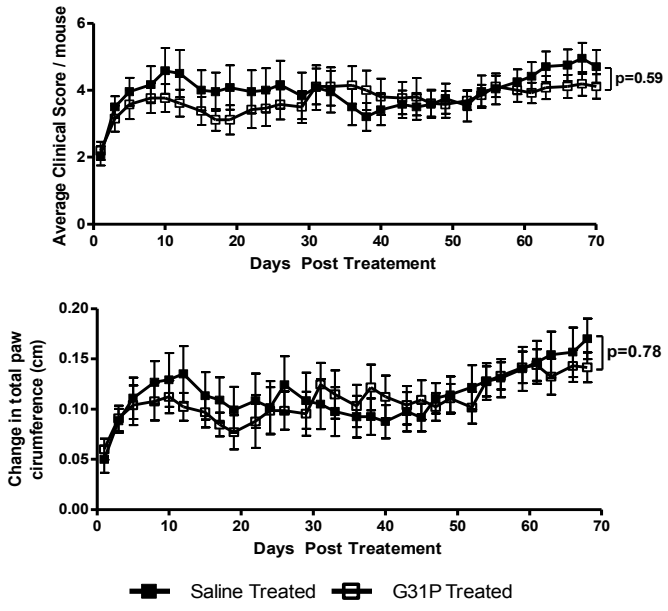



**Figure 4.6 Clinical assessment of the effects of CXCR1/2 blockade on arthritis**. CIA is induced in DBA/1 mice, described in section 3.2 of Materials and Methods. Mice were monitored and the severity of diseases was graded as described in Fig 4.1. Mice were scored in a blinded manner. Values are mean ± SEM. **A**. Experiment 1: Saline-treated (n=10), G31P-treated (n=9). **B**. Experiment 2: Saline-treated (n=12), G31P-treated (n=13). All P values were determined by repeated measures ANOVA. Human G31P (hG31P) referred to as G31P in all figures.

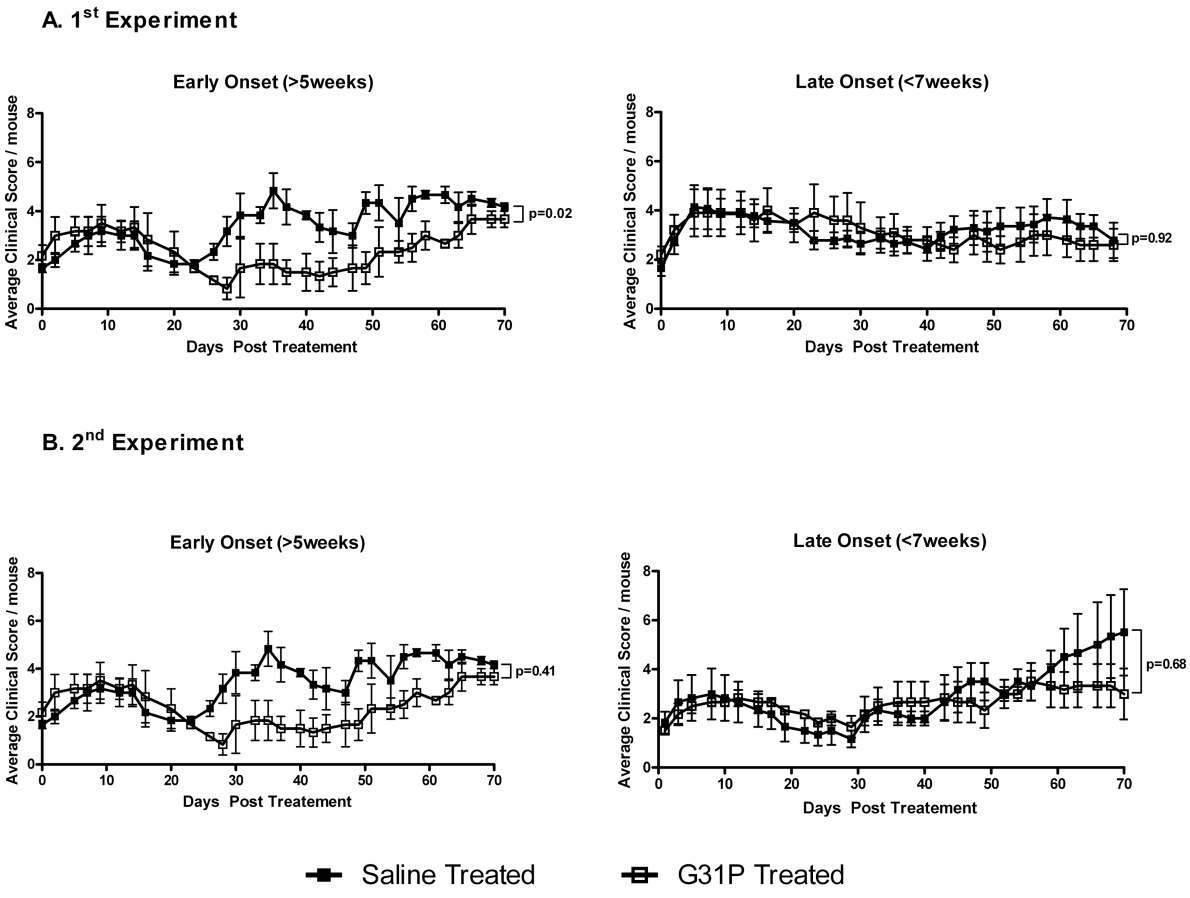



**Figure 4.7 Effects of CXCR1/2 blockade on development of early and late onset arthritis**. CIA is induced in DBA/1 mice as described in section 3.2 of Materials and Methods. Mice were monitored and clinical scores of early onset (< 5weeks) versus late onset (>7 weeks) disease were measured, as described in Fig 4.1. Values are mean ± SEM. **A**. Experiment 1: early onset (n=3), late onset (n=5). **B**. Experiment 2: early onset (n=5), late onset (n=3). All P values were determined by repeated measures ANOVA.

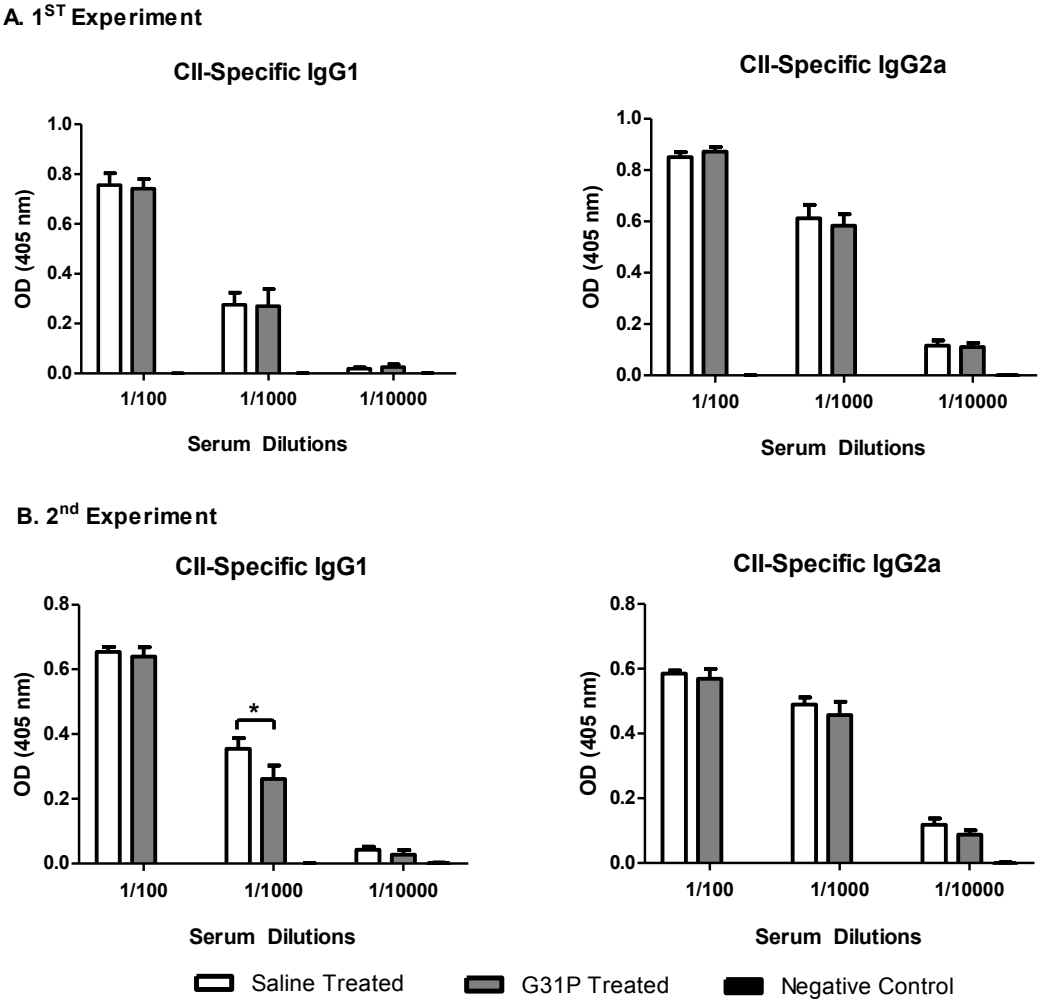



**Figure 4.8 Effects of CXCR1/2 blockade on serum levels of anti-CII antibody**. CIA was induced in DBA/1 mice as described in section 3.2 of Materials and Methods. To examine the production of anti-CII Abs the levels of CII-specific IgG1 and IgG2a were measured in the serum by ELISA. Values are mean ± SEM. **A**. Experiment 1: Saline-treated (n=10), G31P-treated (n=9). **B**. Experiment 2: Saline-treated (n=12), G31P-treated (n=13). All P values were determined by ANOVA, *P<0.05.

### 4.2.3 Inflammatory Cytokine Protein Levels

The effects of CXCR/1 blockade on the levels of inflammatory cytokines in CIA were determined by extracting joint proteins from paw tissues and then using the Luminex magnetic bead-based multiplex assays. It is important to note that even though all paws that were used in the analysis were inflamed at one point during the 10 wk experiment, most were not actively inflamed at the time of harvest and that is a potential confounder. The cytokines measured included IL-1β, IL-6, MIP-2, and KC; their protein levels as determined using saline- and hG31P-treated mice can be seen in Fig. 4.7. In the second experiment there was a statistically significant decrease in MIP-2 levels in the hG31P-treated CIA mice ($p \leq 0.05$) when compared to saline-treated CIA mice. Otherwise, there were no other significant differences in the cytokine protein levels between the hG31P- and saline-treated CIA mice ($p \geq 0.05$).

### 4.2.4 mRNA Expression Levels for Inflammatory Cytokines

The effects of CXCR/1 blockade on the levels of inflammatory cytokine mRNA in the paws of CIA mice were determined using qRT-PCR for IL-1β, TNF, KC and MIP-2. Once again, it is important to note that even though all paws that were used in the analysis were inflamed at one point in the 10 wk experiment, most were not actively inflamed at the time of harvest. In both experiments there were no significant differences in the cytokine expression levels between the hG31P- and saline-treated CIA mice (Fig. 4.8).

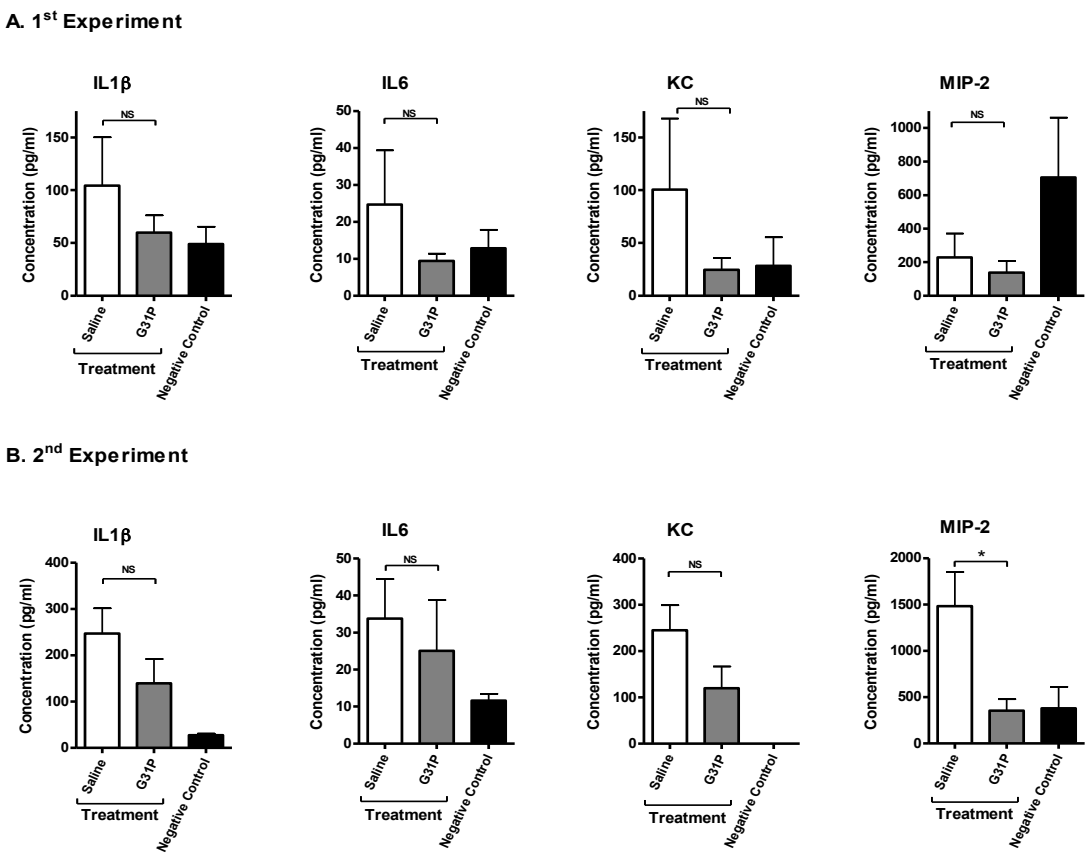



**Figure 4.9 Effects of CXCR1/2 blockade on inflammatory cytokine protein levels**. CIA was induced in DBA/1 mice as described in section 3.2 of Materials and Methods. A multiplex magnetic bead-based assay, containing individual colour-coded fluorescent beads conjugated to monoclonal Abs specific for a target protein was used for cytokine measurement. The cytokines measured were: IL-1β, IL-6, MIP-2, and KC. At the time of harvest mouse paw tissues were snap frozen in liquid nitrogen and stored at -80°C. The tissues were ground with a mortar and pestle, re-suspended in PBS, and the resulting supernatants were used for multiplex analysis. The data is presented as pg/ml, based on a range of recombinant cytokines used to establish standard curves. Each paw was processed individually and only paws that exhibited arthritic symptoms were included. Values are mean ± SEM. **A**. Experiment 1: Saline-treated arthritic (n=34), G31P-treated arthritic (n=29), and Negative control (n=20). **B**. Experiment 2: Saline- or G31P-treated arthritic (n=41), and Negative control (n=20). All P values were determined by ANOVA, **P<0.01.

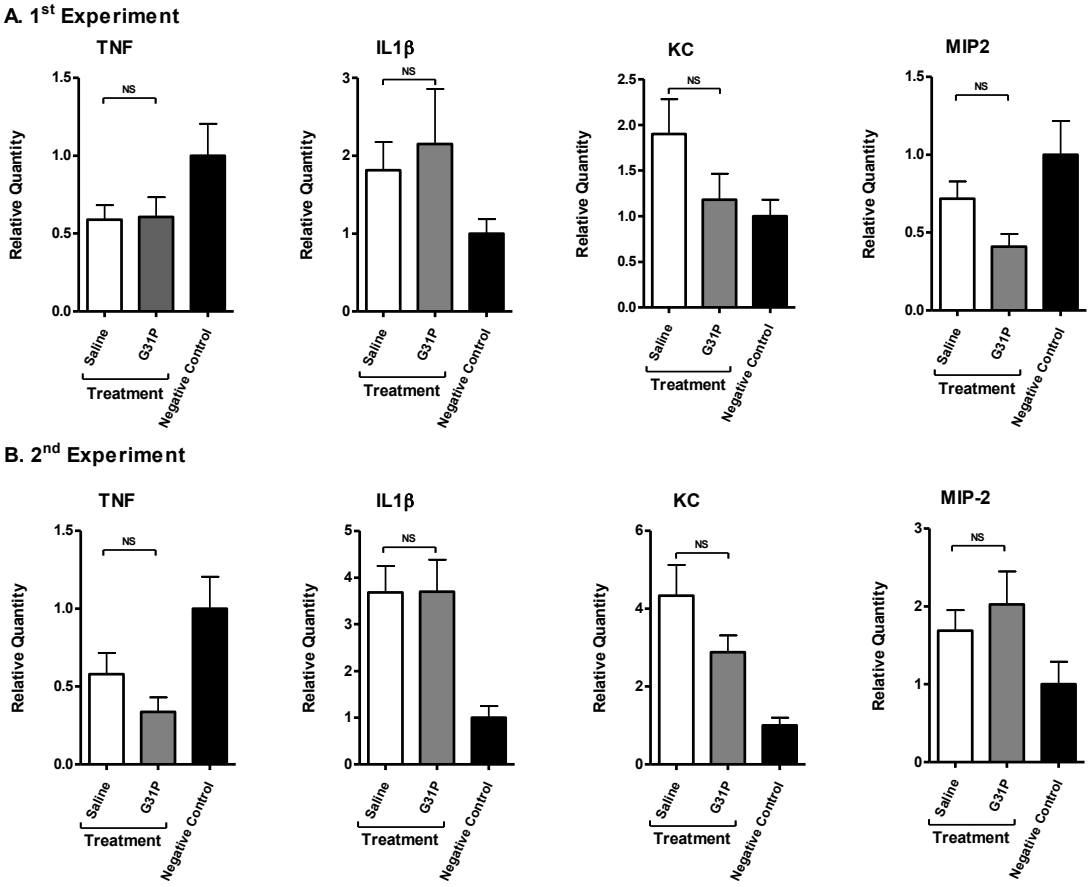



**Figure 4.10 Effects of CXCR1/2 blockade on mRNA expression levels for inflammatory cytokines**. CIA is induced in DBA/1 mice as described in section 3.2 of Materials and Methods. For determination of mRNA expression of IL-1β, TNF, KC, and MIP-2 RNA from each paw was purified and mRNA expression was evaluated using qRT- PCR. The reaction conditions and determination of relative gene expression is described in section 3.6 of the Materials and Methods. Each paw was processed individually and only paws that exhibited arthritic symptoms at some point in the 10 wk experiment were included. Values are mean ± SEM. **A**. Experiment 1: Saline-treated arthritic (n=34), G31P-treated arthritic (n=29), and Negative control (n=20). **B**. Experiment 2: Saline- or G31P-treated arthritic (n=41), and Negative control (n=20). All P values were determined by ANOVA.

### 4.2.5 Serum Levels of anti-G31P Antibody

This is one of the first experiments in our lab where hG31P has been used as a long-term treatment. It is also the first wherein hG31P treatment did not have a substantial and obvious anti-inflammatory impact. Moreover, we were employing human G31P in animals that do not express a murine equivalent of CXCL8. For those reasons we questioned whether or not our mice were producing anti-hG31P Abs. In order to answer this question we developed an ELISA to detect anti-hG31P Ab in serum. We found significant ($P<0.0001$) and very high levels of anti-hG31P Abs in our hG31P-treated CIA mice (Fig. 4.9), while there were negligible levels of anti-hG31P Abs found in our saline-treated CIA mice or our non-arthritic mice. We were also interested to see when this Ab response to hG31P arose in our animals, so we did a time course experiment where we treated DBA/1 mice three times a week with 500 μg/kg of hG31P and took blood samples every two weeks to look for anti-hG31P Ab response (Fig 4.10). We saw quite dramatic results, such that by the second week of treatment with hG31P we observed a fully mature anti-hG31P Ab response.

### 4.2.6 Neutralizing Ability of anti-G31P Antibody

After we determined that there were anti-hG31P Abs found in our hG31P-treated arthritic mice the next step was to establish if these were hG31P-neutralizing Abs. The assay we used to determine this was a $Ca^{2+}$ influx assay. In the $Ca^{2+}$ flux assay human neutrophils are purified and labeled with the $Ca^{2+}$ indicator dye fluo-4 AM. Before challenge we pre-incubated serum samples from saline-treated and hG31P-treated CIA mice with or without 10 ng/ml hG31P for 2 hours and then added these hG31P-containing serums to the fluo-4 AM-labeled PMNs. The cells were then challenged with 100 ng/ml GROα and the $Ca^{2+}$ flux was monitored for 120 seconds using a microplate spectrofluorometer. We converted the output fluorescent data from to area under the curve values. We had previously shown that hG31P reduces GROα-mediated $Ca^{2+}$ influx (Zhao 2009). Therefore, we were interested in determining whether the anti-hG31P Abs in the serum would prevent hG31P inhibition of the GROα's agonist activities. In this experiment our baseline calcium influx is observed when serum from saline-treated CIA

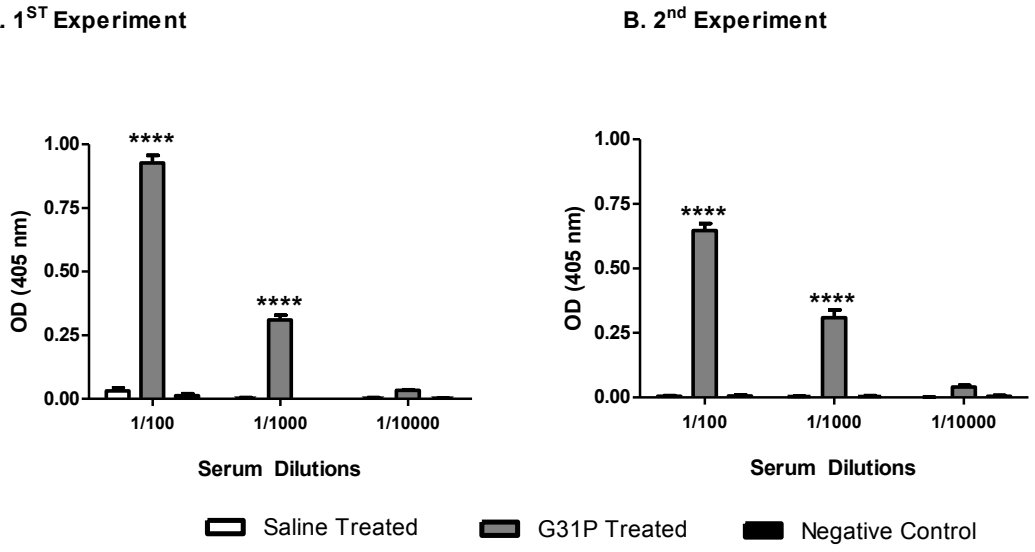



**Figure 4.11 Serum levels of anti-G31P antibody in hG31P-treated CIA mice**. CIA was induced in DBA/1 mice as described in section 3.2 of Materials and Methods. Serum samples were collected at time of harvest and G31P-specific Ab levels were measures by ELISA, as described in section 3.8 of materials and methods. Shows data from 1st experiment (A) and 2nd experiment (B). Values are mean ± SEM. A: Saline-treated (n=10), G31P-treated (n=9), non-arthritic (n=5). B: Saline-treated (n=12), G31P-treated (n=13), non-arthritic (n=5). All P values were determined by ANOVA, ****P<0.0001. Stars indicate P values relative to saline treated and non-arthritic mice.

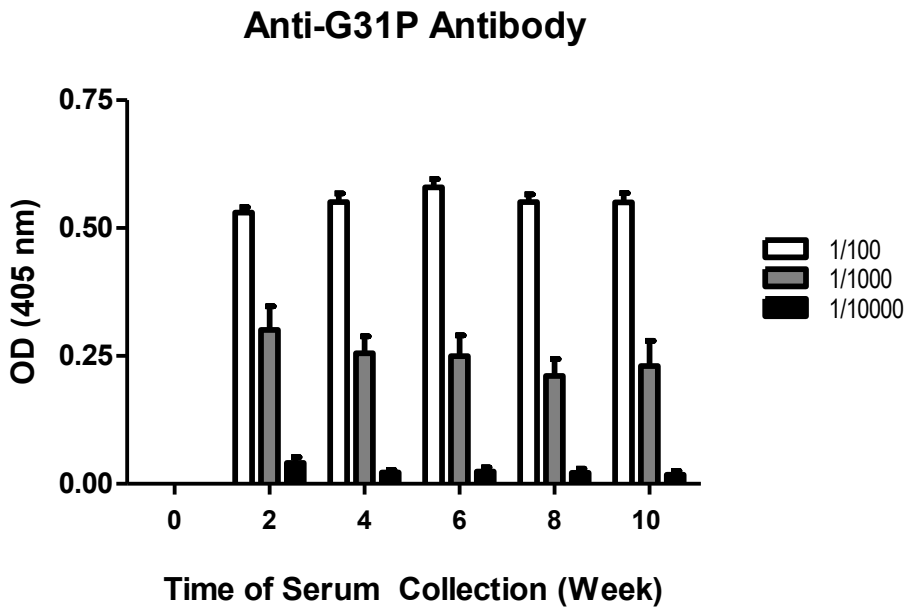



**Figure 4.12 Time course for induction of anti-hG31P antibody responses**. Healthy DBA/1 mice were treated with 500 μg/kg hG31P 3x week for 10 weeks. Serum samples were collected every two weeks until the time of harvest (10 weeks) and hG31P-specific Ab levels were measured by ELISA at 3 different serum dilutions, as previously described. Values are mean ± SEM (n=5).

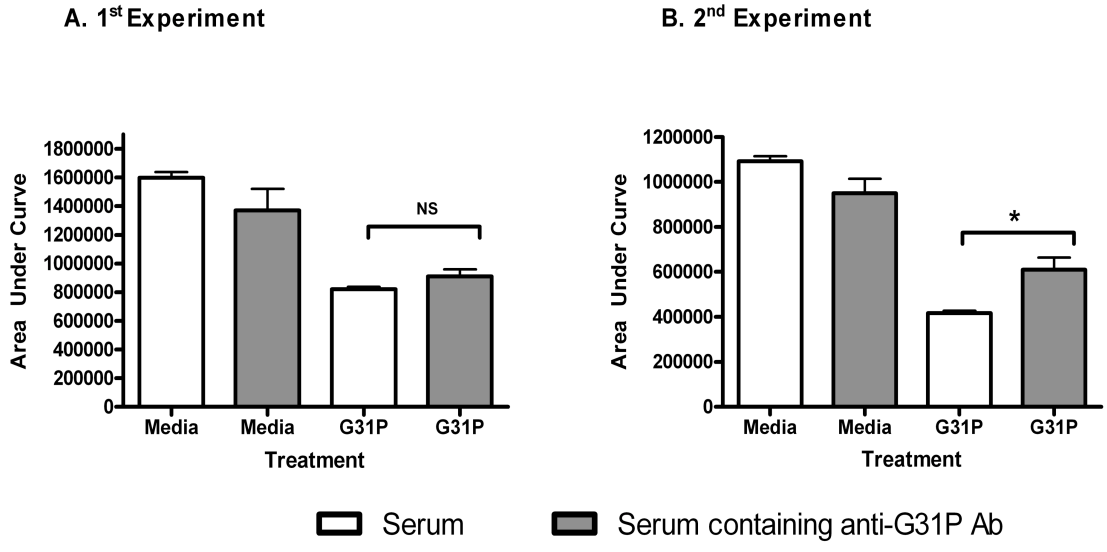



**Figure 4.13 Neutralizing ability of anti-G31P antibody.** CIA was induced in DBA/1 mice as described in section 3.2 of Materials and Methods. Serum samples were collected at the time of harvest from both saline- (serum) and hG31P-treated (Serum containing anti-G31P Ab) CIA mice. For the assay, purified human neutrophils were incubated with the indicated, putatively neutralizing mouse serum (1:250 dilution) +/- hG31P, then challenged with media or agonist (GROα). GRO-induced intracellular $Ca^{2+}$ flux was determined using a spectrofluorometer (emission wavelength 520 nm; excitation wavelength 488 nm). The data is presented as the area under the curve. Values are mean ± SEM for saline- or hG31P-treated (n=6) arthritic or non-arthritic (n=2) mice. Data was not normally distributed, therefore data was ranked and then ANOVA was performed on ranks. All P values were determined by ANOVA (Tukey post-hoc) *$P<0.05$.

mice were incubated with neutrophils. When serum from hG31P-treated CIA mice (containing high levels of anti-hG31P Ab) was incubated with neutrophils in the absence of hG31P the calcium flux within the cells did not decrease significantly from the baseline (in both experiments [Fig 4.11]). When hG31P was incubated with the neutrophils in the presence of serum from saline-treated CIA mice there was a significant decrease in calcium influx (in both experiments; $p>0.05$), due to hG31P's ability to reduce GROα-mediated $Ca^{2+}$ influx. In the first experiment there was no significant difference between the calcium flux induced by serum containing anti-hG31P Abs and serum without Abs (in the presence of hG31P). But, in the second experiment when hG31P is incubated with neutrophils and serum containing the anti-hG31P Ab (from hG31P-treated CIA mice) there was a statistically significant increase in the calcium influx when compared that of saline-treated CIA mice incubated with hG31P ($p>0.05$), which indicates that the effects of hG31P are partially blocked. Therefore, in our 2nd experiment we find a significant increase in calcium influx in hG31P-treated animals, leading us to believe that the anti-hG31P Ab found in the serum of these animals is able to partially neutralize hG31P. This data correlates with our clinical score data. In our 1st experiment we did not find neutralizing anti-hG31P Abs and hG31P was able to statistically significantly reduce clinical scores in mice that developed early onset arthritis (Figure 4.5). Whereas in our 2nd experiment we did find neutralizing anti-hG31P Abs and hG31P was unable to have a statistically significant effect on clinical scores. Therefore, when we do not find neutralizing Abs hG31P was able to have an effect on clinical scores and when we do find neutralizing Abs hG31P was unable to lower clinical scores in arthritic mice.

## 5. DISCUSSION AND CONCLUSION

Our lab has reported that bG31P, hbG31P, and hG31P are all potent antagonists of the ELR$^+$CXC chemokines. They bind both the CXCR1 and the CXCR2, and have been successful in blocking neutrophil infiltration in numerous models, such as in airway endotoxemia in piglets and guinea pigs, aspiration pneumonia in guinea pigs, I/R in rats, prostate cancer in mice, and airway inflammation due to the environmental contaminant benzo(a)pyrene in mice (Gordon 2005; Zhao 2007; Podechard 2008; Gordon 2009; Zhao 2010; Zhao 2010; Liu 2012). Rheumatoid arthritis is characterized by neutrophilic inflammation. Neutrophils can be recruited to sites of inflammation by ELR$^+$CXC chemokines (e.g., IL8), among other agents (e.g., C5a), where the cells release strong effectors of cartilage destruction (Grant 2002; Szekanecz 2003; Cascao 2010). ELR$^+$CXC chemokines mediate their effects through the GPCRs CXCR1 and CXCR2, such that blocking these receptors could significantly diminish neutrophil-related pathology in RA. In the present study we examined the therapeutic activities of human G31P in a mouse model of experimental arthritis.

A previous study had shown that an allosteric inhibitor of CXCR1/CXCR2 had beneficial prophylactic (but also some therapeutic) effects in adjuvant-induced arthritis in mice (Barsante 2008). This highlighted the contributions of ELR+CXC chemokines to the pathophysiology of antigen-induced arthritis that suggested that blocking CXCR1/CXCR2 might be a valid target for treatment of arthritis. Since hG31P not only acts as an antagonist for ELR$^+$CXC chemokines, but also cross-desensitizes other GPCR (e.g., C5aR), we believed that hG31P would have similar, if not more, success than previous CXCR1/CXCR2 inhibitors in arthritis. In this study we did not find conclusive evidence that this was indeed the case, and were unable to prove our hypothesis. At times we did see hG31P having an effect on a couple of the experimental CIA parameters we assessed, but we weren't able to duplicate these results in a subsequent experiment. Thus we do not have evidence to show that hG31P had a therapeutic effect in CIA.

One of the most time-consuming and difficult parts of this research was establishing the CIA model in mice. The CIA model is one of the most widely used

models for RA, as CIA and RA share many pathological and immunological features (Cho 2007; Hegen 2008). We were able to induce CIA in DBA/1 mice, although both the severity and onset of disease varied between mice and the disease incidence was not 100%. We also found different levels of incidence of CIA in DBA/1 mice from different suppliers. Eventually we were able to determine that we had better incidence in mice from Taconic rather than mice from Jackson. We had done several G31P-treatment trials in Jackson mice but the incidence of disease was so low that we were unable to use the outcome data. A future option to alleviate some of the problems with the CIA model could be to increase the number of mice used in the trial, thereby increasing the chance of mice developing CIA. Another option would be to use other animal models of arthritis. Collagen-antibody-induced arthritis (CAIA) uses commercially available monoclonal Abs to CII, which results in disease with a very high incidence as well as a rapid onset of disease (days instead of weeks). Limitations of this model are the high cost of the CII Ab cocktail, and the fact that the rapid onset of disease differs from the chronic processes of RA (Khachigian 2006). The K/BxN model is a genetically-engineered mouse model of arthritis where spontaneous arthritis develops due to auto-Abs to the ubiquitous protein glucose-6-phosphate isomerase. This model shares many features with RA and has a guaranteed high incidence of disease, although a limitation would be the increased cost of the transgenic mice (Ditzel 2004).

One of our initial assessments of the effects of hG31P in CIA was its impact on clinical scores and measurements. In both experiments we were unable to see a significant decrease in both clinical scores and measurements in the hG31P-treated CIA mice (Fig 4.4). Some of the drawbacks of this methodology are in the variability we discovered in the CIA model. The incidence of disease was not 100%, and the mice that developed arthritis did so at varying times after CII sensitization, anywhere from 4 to 9 weeks after the initial CII injection. When we looked at mice that had an earlier onset of arthritis versus those that had a later onset of disease (Fig 4.5) we saw hG31P having a significant effect in one experiment (1$^{st}$ experiment), but we did not observe this in the replicate experiment.

In addition to assessing clinical scores, we looked at serum levels of anti-CII Ab and levels of inflammatory cytokines in the paw tissue of our CIA mice. We saw a

significant reduction in CII-specific IgG1 (Figure 4.6) in CIA mice treated with hG31P in one (2nd experiment), but not both of our experiments. We assayed to see if hG31P would have an effect on B cell responses because hG31P blocks $ELR^+CXC$ chemokine receptors, which should in turn dampen inflammation. Inflammation drives B cell responses (e.g. IL-6 affects B cells), and we know that Ab plays a role in disease, therefore limiting inflammation could in turn affect B cell responses. In the 2nd experiment we also saw a significant reduction in the protein levels of the inflammatory cytokine MIP-2 (Figure 4.7) in hG31P-treated CIA mice. In both experiments we were unable to see any significant effects of hG31P in the expression levels of inflammatory cytokines (Figure 4.8). We had expected after treatment with G31P we might see a reduction in the presence of $ELR^+CXC$ chemokines KC, and MIP-2 as well as the inflammatory cytokines IL-1, TNF-α, and IL-6. G31P, which blocks neutrophil infiltration, should reduce levels of cytokines and chemokines produced by neutrophils, such as IL-1, TNF-α, and IL-6, KC and MIP-2.

The three aforementioned assessments were done at the time of harvest, which was 10 weeks after the mice received their first hG31P treatment and past the peak of disease. We wanted to look at the levels of CII-specific Ab throughout the trial by taking blood from the tail, but were unable to do so due to the deteriorated state of the mice tails as a result of the initial induction of CIA with Freund's complete adjuvant. We would have preferred that blood samples would have been taken throughout the course of treatment with hG31P and particularly at the peak of disease. For the most part we were unable to see any significant differences in either mRNA or protein levels for the inflammatory cytokines. This could have been in part due to the variability of the model. There was significant variability in the severity of CIA between different mice and also between individual paws for each mouse. Due to this variability in the paws we made sure to use the same portion of all 4 paws in each analysis for consistency in our methodology. The lateral portions of the paws were used for histology, the medial proximal portions of all 4 paws were used for protein analysis and the medial distal portions of all 4 paws were used for mRNA analysis. Unfortunately to be able to have a portion of each paw in each analysis we ended up having a small amount of tissue to work with. It is also worth mentioning that since we harvest the mice past the peak of

disease the majority of the paws had a significant reduction in their level of inflammation at time of harvest. So, the variability in the CIA model and our harvesting tissues past the peak of disease are two possible contributing factors for not seeing differences in the levels of these inflammatory mediators between CIA and control mice as well as hG31P-treated and untreated CIA mice.

It is possible the lack of effect of human G31P in CIA was due to the presence of neutralizing anti-hG31P Abs that were found in abundance in the hG31P-treated mice (Figure 4.9). ADAs can be either non-neutralizing Abs, which bind to the biologic (e.g. G31P) without disrupting the interaction with its target (CXCR1 and CXCR2), or neutralizing Abs, which bind to sites that are critical for biologic-target interaction, thereby reducing the biologics activity (Sathish 2013). We assessed the ability of these anti-hG31P Abs to neutralize the activity of hG31P using $Ca^{2+}$ influx assays. In our 1st experiment we did not see any significant changes in our $Ca^{2+}$ flux (Figure 4.11), therefore the Abs found were non-neutralizing Abs. This correlates with our clinical score data where in the same experiment we saw a significant decrease in the score in our hG31P-treated mice that developed arthritis early (< 5 weeks) (Figure 4.5). In our 2nd experiment we saw significant changes in our $Ca^{2+}$ flux assay that indicated that the anti-hG31P Ab found in these animals may have partially neutralized the effects of hG31P (Figure 4.11). The presence of neutralizing Abs in this experiment disrupted the action of hG31P which resulted in no change in clinical scores (Figure 4.5). Therefore, when we found neutralizing Abs we saw no change in clinical scores, and when we found no neutralizing Abs we saw that G31P significantly reduced clinical scores. A possible reason for the difference between our two experiments could be due to the levels of LPS found in the G31P. At the time of my experiment we did not test our G31P for LPS. Since then we have started to test for LPS and we see a great deal of batch to batch variability. Since different batches of G31P were used there could have been varying concentrations of LPS. LPS can act as a B cell adjuvant, therefore if one of our batches of G31P had a high concentration of LPS it would not be surprising that an immune response occurred.

In continuing with this project, there are several potential options to explore. hG31P might have success treating arthritis in an animal model wherein IL-8 is not so

foreign a protein (e.g., guinea pigs, but not mice, express an IL-8 gene), so an Ab response to hG31P wouldn't be as likely to occur. Another possibility would be to create another $ELR^{+}CXC$ chemokine antagonist modeled on a chemokine found in mice, such as KC or MIP-2. Although this is an interesting idea in theory, our long term goal is to use this treatment in therapy for humans, so making a molecule for specific use in mice is not necessarily a favourable idea. Another way to make hG31P a more viable therapy in mouse models of arthritis would be to induce tolerance to hG31P in the mice. Tolerance to OVA has been shown through both oral and i.v. administration of OVA in mice (Kearney 1994; Van Houten 1996). Moreover, our lab has abundant experience with inducing tolerance either through use of tolerogenic dendritic cells or my administration of very low doses of antigen on alum. Therefore, inducing tolerance to G31P in a similar fashion should allow hG31P to be used therapeutically in arthritic mice.

We had plans to include immunohistochemistry and histopathology approaches in this study but were unable to obtain consistent results. These two approaches would have allowed us to look at the presence of neutrophils in our joint tissues. When we were analyzing the paw tissues, sections from non-arthritic mice exhibit normal histological features, with intact articular cartilage and no evidence of inflammation or erosion, whereas arthritic mice showed severe joint damage, as evidenced by cartilage damage, bone erosion, pannus formation, and infiltration of inflammatory cells. We developed a scoring method to assess degrees of joint damage by looking at infiltration of cells, cartilage damage and bone erosion. Using immunohistochemistry we also attempted to use a biotinylated anti-neutrophil Gr-1 Ab to identify neutrophils in paw tissues, but we had a great deal of non-specific staining occurring. In addition, we were unable to consistently get good quality sections. It was difficult to consistently get sections that included the joint and maintained the tissue architecture required to properly visualize changes occurring in the joints. Another option that could be used to quantify neutrophils would be an MPO assay, where MPO is a lysosomal protein stored in the azurophilic granules of the neutrophil.

In conclusion, although at this time we were unable to show evidence of the therapeutic effects of human G31P in our mice we believe we have set up a good foundation for any future investigation in this area and that with further refinement of this

project we have the potential to explore the effects of G31P in a model of experimental arthritis. Ideally one would want to diminish the possible adverse response against G31P by one of the above mentioned possibilities, more than likely the most effective method being the induction of tolerance to G31P. We would also want to test the LPS content in our G31P and use a batch that has a low concentration, as that might be contributing to our anti-G31P Ab response. Harvesting at an earlier time point might also be beneficial in terms of seeing differences in our experimental parameters, such as cytokine levels. It would also be ideal to get a clearer picture of the neutrophils' role in our model of arthritis and its subsequent response to G31P. To do this one would have to refine our initial histochemistry methods to get a more reliable method to assess both the infiltration of cells into the joint, specifically neutrophils, as well as the changes in joint architecture that occur.

## 6. LIST OF REFERENCES


Aarvak, T., and Natvig, J.B. (2001). "Cell-cell interactions in the synovitis. Antigen presenting cells and T cell interation in rheumatoid arthritis." Arthritis Research **3**: 13-17.

Abramson, S. B., and Amin, A. (2002). "Blocking the effects of IL-1 in rheumatoid arthritis protects bone and cartilage." Rheumatology **41**: 972-980.

Afonso, V., Champy, R., Mitrovic, D., et. al. (2007). "Reactive oxygen species and superoxide dismutase: role in joint diseases." Joint Bone Spine **74**: 324-329.

Agarwal, V., and Malaviya, A.N. (2005). "Cytokine network and its manipulation in rheumatoid arthritis." The Journal of the Association of the Physicians of India **13**: 86-91.

Ainola, M. M., Mandelin, J.A., and Liljestrom, M.P., et. al. (2005). "Pannus invasion and cartilage degradation in rheumatoid arthritis: Involvement of MMP-3 and interleukin-1-beta." Clinical and Experimental Rheumatology **23**: 644-650.

Alamanos, Y., Drosos, A.A. (2005). "Epidemiology of adult rheumatoid arthritis." Autoimmunity Reviews **4**: 130-136.

Alawadhi, A., Alawneh, K., and Alzahrani, Z.A. (2012). "The effect of neutralizing antibodies on the sustainable efficacy of biologic therapies: what's in it for African and Middle Eastern rheumatologists." Clinical Rheumatology **31**: 1281-1287.

Albelda, S. M., Smith, C.W., Ward, P.A. (1994). "Adhesion molecules and inflammatory injury." The Journal of the Federation of American Societies for Experimental Biology **8**: 504-512.

Ali, H., Richardson, R.M., Haribabu, B., and Snyderman, R. (1999). "Chemoattractant receptor cross-desensitization." The Journal of Biological Chemistry **274**: 6027-6030.

Ali, S., and Mann, D.A. (2004). "Signal transduction via NF-kB pathway: a targeted treatment modality for infection, inflammation and repair." Cell Biochemistry and Function **22**: 67-79.

Allen, S. J., Crown, S.E., and Handel, T.M. (2007). "Chemokine: receptor, structure, interactions and antagonism." Annual Review of Immunology **25**: 787-820.

Alvaro-Gracia, J. M., Zvaifler, N.J., Brown, C.B., et. al. (1991). "Cytokines in chronic inflammatory arthritis VI. Analysis of the synovial cells involved in granulocyte-macrophage colony-stimulating factor production and gene expression in rheumatoid arthritis and its regulation by IL-1 and tumor necrosis factor-alpha." Journal of Immunology **146**: 3365-3371.

Ambruso, D. R., and Johnston, R.B. (1981). "Lactoferrin enhances hydroxyl radical production by human neutrophils, neutrophil particulate fractions, and an enzymatic generating system." The Journal of Clinical Investigations **67**: 352-360.

Amin, A. R., Attur, M., and Abramson, S.B., et. al. (1999). "Nitric oxide synthase and cyclooxygenases: distribution, regulation, and intervention in arthritis." Current Opinion in Rheumatology **11**: 202-209.

Andreani, M., Olivier, J.L., Berenbaum, F., et. al. (2000). "Transcriptional regulation of inflammatory secreted phospholipases $A_2$." Biochimica et Biophysica Acta **1488**: 149-158.

Aratani, Y., Koyama, H., Nyui, S., et. al. (1999). "Severe impairment in early host defense against *candida albicans* in mice deficient in myeloperoxidase." Infection and Immunity **67**: 1828-1836.

Arner, E. C., Decicco, C.P., Cherney, R., et. al. (1997). "Cleavage of native cartilage aggrecan by neutrophil collagenase (MMP-8) is distinct from endogenous cleavage by aggreganase." The Journal of Biological Chemistry **272**: 9294-9299.

Badolato, R., and Oppenheim, J.J. (1996). "Role of cytokines, acute-phase proteins, and chemokines in the progression of rheumatoid arthritis." Seminars in Arthritis and Rheumatism **26**: 526-538.

Baggiolini, M., Loetscher, P., and Moser, B. (1995). "Interleukin-8 and the chemokine family." International Society for Immunopharmacology **17**: 103-108.

Baggiolini, M., Walz, A., and Kunkel, S.L. (1989). "Neutrohil-activating peptide-1/interleukin-8, a novel cytokine that activates neutrophils." Journal of Clinical Investigation **84**: 1045-1049.

Bainton, D. F., Ullyot, J.L., and Farquhar, M.G. (1971). "The development of neutrophilic polymorphonuclear leukocytes in human bone marrow." Journal of Experimental Medicine **134**: 907-934.

Balsinde, J., Diez, E., Schuller, A., et. al. (1988). "Phospholipase $A_2$ activity in resting and activated human neutrophils." The Journal of Biological Chemistry **263**: 1929-1936.

Barksby, H. E., Milner, J.M., Patterson, A.M., et. al. (2006). "Matrix metalloproteinase 10 promotion of collagenolysis via procollagenase activation." Arthritis and Rheumatism **54**: 3244-3253.

Barsante, M. M., Cunha, T.M., Allegretti, M., et. al. (2008). "Blockade of the chemokine receptor CXCR2 ameliorates adjuvant-induced arthritis in rats." British Journal of Pharmacology **153**: 992-1002.

Bartok, B., and Firestein, G.S. (2010). "Fibroblast-like synoviocytes: key effector cells in rheumatoid arthritis." Immunology Review **233**: 233-255.

Begovich, A. B., Carlton, V.E.H., Honigberg, L.A., et. al. (2004). "A missesnse single-nucleotide polymorphism in a gene encoding a protein tyrosine phosphatase (PTPN22) is associated with rheumatoid arthritis." American Journal of Human Genetics **75**: 330-337.

Belperio, J. A., Keane, M.P., Arenberg, D.A., et. al. (2000). "CXC chemokines in angiogenesis." Journal of Leukocyte Biology **68**: 1-8.

Ben-Baruch, A., Michiel, D., and Oppenheim, J.J. (1995). "Signals and receptors involved in the recruitment of inflammatory cells." The Journal of Biological Chemistry **270**: 11703-11706.

Bento, A. F., Leite, D.F.P., Claudino, R.F., et. al. (2008). "The selective nonpeptide CXCR2 antagonist SB225002 ameliorates acute experimental colitis in mice." Journal of Leukocyte Biology **84**: 1213-1221.

Berenbaum, F., Jacques, C., Thomas, G., et. al. (1996). "Synergistic effect of interleukin -1β and tumor necrosis factor α on $PGE_2$ production by articular chondrocytes does not involve $PLA_2$ stimulation." Experimental Cell Research **222**: 379-384.

Berliner, N. (1998). "Molecular biology of neutophil differentiation." Current Opinion in Hematology **5**: 49-53.

Bertini, R., Allegretti, M., Bizzarri, C., et. al. (2004). "Noncompetitive allosteric inhibitors of the inflammatory chemokine receptors CXCR1 and CXCR2: prevention of reperfusion injury." Proceedings of the National Academy of Sciences **101**: 11791-11796.

Bhatia, M., Brady, M., Zagorski, J., et. al. (2000). "Treatment with neutralizing antibody against cytokine induced neutrophil chemoattractant (CINC) protects rats against acute pancreatitis associated lung injury." Gut **47**: 838-844.

Biagioni, C., Favilli, F., Catarzi, S., et. al. (2006). "Redox state and $O_2$ production in neutrophils of crohn's disease patients " Experimental Biology and Medicine **231**: 186-195.

Bizzarri, C., Beccari, A.R., Bertini, R., et. al. (2006). "$ELR^+$CXC chemokines and their receptors (CXC chemokine receptor 1 and CXC chemokine receptor 2) as new therapeutic targets." Pharmacology and Therapeutics **112**: 139-149.

Borchers, A. T., Keen, C.L., Cheema, G.S., et. al. (2004). "The use of methotrexate in rheumatoid arthritis." Seminars in Arthritis and Rheumatism **34**: 465-483.

Borish, L., and Steinke, J.W. (2003). "Cytokines and chemokines." Journal of Allergy and Clinical Immunology **111**: s441-s445.

Borregaard, N. (2010). "Neutrophils, from marrow to microbes." Immunity **33**: 657-670.

Borregaard, N., Lollike, K., Kjeldsen, L., et. el. (1993). "Human neutrophil granules and secretory vesicles." European Journal of Haematology **51**: 187-198.

Borregaard, N., Sorensen, O.E. and Theilgaard-Monch, K. (2007). "Neutrophil granules: a library of innate immunity proteins." Trends in Immunology **28**: 340-345.

Brand, D. D., Latham, K.A., and Rosloniec, E.F. (2007). "Collagen-induced arthritis." Nature Protocols **2**: 1269-1275.

Breedveld, F. C., and Dayer, J.M. (2000). "Leflunomide: mode of action in the treatment of rheumatoid arthritis." Annals of the Rheumatic Diseases **59**: 841-849.

Brown, K. A. (1988). "The polymorphonuclear cell in rheumatoid arthritis." British Journal of Rheumatology **27**: 150-155.

Brown, L. S., Greene, M.H., Gershon, S.K., et. al. (2002). "Tumor necrosis factor antagonist therapy and lymphoma development: twenty-six cases reported to the Food and Drug Administration." Arthritis and Rheumatism **46**: 3151-3158.

Bucala, R., Ritchlin, C., Winchester, R., et. al. (1991). "Constitutive production of inflammatory and mitogenic cytokines by rheumatoid synovial fibroblasts." Journal of Experimental Medicine **173**: 569-574.

Buch, M., and Emery, P. (2002). "The aetiology and pathogenesis of rheumatoid arthritis." Hospital Pharmacist **9**: 5-10.

Buch, M. H., Vital, E.M., and Emery, P. (2008). "Abatacept in the treatment of rheumatoid arthritis." Arthritis Research and Therapy **10**: (Suppl 1): S5.

Bugatti, S., Codullo, V., Caporali, R., et. al. (2007). "B cells in rheumatoid arthritis." Autoimmunity Reviews **6**: 482-487.

Burrage, P. S., Mix, K.S., and Brinckerhoff, C.E. (2006). "Matrix metalloproteinases: Role in arthritis." Frontiers in Bioscience **11**: 529-543.

Busch-Petersen, J. (2006). "Small molecule antagonists of the CXCR2 and CXCR1 chemokine receptors as therapeutic agents for the treatment of inflammatory diseases." Current Topics in Medical Chemistry **6**: 1345-1352.

Cascao, R., Rosario, H.S., Souto-Carneiro, M.M., et. al. (2010). "Neutrophils in rheumatoid arthritis: more than simple effectors." Autoimmunity Reviews **9**: 531-535.

Cawston, T. E. (1996). "Metalloproteinase inhibitors and the prevention of connective tissue breakdown." Pharmacology and Therapeutics **70**: 163-182.

CDC (2004). "Tuberculosis associated with blocking agents against tumor necrosis fator-alpha -- California, 2002-2003." Morbidity and Mortality Weekly Report **53**: 683-686.

Cedergren, J., Forslund, T., Sundqvist, T., et. al. (2002). "Inducible nitric oxide synthase is expressed in synovial fluid granulocytes." Clinical and Experimental Immunology **130**: 150-155.

Chapman, R. W., Minnicozzi, M., Celly, C.S., et. al. (2007). "A novel, orally active CXCR1/2 receptor antagonist, Sch527123, inhibits neutrophil recruitment, mucus production, and goblet cell hyperplasia in animal models of pulmonary inflammation." The Journal of Pharmacology and Experimental Therapeutics **322**: 486-493.

Chen, M., Lam, B.K., Kanaoka, Y., et. al. (2006). "Neutrophil-derived leukotriene $B_4$ is required for inflammatory arthritis." The Journal of Experimental Medicine **203**: 837-842.

Cho, Y.-G., Cho, M-L., Min, S-Y, and et. al. (2007). "Type II collagen autoimmunity in a mouse model of human rheumatoid arthritis." Autoimmunity Reviews **7**: 65-70.

Chomarat, P., Rissoan, M.C., Pin, J.J., et. al. (1995). "Contribution of IL-1, CD14, and CD13 in the increased IL-6 production induced by in vitro monocyte-synoviocyte interactions." Journal of Immunology **155**: 3645-3652.

Choy, E. H., and Panayi, G.S. (2001). "Cytokine pathways and joint inflammation in rheumatoid arthritis." The New England Journal of Medicine **344**: 907-916.

Christodoulou, C., and Choy, E.H. (2006). "Joint inflammation and cytokine inhibition in rheumatoid arthritis." Clinical and Experimental Medicine **6**(1): 13-19.

Clark-Lewis, I., Dewald, B., Loetscher, M., et. al. (1994). "Structural requirements for interleukin-8 function identified by design of analogs and CXC chemokine hybrids " The Journal of Biological Chemistry **269**: 16075-16081.

Clark-Lewis, I., Kim, K.S., Rajarathnam, K., et. al. (1995). "Structure-activity relationship of chemokines." Journal of Leukocyte Biology **57**: 703-711.

Clark-Lewis, I., Schumacher, C., Baggiolini, M., et. al. (1991). "Structure-activity relationships of interleukin-8 determined using chemically synthesized analogs." The Journal of Biological Chemistry **266**: 23128-23134.

Clemens, R. A., Newbrough, S.A., Chung, E.Y., et. al. (2004). "PRAM-1 is required for optimal integrin-dependent neutrophil function." Molecular and Cellular Biology **24**: 10923-10932.

Cloutier, A., Ear, T., Blais-Charron, E., et. al. (2007). "Differential involvement of NF-κB and MAP kinase pathways in the generation of inflammatory cytokines by human neutorphils." Journal of Leukocyte Biology **81**: 567-577.

Cloutier, A., Guindi, C., Larivee, P., et. al. (2009). "Inflammatory cytokine production by human neutrophils involves C/EBP transcription factors." The Journal of Immunology **182**: 563-571.

Coelho, F. M., Pinho, V., Amaral, F.A., et. al. (2008). "The chemokine receptors CXCR1/CXCR2 modulate antigen-induced arthritis by regulating adhesion of neutrophils to the synovial microvasculature." Arthritis and Rheumatism **58**: 2329-2337.

Coenen, M. J. H., and Gregersen, P.K. (2009). "Rheumatoid arthritis: a view of the current genetic landscape." Genes and Immunity **10**: 101-111.

Combe, B. (2009). "Progression in early rheumatoid arthritis." Best Practice and Research Clinical Rheumatology **23**: 59-69.

Cornish, A. L., Campbell, I.K., McKenzie, B.S., et. al. (2009). "G-CSF and GM-CSF as therapeutic targets in rheumatoid arthritis." Nature Reviews Rheumatology **5**: 554-559.

Crofford, L. J., Wilder, R.L., Ristimaki, A.P., et. al. (1994). "Cyclooxygenase-1 and -2 expression in rheumatoid synovial tissues. Effects of interleukin-1β, phorbol ester, and corticosteroids." Journal of Clinical Investigation **93**: 1095-1101.

Cross, A., Barnes, T., Bucknall, R.C., et. al. (2006). "Neutrophil apoptosis in rheumatoid arthritis is regulated by local oxygen tensions within joints." Journal of Leukocyte Biology **80**: 521-528.

Cunha, T. M., Barsante, M.M., Guerrero, A.T., et. al. (2008). "Treatment with DF 2162, a non-competitive allosteric inhibitor of CXCR1/2, diminishes neutrophil influx and inflammatory hypernociception in mice." British Journal of Pharmacology **154**: 460-470.

Cunnane, G., FitzGerald, O., Hummel, K.M., et. al. (1999). "Collagenase, cathepsin B and cathepsin L gene expression in the synovial membrane of patients with early inflammtory arthritis." Rheumatology **38**: 34-42.

Cunnane, G., FitzGerald, O., Hummel, K.M., et. al. (2001). "Synovial tissue protease gene expression and joint erosion in early rheumatoid arthritis." Arthritis and Rheumatism **44**: 1744-1753.

Cutolo, M. (1997). "Do sex hormones modulate the synovial macrophages in rheumatoid arthritis?" Annals of the Rheumatic Diseases **56**: 281-286.

Dahlgren, C., and Karlsson, A. (1999). "Respiratory burst in human neutrophils." Journal of Immunological Methods **232**: 3-14.

Dale, D. C., Boxer, L., and Liles, W.C. (2008). "The phagocytes: neutrophils and monocytes." Blood **112**: 935-945.

Davidson, E. M., Rae, S.A., and Smith, M.J.H. (1983). "Leukotriene $B_4$, a mediator of inflammation present in synovial fluid in rheumatoid arthritis." Annals of the Rheumatic Diseases **42**: 677-679.

Deighton, C. M., Walker, D.J., Griffiths, I.D., et. al. (1989). "The contribution of HLA to rheumatoid arthritis." Clinical Genetics **36**: 178-182.

Deng, G., Lenardo, M. (2006). "The role of immune cells and cytokines in the pathogenesis of rheumatoid arthritis." Drug Discovery Today: Disease Mechanisms **3**: 163-168.

Ditzel, H. J. (2004). "The K/BxN mouse: a model of human inflammatory arthritis." TRENDS in Molecular Medicine **10**: 40-45.

Doan, T., and Massarotti, E. (2005). "Rheumatoid arthritis: an overview of new and emerging therapies." Journal of Clinical Pharmacology **45**: 751-762.

Dore, R. K., Mathews, S., Schechtman, J., et. al. (2007). "The immunogenicity, safety, and efficacy of etanercept liquid administered once weekly in patients with rheumatoid arthritis." Clinical and Experimental Rheumatology **25**: 40-46.

Dorner, T. (2006). "Crossroads of B cell activation in autoimmunity: Rationale of targeting B cells." Journal of Rheumatology **33**: 3-11.

Downey, G. P. (1994). "Mechanisms of leukocyte motility and chemotaxis." Current Opinion in Immunology **6**: 113-124.

Drakesmith, H., Chain, B., and Beverley, P. (2000). "How can dendritic cells cause autoimmune disease?" Immunology Today **21**(5): 214-217.

Duffy, M. J., Maguire, T.M., Hill, A., et. al. (2000). "Metalloproteinases: role in breast carcinogenesis, invasion and metastasis." Breast Cancer Research **2**: 252-257.

Edwards, S. W., and Hallett, M.B. (1997). "Seeing the wood for the trees: the forgotten role of neutrophils in rheumatoid arthritis." Immunology Today **18**: 320-324.

Elford, P. R., and Cooper, P.H. (1991). "Induction of neutrophil-mediated cartilage degradation by interleukin-8." Arthritis and Rheumatism **34**: 325-332.

Erdem, H., Pay, S., Serdar, M., et. al. (2005). "Different ELR(+) angiogenic CXC chemokine profiles in synovial fluid of patients with Behcet's disease, familial mediterranean fever, rheumatoid arthritis, and osteoarthritis." Rheumatology International **26**: 162-167.

Fan, X., Patera, A.C., Pong-Kennedy, A., et. al. (2007). "Murine CXCR1 is a functional receptor for GCP-2/CXCL6 and interleukin-8/CXCL8." The Journal of Biological Chemistry **282**: 11658-11666.

Farrell, A. J., Blake, D.R., Palmer, R.M.J., et. al. (1992). "Increased concentrations of nitrite in synovial fluid and serum samples suggest increased nitric oxide synthesis in rheumatic diseases." Annals of the Rheumatic Diseases **51**: 1219-1222.

Faurschou, M., and Borregaard, N. (2003). "Neutrophil granules and secretory vesicles in inflammtion." Microbles and Infection **5**: 1317-1327.

Fava, R. A., Olsen, N.J., Spencer-Green, G., et. al. (1994). "Vascular-permeability factor endothelial growth-factor (VPF/VEGF) : accumulation and expression in human synovial fluids and rheumatoid sunovial tissue." Journal of Experimental Medicine **180**: 341-346.

Feely, M. G., Erickson, A., and O'Dell, J.R. (2009). "Therapeutic options for rheumatoid arthritis." Expert Opinion on Pharmacotherapy **10**: 2095-2106.

Feldmann, M., Brennan, F.M., and Maini, R.N. (1996). "Role of cytokines in rheumatoid arthritis." Annual Review of Immunology **14**: 397-440.

Feniger-Barish, R., Ran, M., Zaslaver, A., et. al. (1999). "Differential modes of regulation of CXC chemokine-induced internalization and recycling of human CXCR1 and CXCR2." Cytokine **11**: 996-1009.

Fernandez, E. J., and Lolis, E. (2002). "Structure, function and inhibiton of chemokines." Annual Review of Pharmacology and Toxicology **42**: 469-499.

Fialkow, L., Wang, Y., and Downey, G.P. (2007). "Reactive oxygen and nitrogen species as signaling molecules regulating neutrophil function." Free Radical Biology and Medicine **42**: 153-164.

Filippin, L. I., Vercelino, R., Marroni, N.P., et. al. (2008). "Redox signalling and the inflammatory response in rheumatoid arthritis." Clinical and Experimental Immunology **152**: 415-422.

Firestein, G. S. (2003). "Evolving concepts of rheumatoid arthritis." Nature **423**: 356-361.

Firestein, G. S., and Zvaifler, N.J. (2002). "How important are T cells in chronic rheumatoid synovitis? II. T cell-independent mechanisms from beginning to end." Arthritis and Rheumatism **46**(2): 298-308

Folkerts, G., Kloek, J., Muijsers, R.B.R., et. al. (2001). "Reactive nitrogen and oxygen species in airway inflammation." European Journal of Pharmacology **429**: 251-262.

Fox, D. A., Gizinski, A., Morgan, R., et. al. (2010). "Cell-cell interactions in rheumatoid arthritis synovium." Rheumatic Disease Clinics of North America **36**: 311-323.

Fox, R. I. (1993). "Mechanism of action of hydroxychloroquine as an antirheumatic drug." Seminars in Arthritis and Rheumatism **23**: 82-91.

Furst, D. E. (1996). "Rational disease-modifying anti-rheumatic drug (DMARD) therapy: is it possible?" British Journal of Rheumatology **35**: 707-710.

Furst, D. E. (2004). "Anakinra: Review of recombinant human interleukin-1 receptor antagonist in the treatment of rheumatoid arthritis." Clinical Therapeutics **26**: 1960-1975.

Furze, R. C., and Rankin, S.M. (2008). "The role of the bone marrow in neutrophil clearance under homeostatic conditions in the mouse." The Journal of the Federation of American Societies for Experimental Biology **22**: 3111-3119.

Garrett, I. R., Boyce, B.F., Oreffo, R.O., et. al. (1990). "Oxygen-derived free radicals stimulate osteoclastic bone resorption in rodent bone in vitro and in vivo." The Journal of Clinical Investigations **85**: 632-639.

Gernez, Y., Tirouvanziam, R., and Chanez, P. (2010). "Neutrophils in chronic inflammatory airway diseases: can we target them and how?" European Respiratory Journal **35**: 467-469.

Gevrey, J., Isaac, B.M., and Cox, D. (2005). "Sky is required for monocyte/macrophage chemotaxis to CX3CL1 (fractalkine)." Journal of Immunology **175**: 3737-3745.

Gheorghe, K. R., Korotkova, M., Catrina, A.I., et. al. (2009). "Expression of 5-lipoxygenase and 15-lipoxygenase in rheumatoid arthritis synovium and effects of intraarticular glucocorticoids " Arthritis Research and Therapy **11**: R83.

Gierut, A., Perlman, H., and Pope, R.M. (2010). "Innate immunity and rheumatoid arthritis." Rheumatic Disease Clinics of North America **36**: 271-296.

Goldbach-Mansky, R., Lee, J., McCoy, A., et al. (2000). "Rheumatoid arthritis associated autoantibodies in patients with synovitis of recent onset." Arthritis Research **2**: 236-243.

Goldring, M. B., and Marcu, K.B. (2009). "Catilage homeostasis in health and rheumatic diseases." Arthritis Research and Therapy **11**: 224.

Goldring, S. R. (2003). "Pathogenesis of bone and cartilage destruction in rheumatoid arthritis." Rheumatology **42**: ii11-ii16.

Gonsiorek, W., Fan, X., Hesk, D., et. al. (2007). "Pharmacological characterization of Sch527123, a potent allosteric CXCR1/CXCR2 antagonist " The Journal of Pharmacology and Experimental Therapeutics **322**: 477-485.

Goodacre, J. A., and Pearson, J.P. (1992). "Human cartilage proteoglycans as T cell autoantigens." Annals of the Rheumatic Diseases **51**: 1094-1097.

Gordon, J. R., Li, F., Zhang, X., et. al. (2005). "The combined CXCR1/CXCR2 antagonist CXCL8(3-74)K11R/G31P blocks neutrophil infiltration, pyrexia, and pulmonary vascular pathology in endotoxemic animals." Journal of Leukocyte Biology **78**: 1265-1272.

Gordon, J. R., Zhang, X., Li, F., et. al. (2009). "Amelioration of pathology by ELR-CXC chemokine antagonism in a swine model of airway endotoxin exposure." Journal of Agromedicine **14**: 235-241.

Gorman, C. L., and Cope, A.P. (2008). "Immune-mediated pathways in chronic inflammatory arthritis." Best Practice and Research Clinical Rheumatology **22**: 221-238.

Goronzy, J. J., and Weyland, C.M. (2005). "Rheumatoid arthritis." Immunological Reviews **204**: 55-73.

Grant, E. P., Picarella, D., Burwell, T., et. al. (2002). "Essential Role for the C5a Receptor in Regulating the Effector Phase of Synovial Infiltration and Joint Destruction in Experimental Arthritis." Journal of Experimental Medicine **196**: 1461-1471.

Gravallese, E. M., and Goldring, S.R. (2000). "Cellular mechanisms and the role of cytokines in bone erosion in rheumatoid arthritis." Arthritis and Rheumatism **43**: 2143-2151.

Gravallese, E. M., Manning, C., Tsay, A., et. al. (2000). "Syovial tissue in rheumatoid arthritis is a source of osteoclast differentiation factor." Arthritis and Rheumatism **43**: 250-258.

Greenwald, R. A., and Moak, S.A. (1986). "Degradation of hyaluronic acid by polymorphonuclear leukocytes." Inflammation **10**: 15-30.

Gregersen, P. K. (2005). "Gaining insight into PTPN22 and autoimmunity." Nature Genetics **37**: 1300-1302.

Gregersen, P. K., Sliver, J., and Winchester, R.J. (1987). "The shared epitope hypothesis. An approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis." Arthritis and Rheumatism **30**: 1205-1213.

Grillet, B., Dequeker, J., Paemen, L., et. al. (1997). "Gelatinase B in chronic synovitis: Immunolocalization with a monoclonal antibody." British Journal of Rheumatology **36**: 744-747.

Guerne, P., Zuraw, B.L., Vaughan, J.H., et. al. (1989). "Synovium as a source of interleukin 6 in vitro." Journal of Clinical Investigation **83**: 585-592.

Hall, T. J., Schaeublin, M., Jeker, H., et. al. (1995). "The role of reactive oxygen intermediates in osteoclastic bone resorption " Biochemical and Biophysical Research Communications **207**: 280-287.

Halliwell, B. (1995). "Oxygen radicals, nitric oxide and human inflammatory joint disease." Annals of the Rheumatic Diseases **54**: 505-510.

Hamann, J., Wishaupt, J.O., Vanlier, R.A.W., et. al. (1999). "Expression of the activation antigen CD97 and its ligand CD55 in rheumatoid synovial tissue." Arthritis and Rheumatism **42**: 650-658.

Hammond, M. E. W., Shyamala, V., Siani, M.A., et. al. (1996). "Receptor recognition and specificity of interleukin-8 is determined by residues that cluster near a surface-accessible hydrophobic pocket." The Journal of Biological Chemistry **271**: 8228-8235.

Hampton, M. B., Kettle, A.J., and Winterbourn C.C. (1998). "Inside the neurtrophil phagosome: oxidants, myeloperoxidase, and bacterial killing." Blood **92**: 3007-3017.

Hanemaaijer, R., Sorsa, T., Konttinen, Y.T., et. al. (1997). "Matrix metalloproteinase-8 is expressed in rheumatoid synovial fibroblasts and endothelial cells." The Journal of Biological Chemistry **272**: 31504-31509.

Harada, A., Sekido, N., Akahoshi, T., et. al. (1994). "Essential involvement of interleukin-8 (IL-8) in acute inflammation." Journal of Leukocyte Biology **56**: 559-564.

Haringman, J. J., Ludikhuize, J., and Tak, P.P. (2004). "Chemokines in joint disease: the key to inflammation?" Annals of the Rheumatic Diseases **63**: 1186-1194.

Harrison, B. J. (2002). "Influence of cigarette smoking on disease outcome in rheumatoid arthritis." Current Opinion in Rheumatology **14**: 93-97.

Hashimoto, Y., Kakegawa, H., Narita, Y., et. al. (2001). "Significance of cathepsin B accumulation in synovial fluid of rheumatoid arthritis." Biochemical and Biophysical Research Communications **283**: 334-339.

Hatano, Y., Kasama, T., Iwabuchi, H., et. al. (1999). "Macrophage inflammatory protein 1 alpha expression by synovial fluid neutrophils in rheumatoid arthritis." Annals of the Rheumatic Diseases **58**: 297-302.

Hayashida, K., Shimaoka, Y., Ochi, T., et. al. (2000). "Rheumatoid arthritis synovial stromal cells inhibit apoptosis and up-regulate Bcl-xL expression by B cells in a CD49/CD29-CD106-dependent mechanism." The Journal of Immunology **164**: 1110-1116.

Hebert, C. A., Vitangcol, R.V., and Baker, J.B. (1991). "Scanning mutagenesis of interleukin-8 identifies a cluster of residues required for receptor binding." The Journal of Biological Chemistry **266**: 18989-18994.

Hegen, M., Keith Jr, J.C., Collins, M., et. al. (2008). "Utility of animal models for identification of potential therapeutics for rheumatoid arthritis." Annals of the Rheumatic Diseases **67**: 1505-1515.

Heller, A., Koch, T., Schmeck, J., et. al. (1998). "Lipid mediators in inflammatory disorders." Drugs **55**: 487-496.

Henrotin, Y., Kurz, B., and Aigner, T. (2005). "Oxygen and reactive oxygen species in cartilage degradation: friends or foes?" Osteoarthritis and Cartilage **13**: 643-654.

Henrotin, Y. E., Bruckner, P., and Pujol, JP.L. (2003). "The role of reactive oxygen species in homeostasis and degradation of cartilage." Osteoarthritis and Cartilage **11**: 747-755.

Henrotin, Y. E., DeGroote, D.D., Labasse, A.H., et. al. (1996). "Effects of exogenous IL-1β, TNFα, IL-6, IL-8 and LIP on cytokine production by human articular chondrocytes." Osteoarthritis and Cartilage **4**: 163-173.

Hikiji, H., Takato, T., Shimizu, T., et. al. (2008). "The roles of protanoids, leukotrienes, and platelet-activating factor in bone metabolism and disease." Progress in Lipid Research **47**: 107-126.

Hitchon, C. A., and El-Gabalawy, H.S. (2004). "Oxidation in rheumatoid arthritis." Arthritis Research and Therapy **6**: 265-278.

Hla, T., Bishop-Bailey, D., Liu, C.H., et. al. (1999). "Cyclooxygenase-1 and -2 isoenzymes." The International Journal of Biochemistry & Cell Biology **31**: 551-557.

Hogan, M., Sherry, B., Ritchlin, C., et. al. (1994). "Differential expression of the small inducible cytokines GROα and GROβ by synovial fibroblasts in chronic arthritis: Possible role in growth regulation." Cytokine **6**: 61-69.

Hogg, N., Palmer, D.G., and Revell, P.A. (1985). "Mononuclear phagocytes of normal and rheumatoid synovial membrane identified by monoclonal antibodies." Immunology **56**: 673-681.

Holmes, W. E., Lee, J., Kuang, W.J., et. al. (1991). "Structure and functional expression of a human interleukin-8 receptor." Science **253**: 1278-1280.

Hosaka, S., Akahoshi, T., Wada, C., et. al. (1994). "Expression of the chemokine superfamily in rheumatoid arthritis." Clinical and Experimental Immunology **97**: 451-457.

Hou, W.-S., Li, W., Keyser, G., et. al. (2002). "Comparison of cathepsin K and S expression within the rheumatoid and osteoarthritic synovium." Arthritis and Rheumatism **46**: 663-674.

Hou, W.-S., Li, Z., Gordon, R.E., et. al. (2001). "Cathepsin K is a critical protease in synovial fibroblast-mediated collagen degradation." The American Journal of Pathology **159**: 2167-2177.

Huet, G., Flipo, R.M., Colin, C., et. al. (1993). "Stimulation of the secretion of latent cysteine proteinase activity by tumor necrosis factor alpha and interleukin-1." Arthritis and Rheumatism **36**: 772-780.

Imboden, J. B. (2009). "The immunopathogenesis of rheumatoid arthritis." Annual Review of Pathology: Mechanisms of Disease **4**: 417-434.

Iovine, N. M., Elsbach, P., and Weiss, J. (1997). "An opsonic function of the neutrophil bactericidal/permeability-increasing protein depends on both its N- and C- terminal domains." Proceedings of the National Academy of Sciences **94**: 10973-10978.

Itoh, T., Matsuda, H., Tanioka, M., et. al. (2002). "The role of matrix metalloproteinase-2 and matrix metalloproteinase-9 in antibody-induced arthritis." Journal of Immunology **169**: 2643-2647.

Jahn, B., Von Kempis, J., Kramer, K.L., et al. (1993). "Interaction of the terminal compement components C5b-9 with synovial fibroblasts: binding to the membrane surface leads to increase levels in collagenase-specific mRNA." Immunology **78**: 329-334.

Jang, D., and Murrell, G.A.C. (1998). "Nitric oxide in arthritis." Free Radical Biology and Medicine **24**: 1511-1519.

Janusz, M. J., and Doherty, N.S. (1991). "Degradation of cartilage matrix proteoglycan by human neutrophils involves both elastase and cathepson G." Journal of Immunology **146**: 3922-3928.

Jasin, H. E. (2000). "Mechanisms of joint destruction in rheumatoid arthritis." Journal of Orthopaedic Science **5**: 178-181.

Jenkins, J. K., Hardy, K.J., and McMurray, R.W. (2002). "The pathogenesis of rheumatoid arthritis: A guide to therapy." The American Journal of the Medical Sciences **323**: 171-180.

Jones, S. A., Wolf, M., Qin, S., et. al. (1996). "Different functions for the interleukin 8 receptors (IL-8R) of human neutrophil leukocytes: NADPH oxidase and phospholiapse D ar activated through IL-8R1 but not IL-8R2." Proceedings of the National Academy of Sciences **93**: 6682-6686.

Jordan, J. E., Zhao, Z.Q., and Vinten-Johansen, J. (1999). "The role of neutrophils in myocardial ischemia-reperfusion injury " Cardiovascular Research **43**: 860-878.

Joseph, P. R., Sarmiento, J.M., Mishra, A.K., et. al. (2010). "Probing the role of CXC motif in chemokine CXCL8 for high affinity binding and activation of CXCR1 and CXCR2 receptors." The Journal of Biological Chemistry **285**: 29262-29269.

Jovanovic, D. V., Di Battista, J.A., Martel-Pelletier, J., et. al. (1998). "IL-17 stimulates the production and expression of proinflmmatory cytokines, IL-beta and TNF-alpha, by human macrophages." Journal of Immunology **160**: 3513-3521.

Kakimoto, K., Matsukawa, A., Yoshinaga, M., et. al. (1995). "Suppressive effects of a neutrophil elastase inhibitor on the development of collagen-induced arthritis." Cellular Immunology **165**: 26-32.

Kamradt, T., and Schubert, D. (2005). "The role and clinical implications of G6PI in experimental models of rheumatoid arthriits." Arthritis Research and Therapy **7**: 20-28.

Kaplan, M. K. (2005). "STAT4 a critical regulator of inflammation in vivo." Immunologic Research **32**: 231-241.

Katancik, J. A., Sharma, A., Radel, S.J., et. al. (1997). "Mapping of the extracellular binding regions of the human interleukin-8 type b receptor." Biochemical and Biophysical Research Communications **232**: 663-668.

Kearney, E. R., Pape, K.A., Loh, D.Y., et. al. (1994). "Visualization of peptide-specific T cell immunity and peripheral tolerance induction in vivo." Immunity **1**: 327-339.

Kelly, M., Hwang, J.M., and Kubes, P. (2007). "Modulating leukocyte recruitment in inflammation." Journal of Allergy and Clinical Immunology **120**: 3-10.

Khachigian, L. M. (2006). "Collagen antibody-induced arthritis." Nature Protocols **1**: 2512-2516.

Khurana, R., and Berney, S.M. (2005). "Clincal aspects of rheumatoid arthritis." Pathophysiology **12**: 153-165.

Kim, H. J., and Berek, C. (2000). "B cells in rheumatoid arthritis." Arthritis Research **2**: 126-131.

Kim, N. D., Chou, R.C., Seung, E., et. al. (2006). "A unique requirement for the leukotriene $B_4$ receptor BLT1 for neutrophil recruitment in inflammatory arthritis." The Journal of Experimental Medicine **203**: 829-835.

Kimura, A., and Kishimoto, T. (2010). "IL-6: Regulators of Treg/Th17 balance." European Journal of Immunology **40**(1830-1835).

Kinne, R. W., Brauer, R., Stuhlmuller, B., et. al. (2000). "Macrophages in rheumatoid arthritis." Arthritis Research **2**: 189-202.

Kinne, R. W., Stuhlmuller, B., and Burmester, G-R. (2007). "Cells of the synovium in rheumatoid arthritis: Macrophages." Arthritis Research and Therapy **9**: 224.

Kitayama, J., Carr, M.W., Roth, S.J., et. al. (1997). "Contrasting responses to multiple chemotactic stimuli in transendothelial migration: heterologous desensitization in neutrophils and augmentation of migration in eosinophils." The Journal of Immunology **158**: 2340-2349.

Kitsis, E., and Weissmann, G. (1991). "The role of the neutrophil in rheumatoid arthritis." Clinical Orthopaedics and Related Research **265**: 63-72.

Klareskog, L., Catrina, A.I., and Paget, S. (2009). "Rheumatoid arthritis." The Lancet **373**: 659-672.

Klockars, M., Koskela, R.S., Jarvinen, E., et al. (1987). "Silica exposure and rheumatoid arthritis: a follow up study of granite workers 1940-81." British Medical Journal **294**: 997-1000.

Kobayashi, S. D., Voyich, J.M., and DeLeo, F.R. (2003). "Regulation of the neutrophil-mediated inflammatory response to infection." Microbles and Infection **5**: 1337-1344.

Koch, A. E. (2003). "Angiogenesis as a target in rheumatoid arthritis." Annals of the Rheumatic Diseases **62**: ii60-ii67.

Koch, A. E. (2005). "Chemokines and their receptors in rheumatoid arthritis." Arthritis and Rheumatism **52**: 710-721.

Koch, A. E., Burrows, J.C., Haines, G.K., et. al. (1991). "Immunolocalization of endothelial and leukocyte adhesion molecules in human rheumatoid and osteoarthritic synovial tissues." Laboratory Investigation **64**: 313-320.

Koch, A. E., Kunkel, S.L., Harlow, L.A., et. al. (1992). "Enhanced production of monocyte chemoattractant protein-1 in rheumatoid arthritis." The Journal of Clinical Investigations **90**: 772-779.

Koch, A. E., Kunkel, S.L., Harlow, L.A., et. al. (1994). "Epithelial neutrophil activating peptide-78: a novel chemotactic cytokine for neutrophils in arthritis." Journal of Clinical Investigation **94**: 1012-1018.

Koch, A. E., Kunkel, S.L., Harlow, L.A., et. al. (1994). "Macrophage inflammatory protein-1 alpha. A novel chemotactic cytokine for macrophages in rheumatoid arthritis." Journal of Clinical Investigation **93**: 921-928.

Koch, A. E., Kunkel, S.L., Shah, M.R., et. al. (1995). "Growth-related gene product α. A chemotactic cytokine for neutophils in rheumatoid arthritis." Journal of Immunology **155**: 3660-3666.

Koch, A. E., Volin, M.V., Woods, J.M., et. al. (2001). "Regulation of angiogenesis by the C-X-C chemokines interleukin-8 and epithelial neutophil activating peptide 78 in the rheumatoid joint." Arthritis and Rheumatism **44**: 31-40.

Kosmac, M., Avcin, T., Toplak, N., et. al. (2011). "Exploring the binding sites of anti-infliximab antibodies in pediatric patients with rheumatic diseases treated with inflixiamb." Pediatric Research **69**: 243-248.

Kumar, P., Pai, K., Pandey, H.P., et. al. (2002). "NADH-oxidase, NADPH-oxidase and myeloperoxidase activity of visceral leishmaniasis patients." Journal of Medical Microbiology **51**: 832-836.

Laing, K. J., and Secombes, C.J. (2004). "Chemokines." Developmental and Comparative Immunology **28**: 443-460.

Landino, L. M., Crews, B.C., Timmons, M.D., et. al. (1996). "Peroxynitrite, the coupling product of nitric oxide and superoxide, activates protaglandin biosynthesis." Biochemistry **93**: 15069-15074.

Larche, M. J., Sacre, S.M., and Foxwell, B.M. (2005). "Pathogenic role of TNF-alpha in the pathogenesis of rheumatoid arthritis." Drug Discovery Today: Disease Mechanisms **2**: 367-375.

Lau, C. M., Broughton, C., Tabor, A.S., et al. (2005). "RNA-associated autoantigens activate B cells by combined B cell antigen receptor/Toll-like receptor 7 engagement." The Journal of Experimental Medicine **202**(9): 1171-1177.

Le, Y., Zhou, Y., Iribarren, P., et. al. (2004). "Chemokines and chemokine receptors: their manifold roles in homeostasis and disease." Cellular and Molecular Immunology **1**: 95-104.

Leadbetter, E. A., Rifkin, I.R., Hohlbaum, A.M., et al. (2002). "Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors." Nature **416**: 603-607.

Lee, D. M., and Weinblatt, M.E. (2001). "Rheumatoid arthritis " The Lancet **358**: 903-911.

Lee, H., Whitfeld, P.L., and Mackay, C.R. (2008). "Receptors for complement C5a. The importance of C5aR and the enigmatic role of C5L2." Immunology and Cell Biology **86**: 153-160.

Lemaire, R., Huet, G., Zerimech, F., et. al. (1997). "Selective induction of the secretion of cathepsins B and L by cytokines in synovial fibroblast-like cells." British Journal of Rheumatology **36**: 735-743.

Leong, S. R., Lowman, H.B., Liu, J., et. al. (1997). "IL-8 single-chain homodimers and heterodimers: Interaction with the chemokine receptors CXCR1, CXCR2, and DARC " Protein Science **6**: 609-617.

Levay, P. F., and Viljoen, M. (1995). "Lactoferrin: A general review." Haematologica **80**: 252-267.

Levy, O. (2004). "Antimicrobial proteins and peptides: anti-infective molecules of mammalian leukocytes." Journal of Leukocyte Biology **76**: 909-925.

Ley, K., Laudanna, C., Cybulsky, M.I., et. al. (2007). "Getting to the site of inflammation: the leukocyte adhesion cascade update." Nature Reviews Immunology **7**: 678-689.

Li, F., and Gordon, J.R. (2001). "IL-$8_{(3-73)}$K11R is a high affinity agonist of the neutrophil CXCR1 and CXCR2." Biochemical and Biophysical Research Communications **286**: 595-600.

Li, F., Zhang, X., and Gordon, J.R. (2002). "$CXCL8_{(3-73)}$K11R/G31P antagonizes ligand binding to the neutrophil CXCR1 and CXCR2 receptors and cellular responses to CXCL8/IL-8." Biochemical and Biophysical Research Communications **293**: 939-944.

Li, F., Zhang, X., Mizzi, C., et. al. (2002). "$CXCL8_{(3-73)}$K11R/G31P antagonizes the neutrophil chemoattractants present in pasteurellosis and mastitis lesions and abrogates neutophil influx into intradermal endotoxin challenge sites in vivo." Veterinary Immunology and Immunopathology **90**: 65-77.

Li, X., Okada, Y., Pilbeam, C.C., et. al. (2000). "Knockout of the murine prostaglandin EP2 receptor impairs osteoclastogenesis in vitro." Endocrinology **141**: 2054-2061.

Lindley, I., Aschauer, H., Seifert, J.M., et. al. (1988). "Synthesis and expression in *Escherichia coli* of the gene encoding monocyte-derived neutrophil-activating factor: Biological equivalence between natural and recombinant neutrophil-activating factor." Proceedings of the National Academy of Sciences **85**: 9199-9203.

Lindy, O., Konttinen, Y.T., Sorsa, T., et. al. (1997). "Matrix metalloproteinase 13 (collagenase 3) in human rheumatoid arthritis." Arthritis and Rheumatism **40**: 1391-1399.

Listing, J., Strangfeld, A., Kary, S., et. al. (2005). "Infections in patients with rheumatoid arthritis treated with biologic agents " Arthritis and Rheumatism **52**: 3403-3412.

Liu, X., Peng, J., Sun, W., et. al. (2012). "G31P, an antagonist against CXC chemokine receptors 1 and 2, inhibits growth of human prostate cancer cells in nude mice." The Tohoku Journal of Experimental Medicine **228**: 147-156.

Loetscher, P. (2005). "Chemokines in rheumatoid arthritis." Drug Discovery Today: Disease Mechanisms **2**: 377-382.

Luan, J., Furuta, Y., Du, J., et. al. (2001). "Developmental expression of two CXC chemokines, MIP-2 and KC, and their receptors." Cytokine **14**: 253-263.

Lundy, S. K., Sarkar, S., Tesmer, L.A., et. al. (2007). "T lymphocytes: Cells of the synovium in rheumatoid arthritis." Arthritis Research & Therapy **9**: 202-213.

Luo, H. R., and Loison, F. (2008). "Constitutive neutrophil apoptosis: mechanisms and regulation." American Journal of Hematology **83**: 288-295.

Luross, J. A., and Williams, N.A. (2001). "The genetic and immunopathological processes underlying collagen-induced arthritis." Immunology **103**: 407-416.

Luster, A. D. (1998). "Chemokines - chemotactic cytokines that mediate inflammation." The New England Journal of Medicine **338**: 436-445.

Luster, A. D. (2001). Chemotaxis: role in immune response. Encyclopedia of Life Sciences, Nature Publishing Group.

Luster, A. D., Alon, R., and von Andrian, U.H. (2005). "Immune cell migration in inflammation: present and future therapeutic targets." Nature Immunology **6**: 1182-1190.

Ma, Y., Pope, R.M. (2005). "The role of macrophages in rheumatoid arthritis." Current Pharmaceutical Design **11**: 569-580.

MacDonald, K. P., Nishioka, Y., Lipsky, P.E., et. al. (1997). "Functional CD40 ligand is expressed by T cells in rheumatoid arthritis." Journal of Clinical Investigation **100**: 2404-2414.

MacGregor, A. J., Snieder, H., Rigby, A.S., et. al. (2000). "Characterizing the quantitative genetic contribution to rheumatoid arthritis using data from twins." Arthritis and Rheumatism **43**: 30-37.

Malfait, A. M., Butler, D.M., Presky, D.H., et. al. (1998). "Blockade of IL-12 during the induction of collagen-induced arthritis (CIA) markedly attenuates the severity of the arthritis." Clinical and Experimental Immunology **111**: 377-383.

Marcus, A. J. (1984). "The eicosanoids in biology and medicine." Journal of Lipid Research **25**: 1511-1516.

Martel-Pelletier, J., Pelletier, J.P., and Fahmi, H. (2003). "Cyclooxgenase-2 and prostaglandins in articular tissues." Seminars in Arthritis and Rheumatism **33**: 155-167.

Maruotti, N., Cantatore, F.P., and Crivellato, E., et. al. (2006). "Angiogenesis in rheumatoid arthritis." Histology and Histophathology **21**: 557-566.

Mathis, S., Jala, V.R., and Haribabu, B. (2007). "Role of leukotriene $B_4$ receptors in rheumatoid arthritis." Autoimmunity Reviews **7**: 12-17.

Matsushima, K., Morishita, K., Yoshimura, T., et. al. (1988). "Molecular cloning of a human monocyte-derived neutophil chemotactic factor (MDNCF) and the induction of MDNCF mRNA by interleukin 1 and tumor necrosis factor " Journal of Experimental Medicine **167**: 183-1893.

Mauch, C. (1998). "Regulation of connective tissue turnover by cell-matrix interactions." Archives of Dermatological Research **290**: S30-S36.

Mauri, C., and Ehrenstein, M.R. (2007). "Cells of the synovium in rheumatoid arthritis: B cells." Arthritis Research and Therapy **9**: 205.

McInnes, I. B., and Schett, G. (2007). "Cytokines in the pathogenesis of rheumatiod arthritis." Nature Reviews Immunology **7**: 429-442.

Middleton, J., Americh, L., Gayon, R., et. al. (2004). "Endothelial cell phenotypes in the rheumatoid synovium: activated, angiogenic, apoptotic and leaky." Arthritis Research and Therapy **6**: 60-72.

Miossec, P. (2003). "Interlenkin-17 in rheumatoid arthritis. If T cells were to contribute to inflammation and destruction through synergy." Arthritis and Rheumatism **48**(3): 594-601.

Moepps, B., Nuesseler, E., Braun, M., et. al. (2006). "A homolog of the human chemokine receptor CXCR1 is expressed in the mouse." Molecular Immunology **43**: 897-914.

Mohr, W., Westerhellweg, H., and Wessinghage, D. (1981). "Polymorphonuclear granulocytes in rheumatic tissue destruction. III. An electron microscopic study of PMNs at the pannus-cartilage junction in rheumatoid arthritis." Annals of the Rheumatic Diseases **40**: 396-399.

Mor, A., Abramson, S.B., and Pillinger, M.H. (2005). "The fibroblast-like synovial cell in rheumatoid arthritis: a key player in inflammation and joint destruction." Clinical Immunology **115**: 118-128.

Moreland, L. W. (1999). "Recent advances in anti-tumor necrosis factor (TNF) therapy in rheumatoid arthritis - Focus on the soluble TNF receptor p75 fusion protein, etanercept." BioDrugs **11**: 201-210.

Moser, B., and Loetscher, P. (2001). "Lymphocyte traffic control by chemokines." Nature Immunology **2**: 123-128.

Moser, B., and Willimann, K. (2004). "Chemokines: role in inflammation and immune surveillance." Annals of the Rheumatic Diseases **63**: ii84-ii89.

Mukaida, N. (2003). "Pathophysiological roles of interleukin-8/CXCL8 in pulmonary diseases." American Journal of Physiology: Lung Cellular and Molecular Physiology **284**: 566-577.

Mukherjee, P., Wu, B., Mayton, L., et. al. (2003). "TNF receptor gene therapy results in suppression of IgG2a anticollagen antibody in collagen induced arthritis." Annals of the Rheumatic Diseases **62**: 707-714.

Muller-Ladner, U., and Gay, S. (2002). "MMPs and rheumatoid synovial fibroblasts: Siamese twins in joint destruction?" Annals of the Rheumatic Diseases **61**: 957-959.

Muller-Ladner, U., Ospelt, C., Gay, S., et. al. (2007). "Cells of the synoivum in rheumatoid arthritis. Synoival fibroblasts." Arthritis Research and Therapy **9**: 223-233.

Muller, W. A. (2003). "Leukocyte - endothelial-cell interactions in leukoctye transmigration and the inflammatory response." Trends in Immunology **24**: 326-333.

Murphy, G., and Lee, M.H. (2005). "What are the roles of metalloproteinases in cartilage and bone damage?" Annals of the Rheumatic Diseases **64**: iv44-iv47.

Murphy, G., Stanton, H., Cowell, S., et. al. (1999). "Mechanisms for pro matrix metalloproteinase activation." ACTA Pathologica, Microbiologica et Immunologica Scandinavica **107**(38-44).

Murrell, G. A. C., Jang, D., and Williams, R.J. (1995). "Nitric oxide activates metalloprotease enzymes in articular cartilage." Biochemical and Biophysical Research Communications **206**: 15-21.

Myers, L. K., Rosloniec, E.F., Cremer, M.A., et. al. (1997). "Collagen-induced arthritis, an animal model of autoimmunity." Life Sciences **61**: 1861-1878.

Nagase, H., and Kashiwagi, M. (2002). "Aggrecanases and cartilage matrix degradation." Arthritis Research and Therapy **5**: 94-103.

Nakashima, T., and Takayanagi, H. (2009). "Osteoclasts and the immune system." Journal of Bone and Mineral Metabolism **27**: 519-529.

Nandakumar, K. S., Svensson, L., and Holmdahl, R. (2003). "Collagen type II-specific monoclonal antibody-induced arthritis in mice." American Journal of Pathology **163**: 1827-1837.

Nash, J. A., Ballard, T.N.S., Weaver, T.E., et. al. (2006). "The peptidoglycan-degrading property of lysozyme is not required for bactericidal activity in vivo." The Journal of Immunology **177**: 519-526.

Nasser, M. W., Marjoram, R.J., Brown, S.L., et. al. (2005). "Cross-desensitization among CXCR1, CXCR2, and CCR5: role of protein kinase C." The Journal of Immunology **174**: 6927-6933.

Nasser, M. W., Raghuwanshi, S.K., Malloy, K.M., et. al. (2007). "CXCR1 and CXCR2 activation and regulation: role of aspartate 199 of the second extracellular loop of CXCR2 in CXCL8-mediated rapid receptor internalization." The Journal of Biological Chemistry **282**: 6906-6915.

Nasu, Y., Nishida, K., Abe, N., et al. (2008). "Trichostatin A, and histone deacetylase inhibitor, suppresses synovial inflammation and subsequent cartilage destruction in a collagen antibody-induced arthritis mouse model." Osteoarthritis and Cartilage **16**: 723-732.

Nathan, C. (2006). "Neutrophils and immunity: challenges and opportunities." Nature Reviews Immunology **6**: 173-182.

Nawroth, P. P., Bank, I., Handley, D., et. al. (1986). "Tumor necrosis factor/cachectin interacts with endothelial-cell receptors to induce release of interleukin 1." Journal of Experimental Medicine **163**: 1363-1375.

Nerusu, K. C., Warner, R.L., Bhagavathula, N., et. al. (2007). "Matrix metalloproteinase-3 (stromelysin-1) in acute inflammatory tissue injury." Experimental and Molecular Pathology **83**: 169-176.

Niedermeier, M., Pap, T., and Korb, A. (2010). "Therapeutic opportunities in fibroblasts in inflammatory arthritis." Best Practice and Research Clinical Rheumatology **24**: 527-540.

Novack, D. V., and Teitelbaum, S.L. (2008). "The osteoclat: friend or foe?" The Annual Review of Pathology: Mechanisms of Disease **3**: 457-484.

Ochoa, L., Waypa, G., Mahoney, J.R., et. al. (1997). "Contrasting effects of hypochlorous acid and hydrogen peroxide on endothelial permeabiltiy." American Journal of Respiratory and Critical Care Medicine **156**: 1247-1255.

Okroj, M., Heinegard, D., Holmdahl, R., et. al. (2007). "Rheumatoid arthritis and the complement system." Annals of Medicine **39**: 517-530.

Onodera, S., Nishihira, J., Koyama, Y., et al. (2004). "Macrophage migration inhibitory factor up-regulates the expression of interleukin-8 messenger RNA in synovial fibroblasts of rheumatoid arthritis patients." Arthritis and Rheumatism **50**: 1437-1447.

Orozco, G., and Barton, A. (2010). "Update on the genetic risk factors for rheumatoid arthritis." Expert Review in Clinical Immunology **6**: 61-75.

Osman, M. O., Kristensen, J.U., Jacobsen, N.O., et. al. (1998). "A monoclonal anti-interleukin 8 antibody (WS-4) inhibits cytokine response and acute lung injury in experimental severe acute necrotising pancreatitis in rabbits." Gut **43**: 232-239.

Otero, M., and Goldring, M.B. (2007). "Cells of the synovium in rheumatoid arthritis. Chondrocytes." Arthritis Research and Therapy **9**: 220.

Owen, C. A., and Campbell, E.J. (1999). "The cell biology of leukocyte-mediated proteolysis." Journal of Leukocyte Biology **65**: 137-150.

Pablos, J. L., Santiago, B., Galindo, M., et. al. (2003). "Synoviocyte-derived CXCL12 is displayed on endothelium and induces angiogenesis in rheumatoid arthritis." The Journal of Immunology **170**: 2147-2152.

Paleolog, E. W. (2002). "Angiogenesis in rheumatoid arthritis." Arthritis Research **4**( ): iii81-iii90.

Palmer, R. M. J., Hickery, M.S., Charles, I.G., et. al. (1993). "Induction of nitric oxide synthase in human chondrocytes." Biochemical and Biophysical Research Communications **193**: 398-405.

Pap, T., Muller-Ladner, U., Gay, R.E., and Gay, S. (2000). "Fibroblast biology - Role of synovial fibroblasts in the pathogenesis of rheumatoid arthritis." Arthritis Research **2**: 361-367.

Pap, T., Shigeyama, Y., Kuchen, S., et. al. (2001). "Differential expression patterns of membrane-type matrix metalloproteinases in rheumatoid arthritis." Arthritis and Rheumatism **43**: 1226-1232.

Park, J. Y., Pillinger, M.H., and Abramson, S.B. (2006). "Prostaglandin E2 synthesis and secretion: the role of PGE2 synthase." Clinical Immunology **119**: 229-240.

Parry, C. M., Simas, J.P., Smith, V.P., et. al. (2000). "A broad specturum secreted chemokine binding protein encoded by a herpesvirus." Journal of Experimental Medicine **191**: 573-578.

Patterson, A. M., Schmutz, C., Davis, S., et. al. (2002). "Differential binding of chemokines to macrophages and neutrophils in the human inflamed synovium." Arthritis Research **4**: 209-214.

Penfold, M. E., Dairaghi, D.J., Duke, G.M., et. al. (1999). "Cytomegalovirus encodes a potent α chemokine." Proceedings of the National Academy of Sciences **96**: 9839-9844.

Pennathur, S., Maitra, D., Byun, J., et. al. (2010). "Potent antioxidative activity of lycopene: a potential role in scavenging hypochlorous acid." Free Radical Biology and Medicine **49**: 205-213.

Perdriger, A. (2009). "Infliximab in the treatment of rheumatoid arthritis." Biologics: Targets and Therapy **3**: 183-191.

Pettersen, C. A., and Adler, K.B. (2002). "Airways inflammation and COPD: epithelial-neutrophil interactions." Chest **121**: s142-s150.

Pham, C. T. N. (2006). "Neutrophil serine proteases: specific regulators of inflammation." Nature Reviews Immunology **6**: 541-550.

Pillinger, M. H., and Abramson, S.B. (1995). "The neutrophil in rheumatoid arthritis." Rheumatic Disease Clinics of North America **21**: 691-714.

Plenge, R. M., Cotsapas, C., Davies, L., et. al. (2007). "Two independent alleles at 6q23 associated with risk of rheumatoid arthritis." Nature Genetics **39**: 1477-1482.

Plenge, R. M., Seielstad, M., Padyukov, L., et. al. (2007). "TRAF1-C5 as a risk locus for rheumatoid arthritis - a genomewide study." The New England Journal of Medicine **357**: 1199-1209.

Pober, J. S., and Sessa, W.C. (2007). "Evolving functions of endothelial cells in inflammation." Nature Reviews Immunology **7**: 803-815.

Podechard, N., Lecureur, V., Le Ferrec, E., et. al. (2008). "Interleukin-8 induction by the environmental contaminant benzo(a)pyrene is aryl hydrocarbon receptor-dependent and leads to lung inflammation." Toxicology Letters **177**: 130-137.

Pratt, A. G., Isaacs, J.D., and Mattey, D.L. (2009). "Current concepts in pathogenesis of early rheumatoid arthritis." Best Practice and Research Clinical Rheumatology **23**: 37-48.

Proudman, S. M., Cleland, L.G., and Mayrhofer, G. (1999). "Effects of tumor necrosis factor-α, interleukin 1β, and activate peripheral blood mononuclear cells on the expression of adhesion molecules and recruitment of leukocytes in rheumatoid synovial xenografts in SCID mice." Journal of Rheumatology **26**: 1877-1889.

Pullar, J. M., Vissers, M.C.M., and Winterbourn, C.C. (2000). "Living with a killer: the effects of hypochlorous acid on mammalian cells." IUBMB Life **50**: 259-266.

Rediske, J. J., Koehne, C.F., Zhang, B., et. al. (1994). "The inducible production of nitric oxide by articular cell types." Osteoarthritis and Cartilage **2**: 199-206.

Remmers, E. F., Plenge, R.M., Lee, A.T., et. al. (2007). "STAT4 and the risk of rheumatoid arthritis and systemic lupus erythematosus." The New England Journal of Medicine **357**: 977-986.

Remmers, E. F., Sano, H., and Wilder, R.L. (1991). "Platelet-derived growth factors and heparin-binding (fibroblast) growth factors in the synovial tissue pathology of rheumatoid arthritis." Seminars in Arthritis and Rheumatism **21**: 191-199.

Richardson, R. M., Haribabu, B., Ali, H., et. al. (1996). "Cross-desensitization among receptors for platelet activating facor and peptide chemoattractants. Evidence for independent regulatory pathways. ." The Journal of Biological Chemistry **271**: 28717-28724.

Richardson, R. M., Pridgen, B.C., Haribabu, B., et. al. (1998). "Differential cross-regulation of the human chemokine receptors CXCR1 and CXCR2. Evidence for time-dependent signal generation." The Journal of Biological Chemistry **273**: 23830-23836.

Rinaldi, N., Schwarz-Eywill, M., Weis, D., et. al. (1997). "Increased expression of integrins on fibroblast-like synoviocytes from rheumatoid arthritis in vitro correlates with enhanced binding to extracellular matrix proteins." Annals of the Rheumatic Diseases **56**: 45-51.

Robinson, D. R., Tashjian, A.H., and Levine, L. (1975). "Prostaglandin-stimulated bone resorption by rheumatoid synovia. A possible mechanism for bone destruction in rheumatoid arthritis." Journal of Clinical Investigation **56**: 1181-1188.

Robinson, J. M. (2008). "Reactive oxygen species in phagocytic leukocytes." Histochemistry and Cell Biology **130**: 281-297.

Rollins, B. J. (1997). "Chemokines." Blood **90**: 909-928.

Rosengren, S., Corr, M., and Boyle, D.L. (2010). "Platelet-derived growth factor and transforming growth factor beta synergistically potentiate inflammatory mediator synethesis by fibroblast-like synoviocytes." Arthritis Research and Therapy **12**: R65.

Roux, S., and Orcel, P. (2000). "Bone loss - Factors that regulate osteoclast differentiation: an update." Arthritis Research **2**: 451-456.

Rovai, L. E., Herschman, H.R., and Smith, J.B. (1998). "The murine neutrophil-chemoattractant chemokines LIX, KC, and MIP-2 have distinct induction kinetics, tissue distributions, and tissue-specific sensitivities to glucocorticoid regulation in endotoxemia." Journal of Leukocyte Biology **64**: 494-502.

Rubin, E., and Reisner, H.M., Ed. (2009). Essentials of Rubin's Pathology. Philadelphia, PA, Lippincott Williams & Wilkins.

Ruth, J. H., Park, C.C., Amin, M.A., et. al. (2010). "Interleukin-18 as an in vivo mediator of monocyte recruitment in rodent models of rheumatoid arthritis." Arthritis Research and Therapy **12**: R118.

Ruth, J. H., Volin, M.V., Haines, G.K., et. al. (2001). "Fractalkine, a novel chemokine in rheumatoid arthritis and in rat adjuvant-induced arthritis." Arthritis and Rheumatism **44**: 1568-1581.

Saksela, O., and Rifkin, D.B. (1988). "Cell-associated plasminogen activation: Regulation and physiological functions." Annual Review of Cell Biology **4**: 93-126.

Salminen-Mankonen, H. J., Morko, J., and Vuorio, E. (2007). "Role of cathepsin K in normal joints and in the development of arthritis." Current Drug Targets **8**: 315-323.

Sampey, A. V., Hall, P.H., Mitchell, R.A., et. al. (2001). "Regualtion of synoviocyte phospholipase A2 and cyclooxygenase 2 by macrophage migration inhibitory factor." Arthritis and Rheumatism **44**: 1273-1280.

Sano, H., Engleka, K., Mathern, P., et. al. (1993). "Coexpression of phosphotyrosine-containing proteins, platelet-derived growth factor-B, and fibroblast growth factor-1 in situ in synovial tissues of patients with rheumatoid arthritis and lewis rats with adjuvant or streptococcal cell wall arthritis." The Journal of Clinical Investigations **91**: 553-565.

Santos, L. L., Morand, E.F., Yang, Y., et. al. (1997). "Suppression of adjuvant arthritis and synovial macrophage inducible nitric oxide by n-iminoethyl-l-ornithine, a nitric oxide synthase inhibitor." Inflammation **21**: 299-311.

Sathish, J. G., Sethu, S., Bielsky, M.C., et. al. (2013). "Challenges and approaches for the development of safer immunomodulatory biologics." Nature Reviews Drug Discovery **12**: 306-324.

Scapini, P., Lapinet-Vera, J.A., Gasperini, S., et. al. (2000). "The neutrophil as a cellular source of chemokines." Immunological Reviews **177**: 195-203.

Schett, G. (2006). "Arthritis - a nightmare for bone." Wiener Klinische Wochenschrift **118**: 11-15.

Schett, G. (2007). "Cells of the synovium in rheumatoid arthritis - Osteoclasts." Arthritis Research and Therapy **9**: 203.

Schlesinger, P. H., Blair, H.C., Teitelbaum, S.L., et. al. (1997). "Characterization of the osteoclast ruffled border chloride channel and its role in bone resorption." The Journal of Biological Chemistry **272**: 18636-18643.

Schmittgen, T. D., and Livak, K.J. (2008). "Analyzing real-time PCR data by the comparative $C_T$ method." Nature Protocols **3**: 1101-1108.

Scrivo, R., Di Franco, M., Spadaro, A., et. al. (2007). "The immunology of rheumatoid arthritis." Annals of the New York Academy of Sciences **1108**: 312-322.

Seitz, M., Loetscher, P., Fey, M.F., et. al. (1994). "Constitutive messenger-RNA and protein production of macrophage colony stimulating factor but not of other cytokines by synovial fibroblasts from rheumatoid arthritis and osteoarthritis patients." British Journal of Rheumatology **33**: 613-619.

Sekido, N., Mukaida, N., Harada, A., et. al. (1993). "Prevention of lung reperfusion injury in rabbits by a monoclonal antibody against interleukin-8." Nature **365**: 654-657.

Seldin, M. F., Amos, C.I., Ward, R., et. al. (1999). "The genetics revolution and the assault on rheumatoid arthritis." Arthritis and Rheumatism **42**: 1071-1079.

Sethu, S., Govindappa, K., Alhaidari, M., et. al. (2012). "Immunogenicity to biologics: Mechanisms, prediction and reduction." Archivum Immunologiae et Therapiae Experimentalis **60**: 331-344.

Sewell, K. L., and Trentham, D.E. (1993). "Pathogenesis of rheumatoid arthritis." The Lancet **341**: 283-286.

Shadidi, K. R., Aarvak, T., Henriksen, J.E., et al. (2003). "The chemokines CCL5, CCL2, and CXCL12 play significant roles in the migration of Th1 cells into rheumatoid synovial tissue." Scandinavian Journal of Immunology **57**: 192-198.

Shahrara, S., Pickens, S.R., Dorfleutner, A., et. al. (2009). "IL-17 induces monocyte migration in rheumatoid arthritis." Journal of Immunology **182**: 3884-3891.

Shaw, T., Quan, J., and Totoritis, M.C. (2003). "B cell therapy for rheumatoid arthritis: the rituximab (anti-CD20) experience." Annals of the Rheumatic Diseases **62**: ii55-ii59.

Shigeyama, Y., Pap, T., Kunzler, P., et. al. (2000). "Expression of osteoclast differentiation factor in rheumatoid arthritis." Arthritis and Rheumatism **43**: 2523-2530.

Shingu, M., Nagai, Y., Isayama, T., et. al. (1993). "The effects of cytokines on metalloproteinase inhibitors (TIMP) and collagenase production by human chondrocytes and TIMP production by synovial cells and endothelial cells." Clinical and Experimental Immunology **94**: 145-149.

Silverman, G. J., and Carson, D.A. (2003). "Roles of B cells in rheumatoid arthritis." Arthritis Research and Therapy **5**: S1-S6.

Simoes, S. I. D., Eleuterio, C.V, Cruz, M.E.M, et. al. (2003). "Biochemical changes in arthritis rats: dehydroascorbic and ascorbic acid levels." European Journal of Pharmaceutical Sciences **18**: 185-189.

Smith, J. A. (1994). "Neutrophils, host defense, and inflammation: a double-edged sword." Journal of Leukocyte Biology **56**: 672-686.

Smolen, J. S., and Maini, R.N. (2006). "Interleukin-6: a new therapeutic target." Arthritis Research and Therapy **8**: S5.

Snowden, N., Reynolds, I., Morgan, K., et. al. (1997). "T cell response to human type II collagen in patients with rheumatoid arthritis and healthy controls." Arthritis and Rheumatism **40**: 1210-1218.

So, A., Chamot, A.M., Peclat, V., et. al. (1999). "Serum MMP-3 in rheumatoid arthritis: correlation with systemic inflammation but not with erosive status." Rheumatology **38**: 407-410.

Speyer, C. L., and Ward, P.A. (2011). "Role of endothelial chemokines and their receptors during inflammation." Journal of Investigative Surgery **24**: 18-27.

Splettstoesser, W. D., and Schuff-Werner, P. (2002). "Oxidative stress in phagocytes - "the enemy within"." Microscopy Research and Techniques **57**: 441-455.

St. Claire, E. M., Pisetsky, D.S., Haynes, B.F., Ed. (2004). Rheumatoid Arthritis Philadelphia, PA, Lippincott Williams & Wilkins.

Stolt, P., Kallberg, H., Lundberg, I., et. al. (2005). "Silica Exposure is associated with increased risk of developing rheumatoid arthritis: results from the Swedish EIRA study." Annals of the Rheumatic Diseases **64**: 582-586.

Strieter, R. M., Belperio, J.A., Burdick, M.D., el. al. (2004). "CXC chemokines: angiogenesis, immunoangiostasis, and metastases in lung cancer." Annals of the New York Academy of Sciences **1028**: 1-10.

Sverdrup, B., Kallberg, H., Bengtsson, C., et al. (2005). "Association between occupational exposure to mineral oil and rheumatoid arthritis: results from Swedish EIRA case-control study." Arthritis Research and Therapy **7**: 1296-1303.

Sweeney, S. E., and Firestein, G.S. (2004). "Rheumatoid arthritis: regulation of synovial inflammation." The International Journal of Biochemistry & Cell Biology **36**: 372-378.

Symmons, D. P., Bankhead C.R., Harrison, B.J. et al. (1997). "Blood transfusion, smoking, and obesity as risk factors for the development of rheumatoid arthritis: Results from a primary care-based incident case-control study in Norfolk, England." Arthritis and Rheumatism **40**: 1955-1961.

Szekanecz, Z., and Koch, A.E. (2000). "Cell-cell interactions in synovitis: Endothelial cells and immune cell migration." Arthritis Research **2**: 368-373.

Szekanecz, Z., and Koch, A.E. (2007). "Macrophages and their products in rheumatoid arthritis." Current Opinion in Rheumatology **19**: 289-295.

Szekanecz, Z., Gaspar, L., and Koch, A.E. (2005). "Angiogenesis in rheumatoid arthritis." Frontiers in Bioscience **10**: 1739-1753.

Szekanecz, Z., Kim, J., and Koch, A.E. (2003). "Chemokines and chemokine receptors in rheumatoid arthritis." Seminars in Immunology **15**: 15-21.

Szekanecz, Z., Vegvari, A., Szabo, Z., et. al. (2010). "Chemokines and chemokine receptors in arthritis." Frontiers in Bioscience **2**: 153-67.

Tak, P. P., Thurkow, E.W., Daha, M.R., et. al. (1995). "Expression of adhesion molecules in early rheumatoid synovial tissue." Clinical Immunology and Immunopathology **77**: 236-242.

Takemura, S., Klimiuk, P.A., Braun, A., et al. (2001). "T cell activation in rheumatoid synovium is B cell dependent." The Journal of Immunology **167**: 4710-4718.

Tamoto, K., Tada, M., Shimada, S., et. al. (1993). "Effects of high-molecular-weight hyaluronates on the functions of guinea pig polymorphonuclear leukocytes." Seminars in Arthritis and Rheumatism **22**: 4-8.

Taskiran, D., Stefanovic-Racic, M., Georgescu, H., et. al. (1994). "Nitric oxide mediated suppression of cartilage proteoglycan synthesis by interleukin-1." Biochemical and Biophysical Research Communications **200**: 142-148.

Tedder, T. F., Steeber, D.A., Chen, A., et. al. (1995). "The selectins: vascular adhesion molecules." The Journal of the Federation of American Societies for Experimental Biology **9**: 866-873.

Tetlow, L. C., Lees, M., Ogata, Y., et. al. (1993). "Differential expression of gelatinase B (MMP-9) and stomelysin-1 (MMP-3) by rheumatoid synovial cells in vitro and in vivo." Rheumatology International **13**: 53-59.

Theilgaard-Monch, K., Porse, B.T., and Borregaard, N. (2006). "Systems biology of neutorphil differentiation and immune response." Current Opinion in Immunology **18**: 54-60.

Thomas, R. (1998). "Antigen-presenting cells in rheumatoid arthritis." Springer Seminars in Immunopathology **20**: 53-72.

Thomson, W., Barton, A., Ke, X., et. al. (2007). "Rheumatoid arthritis association at 6q23." Nature Genetics **39**: 1431-1433.

Tolboom, T. C. A., Pieterman, E., van der Laan, W.H., et. al. (2002). "Invasive properties of fibroblast-like synoviocytes: correlation with growth characteristics and expression of MMP-1, MMP-3, and MMP-10." Annals of the Rheumatic Diseases **61**: 975-980.

Tomhave, E. D., Richardson, R.M., Didsbury, J.R., et. al. (1994). "Cross-desensitization of receptors for peptide chemoattractants." The Journal of Immunology **153**: 3267-3275.

Udagawa, N. (2003). "The mechanism of osteoclast differentiation from macropahges: possible roles of T lymphocytes in osteoclastogenesis." Journal of Bone and Mineral Metabolism **21**: 337-343.

van Dinther-Janssen, A. C. H. M., Horst, E., Koopman, G., et. al. (1991). "The VLA-4/VCAM-1 pathway is involved in lymphocyte adhesion to endothelium in rheumatoid synovium." Journal of Immunology **147**: 4207-4210.

Van Houten, N., and Blake, S.F. (1996). "Direct measurement of anergy of antigen-specific T cells following oral tolerance induction." Journal of Immunology **157**: 1337-1341.

van Schouwenburg, P. A., van de Stadt, L.A., de Jong, R.N., et. al. (2013). "Adalimumab elicits a restricted anti-idiotypic antibody response in autoimmune patients resulting in functional neutralization." Annals of the Rheumatic Diseases **72**: 104-109.

van Vollenhoven, R. F. (2009). "Treatment of rheumatoid arthritis: state of the art 2009." Nature Reviews Rheumatology **5**: 531-541.

Vang, T., Congia, M., Macis, M.D., et. al. (2005). "Autoimmune-associated lyphoid tyrosine phosphatase is a gain-of-function variant." Nature Genetics **37**: 1317-1319.

Vaughan, J. H., and Chihara, T. (1975). "Lymphocyte function in rheumatic disorders." Archives of Internal Medicine **135**: 1324-1328.

Veihelmann, A., Hofbauer, A., Krombach, F., et. al. (2002). "Differential function of nitric oxide in murine antigen-induced arthritis." Rheumatology **41**: 509-517.

Vergunst, C. E., van de Sande, M.G.H., Lebre, M.C., et. al. (2005). "The role of chemokines in rheumatoid arthritis and osteoarthritis." Scandinavian Journal of Immunology **34**: 415-425.

Verheijden, G. F. M., Rijnders, A.W.M., Bos, E., et al. (1997). "Human cartilage glycoprotein-39 as a candidate autoantigen in rheumatoid arthritis." Arthritis and Rheumatism **40**: 1115-1125.

Villar-Suarez, V., Colaco, B., Calles-Venal, I., et. al. (2005). "Effects of extracellular matrix on molecular morphology and behaviour of rabbit auricular chondrocytes in culture." Journal of Biomedicine and Biotechnology **4**: 364-373.

Vincenti, M. P., and Brinckerhoff, C.E. (2002). "Transcriptional regulation of collagnase (MMP-1, MMP-13) genes in arthritis: integration of complex signaling pathways for the recruitment of gene-specific transcripation factor." Arthritis Research **4**: 157-164.

Volin, M. V., Shah, M.R., Tokuhira, M., et. al. (1998). "RANTES expression and contribution to monocyte chemotaxis in arthritis." Clinical Immunology and Immunopathology **89**: 44-53.

Volin, M. V., Woods, J.M., Amin, M.A., et. al. (2001). "Fractalkine: a novel angiogenic chemokine in rheumatoid arthritis." American Journal of Pathology **159**: 1521-1530.

Voll, R. E., and Kalden, J.R. (2006). "Do we need new treatment that goes beyond tumor necrosis factor blockers for rheumatoid arthritis?" Annals of the New York Academy of Sciences **1051**: 799-810.

Voulgari, P. V., and Droso, A.A. (2006). "Adalimumab for rheumatoid arthritis." Expert Opinion on Biological Therapy **6**: 1349-1360.

Wagner, J. G., and Roth, R.A. (2000). "Neutrophil migration mechanisms, with an emphasis on the pulmonary vasculature." Pharmacological Reviews **52**: 349-374.

Walsh, N. C., Crotti, T.N., Goldring, S.R., et. al. (2005). "Rheumatic diseases: the effects of inflammation on bone." Immunological Reviews **208**: 228-251.

Ward, P. A., and Marks, R.M. (1989). "The acute inflammatory reaction." Current Opinion in Immunology **2**: 5-9.

Watson, W. C., and Townes, A.S. (1985). "Genetic susceptibility to murine collagen II autoimmune arthritis. Proposed relationship to the IgG2 autoantibody subclass response, complement C5, major histocompatibility complex (MHC) and non-MHC loci." Journal of Experimental Medicine **162**: 1878-1891.

Weiss, S. J., Peppin, G., Oritz, X., et. al. (1985). "Oxidative autoactivation of latent collagenase by human neutrophils." Science **227**: 747-749.

Werz, O. (2002). "5-Lipoxygenase: Cellular biology and molecular pharmacology." Current Drug Targets - Inflammation and Allergy **1**: 23-44.

White, J. R., Lee, J.M., Young, P.R., et. al. (1998). "Identification of a potent, selective non-peptide CXCR2 antagonist that inhibits interleukin-8-induced neturophil migration." The Journal of Biological Chemistry **273**: 10095-10098.

Wiedow, O., and Meyer-Hoffert, U. (2005). "Neutrophil serine proteases: potential key regulators of cell signaling during inflammation." Journal of Internal Medicine **257**: 319-328.

Williams, G., Borkakoti, N., Bottomley, G.A., et. al. (1996). "Mutagenesis studies of interleukin-8: Identification of a second epitope involved in receptor binding." The Journal of Biological Chemistry **271**: 9579-9586.

Williams, J. G. (1990). "Phagocytes, toxic oxygen metabolites and inflammatory bowel disease: implications for treatment." Annals of the Royal College of Surgeons of England **72**: 253-262.

Williams, R. O. (2007). "Collagen-induced arthritis in mice: A major role for tumor necrosis factor-alpha." Methods in Molecular Biology **361**: 265-284.

Wipke, B. T., and Allen, P.M. (2001). "Essential role of neutrophils in the initiation and progression of a murine model of rheumatoid arthritis." The Journal of Immunology **167**: 1601-1608.

Witko-Sarsat, V., Rieu, P., Descamps-Latscha, B., et. al. (2000). "Neutrophils: moluecules, functions and pathophysiological aspects." Laboratory Investigation **80**: 617-653.

Wolbink, G. J., Vis, M., Lems, W., et. al. (2006). "Development of anti-infliximab antibodies and relationship to clinical response in patients with rheumatoid arthritis." Arthritis and Rheumatism **54**: 711-715.

Wright, H. L., Moots, R.J., Bucknall, R.C., et. al. (2010). "Neutrophil function in inflammation and inflammatory diseases." Rheumatology **49**: 1618-1631.

Yamanaka, H., Makino, K., Takizawa, M., et. al. (2000). "Expression and tissue localization of membrane-types 1, 2, and 3 matrix metalloproteinases in rheumatoid synovium." Laboratory Investigation **80**: 677-687.

Zarbock, A., Allegretti, M., and Ley, K. (2008). "Therapeutic inhibition of CXCR2 by Reparixin attenuates acute lung injury in mice." British Journal of Pharmacology **155**: 357-364.

Zhao, X., Li, F., Town, J.R., et. al. (2007). "Humanized forms of the CXCR1/CXCR2 antagonist, bovine $CXCL8_{(3-74)}$K11R/G31P, effectively block ELR-CXC chemokine activity and airway endotoxemia pathology." International Immunopharmacology **7**: 1723-1731.

Zhao, X., Town, J.R., Li, F., et. al. (2009). "ELR-CXC chemokine receptor antagonism targets inflammatory responses at multiple levels." The Journal of Immunology **182**: 3213-3222.

Zhao, X., Town, J.R., Li, F., et. al. (2010). "Blockade of neutrophil responses in aspiration pneumonia via ELR-CXC chemokine antagonism does not predispose to airway bacterial outgrowth." Pulmonary Pharmacology and Therapeutics **23**: 22-28.

Zhao, X., Town, J.R., Yang, A., et. al. (2010). "A novel ELR-CXC chemokine antagonist reduces intestinal ischemia reperfusion-induced mortality, and local and remote organ injury." Journal of Surgical Research **162**: 264-273.

Zlotnik, A., Yoshie, O., and Nomiyama, H. (2006). "The chemokine and chemokine recpetor superfamilies and their molecular evolution." Genome Biology **7**: 243.