Anti-CD44 and Anti-Platelet Antibodies Have Similar But Distinct Effects in the Treatment of a Mouse Model of Arthritis

by

Patrick Joseph Mott

A thesis submitted in conformity with the requirements for the degree of Master's of Science
Laboratory Medicine and Pathobiology
University of Toronto

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2012

Abstract

Rheumatoid Arthritis (RA) is an autoimmune disease characterized by inflammation and eventual destruction of the synovial joints. The role of platelets in the pathophysiology of arthritis has only recently been established. Because antibodies to CD44 can deplete platelets, we hypothesized that these antibodies might be effective in arthritis through a platelet-depletion mechanism. We examined the K/BxN passive transfer mouse model of arthritis and found that most antibodies against CD44 were capable of depleting platelets. However, anti-CD44 treatment is effective when administered during developing arthritis, while anti-platelet treatment was not. While CD44 antibodies may be therapeutic through platelet-dependant and independent mechanisms, the ability of CD44 antibodies to decrease platelet counts does not seem to be the critical factor in resolving arthritis in the K/BxN model.
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<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACCP</td>
<td>anti-Cyclic Citrullinated Proteins</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine Diphosphate</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>CCR</td>
<td>C-C chemokine receptor</td>
</tr>
<tr>
<td>CD40L</td>
<td>CD40 ligand</td>
</tr>
<tr>
<td>CD44</td>
<td>Hyaluronan receptor, Pgp-1</td>
</tr>
<tr>
<td>CFA</td>
<td>Complete Freund's Adjuvant</td>
</tr>
<tr>
<td>CIA</td>
<td>Collagen Induced Arthritis</td>
</tr>
<tr>
<td>CII</td>
<td>type II collagen</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence Assisted Cell Sorting</td>
</tr>
<tr>
<td>FLS</td>
<td>Fibroblast Like Synoviocyte</td>
</tr>
<tr>
<td>GPIb</td>
<td>Glycoprotein Ib</td>
</tr>
<tr>
<td>GPI</td>
<td>Glucose-6-phosphate Isomerase</td>
</tr>
<tr>
<td>GPIIb-IIIa</td>
<td>Glycoprotein lib-lia complex</td>
</tr>
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<td>Glycoprotein VI</td>
</tr>
<tr>
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<td>Hyaluronan</td>
</tr>
<tr>
<td>IL1</td>
<td>Interleukin 1</td>
</tr>
<tr>
<td>ITP</td>
<td>Immune thrombocytopenia</td>
</tr>
<tr>
<td>IVM</td>
<td>Intravital Video Microscopy</td>
</tr>
<tr>
<td>K/BxN</td>
<td>KRNxNOD mice, or the model they produce</td>
</tr>
<tr>
<td>KC</td>
<td>chemokine in mice homologous to IL8 in humans</td>
</tr>
<tr>
<td>KRN</td>
<td>the KRN transgene, encoding the Vβ6 T-cell receptor</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metalloproteinase</td>
</tr>
<tr>
<td>MP</td>
<td>micrparticle</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet Derived Growth Factor</td>
</tr>
<tr>
<td>PGI2</td>
<td>Prostacyclin</td>
</tr>
<tr>
<td>PGIA</td>
<td>Proteoglycan Induced Arthritis</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear leukocytes</td>
</tr>
<tr>
<td>PSGL</td>
<td>P-selectin Glycoprotein Ligand</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid Arthritis</td>
</tr>
<tr>
<td>RF</td>
<td>Rheumatoid Factor</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe Combined Immunodeficient</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor Necrosis Factor α</td>
</tr>
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<td>Treg</td>
<td>Regulatory T-cell</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>VWF</td>
<td>von Willebrand Factor</td>
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Chapter 1

1 RHEUMATOID ARTHRITIS

1.1 Introduction to Rheumatoid Arthritis

Rheumatoid Arthritis (RA) is an autoimmune disease that eventually leads to chronic inflammation in synovial tissue surrounding articular joints (Figure 1), gradual loss of flexibility (synovitis), and can be characterized in the late stages of the disease by loss of cartilage and subchondral bone. Even though RA affects approximately 1% of the population, and is classified as an autoimmune disease, the molecular events which initiate the evasion of tolerance is speculative and unconfirmed. The innate immune and adaptive immune responses are both thought to contribute to arthritis, with bacterial infections gaining support as the event that triggers the innate and adaptive immune responses. Current methods of detecting RA include assessing the presence and levels of autoantibody in the circulation and in the synovial fluid. The autoantibody Rheumatoid Factor (RF) is a common diagnostic marker, though presence of RF is not necessarily indicative of disease, while autoantibodies to cyclic citrullinated proteins (ACCP) are another common marker, though these are only found in a portion of RA patients. Given the heterogeneity of autoantigens in RA, current work focuses on the effector phase of the condition; attempting to treat the disease by modification or alleviation of the inflammation and joint destruction.

Figure 1: Normal synovial tissue (a) compared to inflamed synovial tissue and cellular infiltrates in the rheumatoid joint (b). From REF(9)
Though the molecular events that initiate evasion of tolerance are not understood, the molecular mechanisms of inflammation in RA are widely studied, including the role of T and B-lymphocytes, macrophages, mast cells, and synovial fibroblasts, which have all been shown to have a role in the progression of inflammation and bone destruction. Figure 2 shows a summary of the various cellular mechanisms thought to control the progression of the disease in rheumatoid patients. An unidentified initiating event(s) results in the breakdown of tolerance and recruitment of lymphocyte to the joint, where cytokine and/or chemokine production attracts circulating leukocytes into the synovial tissue or activate resident synovial cells, concurrent with fibroblast activation and adhesion to cartilage through the formation of a pannus. Destruction of the joint itself can be readily explained by pro-inflammatory signals activating osteoclasts and fibroblasts to release matrix-metalloproteases (MMPs), directly degrading joint cartilage and subchondral bone in late-stage RA.

Figure 2: General inflammatory pathways mediating the development of arthritis. REF(9)
1.2 Molecular Mechanisms of Rheumatoid Arthritis

1.2.1 T-cells

T-cells appear to play a key role in initiating and maintaining the chronic joint inflammation found in RA patients, as reviewed by Panayi et al. While CD8+ T-cells are present in the inflamed synovium, the majority of T-cells are CD4+/Th1 cells, leading to local activation of macrophages and B-cells (Figure 2). T-cells within the rheumatoid synovium do not proliferate, requiring the constant recruitment of new cells to the synovium, the majority of which are of the CD4+ Th1 phenotype, though CD4+ Th2 and CD8+ cytotoxic T-cells are present in minor populations. Interestingly, while abundant in the rheumatoid synovial tissue, T-cells are found infrequently in the synovial fluid of RA, which is almost exclusively populated by neutrophils. Recent work examining the role of Th17 cells in RA has lead to debate as to whether RA should be understood to be a Th1 or Th17 disease (reviewed in REF). While Th1 and Th17 are thought to be distinct lineages of cells, data from Experimental Autoimmune Encephalitis (EAE) and Collagen Induced Arthritis (CIA) showed that some T-cells are capable of producing Interferon-γ (Th1) as well as Interleukin-17 (IL-17). As such, current opinion appears to favor the understanding that while these two lineages are capable of counter-regulating each other, there are instances where they are capable of co-operating in promoting these diseases.

T-regulatory cells (T_{reg}, CD4+CD25highFoxP3+) also appear to play a role in RA, albeit in an unconventional manner. CD4+CD25+ T_{reg} isolated from RA patients are capable of inhibiting the proliferation of effector T-cells in-vitro, but are incapable of preventing cytokine secretion from activated T-cells. The same study found that T_{reg} from RA patients express much lower FoxP3, and that both FoxP3 expression and suppressive action of these T_{reg} can be restored through administration of anti-TNF-α treatment. The development of HIV in an RA patients did not interrupt established disease, pointing towards the involvement of T-cell independent mechanisms contributing to joint destruction.
1.2.2 B-cells and Autoantibodies

The local response of resident B-cells in the synovial tissue is so strong that an early study comparing the immunoglobulin synthesis from synovial tissue of RA patients concluded that it is comparable to the production in normal human spleens. Though B-cells are understood mainly in their role as precursors for antibody producing plasma cells, they may also function as antigen-presenting cells (APCs) for local activation of T-cells, as shown by transfer of naive T-cells and rheumatoid B-cells into SCID mice, resulting in activation of T-cells in the synovial tissue. While the exact role of these B-cells is unconfirmed, histological examination has noted that they develop follicle-like structures in close proximity to CD4+ and CD8+ T-cells within the synovial tissue, and that these bear a phenotypic resemblance to germinal centres in the peripheral lymphoid tissue. Anti-B-cell treatments (such as Rituximab), are thought to be therapeutic by depleting peripheral and synovial B-cells, thereby interfering with both antibody production and by interrupting local B-cell dependant T-cell activation.

The role of autoantibodies can be traced back to the 1940s, when two different groups noted the presence of an agglutination factor in the serum of patients with RA. The so-called “rheumatoid factor” (RF) is IgM antibodies targeting the Fc-portion of IgG, forming immune complexes with endogenous IgG, and is detectable in approximately 80% of RA patients. RF produced by plasma cells in synovial tissue, is known to fix and activate complement locally within the synovium, and the periodic flares of clinical pathology in RA patients appears to correlate with an increased number of RF-secreting cells in the synovium of patients. A second class of autoantibodies are directed against cyclic citrulline-modified proteins and peptides (ACCP). Citrulline-modified proteins may be created through abnormal protein metabolism in the synovium, resulting in the antigenic peptidylarginine conversion to citrulline through deamination. While their exact cause is speculative, the presence of ACCPs is highly correlated with a diagnosis of RA. The exact cause of citrulline-modification of proteins in RA is not fully understood, though a popular theory proposes that citrullinated proteins occur in synovial macrophages and monocytes. More recent research notes that a pathogenic strain of bacteria (P. gingivalis) has been linked to RA, and when present in the oral and intestinal cavities is capable of citrullinating proteins.
1.2.3 The Complement Cascade

Following the discovery of the role of immune complexes in RA, complement became another suspected contributor to inflammation in RA, which was confirmed in 1991\(^33\). While complement components in the serum can be maintained by synthesis in the liver, local synthesis in rheumatoid joints has also been demonstrated by resident synovial macrophages and fibroblasts\(^34\), as well as endothelial cells and the synovial membrane\(^35\). Immune complexes formed of RF-IgM can activate the classical complement pathway\(^36\), while ACCP from RA patients can activate both the classical and alternative pathways *in-vitro*\(^37\). Various cartilage molecules are capable of binding complement component C1q, though their effects are varied. Binding of C1q to decorin or biglycan (modifications attached to intact cartilage) results in the inhibition of complement activation when normal cartilage structure is maintained\(^38\). However, under inflammatory conditions cartilages can be degraded to produce fibromodulin, which is capable of activating the classical complement pathway\(^39\).
1.2.4 Mast Cells

Mast cells have been described as “immune sentinels” in arthritis due to their proximity to the vasculature in rheumatic joints\(^5\), and the histological evidence for their degranulation\(^40\). In the context of arthritis, mast cells can be activated by immune complexes through Fc\(\gamma\)RIII\(^41\) and complement component 5a (C5a)\(^42\), resulting in degranulation and release of chymase\(^43\) and tryptase capable of converting C5 to C5a\(^44\). Degranulation of mast cells also results in the release of chemokines for the recruitment of leukocytes into the joint, release of cytokines such as Interleukin 1 and 4 (IL-1/4), Tumor Necrosis Factor \(\alpha\) (TNF-\(\alpha\)), as well as the release of various growth factors such as Vascular Endothelial Growth Factor (VEGF) and basic Fibroblast Growth Factor (bFGF) (Figure 3 below, reviewed in \textit{REF}^45). The high levels of pre-formed histamine\(^46\) and proteases\(^47\) in mast cell granules may provide the “kickstart” in the joint by inducing vascular permeability and activating nearby synovial fibroblasts.

\textbf{Figure 3:} Mast cells in synovial inflammation. Activation of mast cells through their Fc\(\gamma\) or C5a receptors results in degranulation, releasing proinflammatory cytokines, chemokines, and growth factors into the synovial tissue. Mast cells can recruit and activate leukocytes, activate fibroblasts, drive the differentiation of osteoclasts, and promote angiogenesis in the inflamed synovium. From \textit{REF}(5)
1.2.5 Synovial Fibroblasts and Monocytes

One of the cells activated by mast cells are synovial fibroblasts residing in the synovial tissue and the synovial lining, which can be activated by IL-1, IL-4, TNF-α, tryptase and histamine. Interestingly, activated fibroblasts can “talk-back” to mast cells, inducing mast cell accumulation of tryptase through production of IL-33. While fibroblast’s normal function is to produce extracellular matrix (ECM) components such as hyaluronan and lubricin, a hyperplastic synovial lining can produce IL1, as well as induce monocyte maturation into osteoclasts (Figure 4 below). The proliferation of fibroblasts is also proposed to induce MMP production through engagement of cadherin 11 on adjacent activated fibroblasts. When their various molecular mechanisms are taken into account, the evidence appears to point to the synergistic effect of mast cells and Fibroblast Like Synoviocytes (FLS) to promote synovial inflammation.

Monocytes involved in RA can be divided into two classes: those expressing CD14, L-selectin, and CCR2, which migrate into and become activated at sites of inflammation; and CD16+CCR2-monocytes that enter synovial tissue and become resident macrophages independent of inflammatory stimuli. Of these two types, only the first (CD16-) are capable of differentiation into osteoclasts, contributing to the direct destruction of mineralized cartilage and subchondral bone. Osteoclasts have been identified as the primary cell responsible for bone resorption, and in healthy synovial tissue maintain a careful balance with osteoblasts for renewal of bone structures. In arthritis, osteoclasts are found to significantly outnumber osteoblasts in the joint, with their presence correlating with bone destruction in RA patients. The maturation of monocytes into osteoclasts is driven by RANKL on fibroblasts, TNF-α, IL-1, and Th17 cells producing IL-17, allowing developing inflammation to drive bone destruction.
1.3 Role of Angiogenesis in Rheumatoid Arthritis

Interestingly, angiogenesis has also been shown to be a significant event in RA\(^6^3\), lending credibility to the concept that the various steps of angiogenesis may be critical in the development of RA. An early review suggests that the development of the pannus initially requires the development of new vessels within the forming pannus, but that in late-stage RA the pannus has changed into a “relatively avascular, fibrotic tissue”\(^6^4\). Supporting this, an early study found that only inflamed joints in RA patients showed vascular proliferation, as compared to un-inflamed joints from the same patient\(^6^5\). Vascular proliferation appears to be RA-specific, as osteoarthritic patients showed very little endothelial cell proliferation compared to RA patients\(^6^6\). Severe inflammation of the synovium increases to volume of the tissue surrounding the joint, which would create a hypoxic local “environment” that the body would attempt to counteract through the formation of new vessels\(^6^7\).

There are several factors mediating angiogenesis in RA, including Platelet Derived Growth Factor (PDGF), TNF-\(\alpha\), and IL-8\(^6^7\), all of which have been demonstrated to have significant influences on the progression of mouse models of arthritis (discussed in later chapters). High levels of Vascular Endothelial Growth Factor (VEGF) can be found in severely inflamed joint tissue of mice, likely produced by local activation of cells in the synovium. Treatment with soluble VEGF receptor was able to reduce the level of inflammation and vascularity in a mouse model of inflammation\(^6^8\). Fibroblasts and mast cells can produce various growth factors when activated, including vascular endothelial growth factor\(^7^,4^8\), allowing them to not only promote the influx of cells into the joint, but also to support the increase in vascular supply to the inflamed tissue through vascular remodeling. While examination of the active participation of the circulatory system has only been deemed important over the last few decades, this evidence has undoubtedly opened a new field of study in RA.
2 MOUSE MODELS OF INFLAMMATORY ARTHRITIS

2.1 The Collagen Induced Arthritis Model

The Collagen Induced Arthritis (CIA) model of RA was initially developed in rats, but by 1980 was also developed in certain mouse strains through injecting mice with heterologous type II collagen (CII) and Complete Fruend’s Adjuvant (CFA). The pathology of CIA resembles RA in that it is characterized by severe synovial inflammation in early development, and erosion of bone and cartilage in the later stage. Expression of a compatible MHC II molecule (e.g., 1-Aq haplotype on the DBA/1 background) is required for induction of CIA using chick CII, and while other strains of mice develop immune responses to chick CII, they do not develop arthritis. Cyanogen Bromide has been used to digest CII, and the 11th peptide produced was found to contain the antigenic determinant, which was further discovered to induce a strong CD4+ T-cell response in susceptible mice. Depletion of T-cells using specific anti-serum resulted in a delayed onset, and reduced pathology, of arthritis in rats. These T-cells promote the production of anti-CII autoantibodies, resulting in IgG immune complexes and complement component 3 (C3) deposits on articular cartilage surface before the development of inflammation of the joint. Interestingly, while single monoclonal antibodies to CII are not effective on their own, a cocktail of mAbs to CII has been shown to induce arthritis in mice that are not considered susceptible to CIA, supporting a role for immune complexes in this model. A possible explanation for this phenomenon is the fact that the Fc Receptor γ-chain (FcRγ) is required on macrophages from DBA/1 mice to produce an effector response upon stimulation with CII immune complexes. The FcγR’s appear to play a critical role in the development of arthritis, as FcR-gamma chain knockouts are resistant to CIA and do not develop edema in the paws. In contrast, FcγRIIB knock-out mice develop a more severe arthritis, with higher titers of anti-CII IgG, compared to controls.
Figure 5 illustrates various pathways and processes which occur after antibodies enter the joint, such as initiation of the alternative complement cascade, extravasation of monocytes, neutrophils, and lymphocytes, which are all observed in conjunction with inflammation of the synovial tissue. Neutrophils were shown to be an extremely important contributor to arthritis in this model, as depletion of neutrophils reduced inflammation by almost half. The production of proinflammatory cytokines by these cells is an important factor in the CIA model, and has been reviewed by Myers et al, specifically examining the roles of TNF-α and IL-1. Though not sufficient to establish autoimmune arthritis on its own, IL-1β does induce synovial inflammation when injected into the joints of naive mice, and accelerated the onset of arthritis when administered prior to immunization. Similarly, TNF-α is also capable of inducing synovitis when injected into the joint, and treatment with TNF-α neutralizing antibodies reduced arthritis severity in CIA, while not affecting anti-CII IgG production. These cytokines appear to work in series towards the development of arthritis, as a TNF-α transgenic mice spontaneously develop chronic inflammatory arthritis. The arthritis appears to be the product of a self-
perpetuating cycle of TNF-α-induced production of IL-1β, inducing further inflammation and
production of TNF-α, which can be prevented by treatment with antibodies against TNF-α or
with soluble IL-1 receptor\textsuperscript{87}. However, while TNFα receptor knockout mice are less susceptible
to the development of arthritis, joints that do develop inflammation are comparable to wild-type
controls\textsuperscript{88}. Therefore, TNF-α appears to promote the autoimmune reaction of CIA, as well as
local inflammation, which in also be controlled by IL-1 in a TNF-α-independent mechanism.
2.2 The Proteoglycan Induced Arthritis Model

Proteoglycan Induced Arthritis (PGIA) is a model of arthritis that shares many similarities with the CIA model of arthritis. Instead of collagen, immunization of susceptible BALB/c mice with the cartilage proteoglycan (PG) aggrecan in adjuvant induces a progressive arthritis in these mice\(^8^9\). These mice develop a strong T-cell response to PG, promoting B-cell production of anti-PG antibodies\(^9^0\). Similar to CIA, administration of a T-cell depleting antibody in these mice protects them against the development of arthritis, while T-cell transfer from arthritic mice to naive recipients induces arthritis\(^9^1\), as can a PG-specific T-cell hybridoma\(^9^2\). While T-cells are required for initiation of the autoimmune response, and possibly contribute to maintenance, polymorphonuclear neutrophils (PMNs) are the main cells recruited to the synovial tissue during inflammation as monitored by Intravital Video Microscopy (IVM)\(^9^3\). Fc\(\gamma\)RIII is absolutely required for arthritis development in this model, suggesting that stimulation of synovial cells through immune complex cross linking of this receptor may play a critical step in establishing inflammation in the joint\(^9^4\). As in CIA, elevated levels of TNF-\(\alpha\), IL-1\(\beta\), and anti-PG antibodies can be detected in the sera of mice exhibiting PGIA\(^9^5\).
2.3 The K/BxN Passive Transfer Model

The K/BxN mouse model of arthritis was discovered after the observation that crossing of KRNxNOD (K/BxN) mice results in F1 progeny that spontaneously develop severe inflammatory arthritis in all distal joints within weeks. Further research from the same lab showed that transfer of either splenocytes or serum from K/BxN F1 mice results in severe arthritis developing relatively quickly (8 days and 2 days respectively). While the identity of the re-arranged T-cell receptor (TCR, Vβ6) had been known since the discovery of the K/BxN strain, later screening of various tissue extracts with K/BxN serum showed a ubiquitous molecular target, identified as glucose-6-phosphate isomerase (GPI). GPI is a cytoplasmic enzyme which converts glucose to fructose in glycolysis, and can be released after cellular damage, or presented on the surface of the synovial lining. Basu et al examined the exact epitope of GPI presented, and determined that the GPI\textsubscript{282-294} peptide was responsible for the autoimmune reaction observed in the K/BxN strain. The role of GPI and anti-GPI IgG antibodies appears to be clinically relevant, as a portion of RA patients have been identified with elevated anti-GPI IgG titers compared to non-arthritic or osteoarthritic controls.

Although there is no joint-specific isoform of GPI, GPI was found to circulate at low concentrations, and is highly concentrated on the articular cartilage surface. They also note that these GPI concentrations co-localize with IgG immune complexes and complement component 3 (C3) in the joint, and that accumulation occurs early (well before fully developed ankle inflammation). They suggest that some unique feature of the joint (perhaps the lack of membrane-bound inactivators of the alternative complement cascade) allows for the joint-specific response to a ubiquitous antigen. Further work showed that anti-GPI antibodies can be found deposited in the joint within minutes of injection, which lends support to the idea that the vasculature of the joint has an critical role in allowing or promoting the initiation of passively induced arthritis, as opposed to an adaptive molecular response to the injection of anti-GPI antibodies. The transfer of K/BxN serum can induce arthritis independent of lymphocytes, as shown by transfer of K/BxN serum into lymphocyte deficient mice.

The actual molecular mechanism of the passive transfer model remains incompletely understood, though there does appear to be 3 distinct phases in arthritis progression. The first stage appears to involve the permeabilization of the vasculature of the joint, mediated by the arthritis serum.
Injection of K/BxN serum or heat-aggregated control IgG results in leakage of an immune-complex sized fluorescent marker from the vasculature into the joint within 10 minutes of serum injection, though this leakage is not observed in other tissues. Using genetically deficient mice and a granulocyte depleting antibody, this group also identified mast cells, neutrophils, and the low-affinity FcγRIII as critical for this leakage, though complement (both C3 and C5) and IL1 do not appear to play a role at this stage. Interestingly, injection of serotonin and histamine also induce localized leakage at the joint, even in the absence of mast cells, neutrophils and the FcR-γ chain. In addition, the vascular leakage in susceptible mice results in an outwardly visible “flare” (edema) in the ankle which becomes apparent within 30 minutes of injection of K/BxN serum but then fades within several hours.

The second phase (pictured in Figure 6, below) could then be the result of immune complex and/or deposited serum in the joint, activating complement, resident mast cells, neutrophils, and macrophages, causing the release of proinflammatory cytokines, such as TNF-α and IL-1β. Neither C5, nor FcR-γ deficient mice develop arthritis when injected with K/BxN serum, while arthritic mice actually undergo rapid reverse in inflammation when treated with an anti-C5 mAb. Further experiments showed that a lack of C4 does not prevent arthritis development, suggesting that the alternative compliment pathway is responsible for the development of arthritis in this model. In the alternative pathway, C3 is degraded into C3a and C3b, which when bound to Factor B forms the C5 convertase, capable of creating C5a (stimulating resident mast cells).
The articular surface is lacking in resident complement regulators, which may explain why GPI and C3 co-localize to this surface, but not others, early in the development of arthritis. Neutrophils and C5a are both absolutely required for arthritis development in this model, are assumed to be the reason this model progresses independently of lymphocytes. C5a has both neutrophil chemotactic and degranulation activity; and neutrophils are capable of producing properdin, C3, and factor B, creating the possibility for a positive-feedback loop driving inflammation. In addition to activating neutrophils, C5a (and to a lesser extend C3a) are also capable of activating mast-cells, which may also contribute to a positive-feedback by releasing tryptase (also capable of cleaving C5 into C5a) and histamine (further activating the endothelium). Stimulation of neutrophils and mast cells also allows for the production of many pro-inflammatory cytokines, including TNF-α and IL-1β, which have been shown to be important in the development of arthritis in the K/BxN model.

Immune complex activation of Fcγ receptors also appears to play a critical role in the development of K/BxN arthritis, as the FcRγ chain is absolutely required for the development of arthritis. Given the predominance of IgG1 anti-GPI antibodies in K/BxN serum, signaling was initially assumed to occur mainly through the low affinity IgG FcγRIIIA, which reduces inflammation when knocked-out. Additional proof was thought to lie in the fact stimulation of mast-cells using immune complexes or monoclonal antibodies results in the release of IL1β from mast cells. However, recent work using a mouse strain deficient in FcγRI,IIb,IIIA and FcεRI,II demonstrated that large amounts of IgG2 from K/BxN serum can signal through the high affinity FcγRIV on neutrophils and monocytes and develop arthritis normally, though the authors acknowledge that IgG1 antibodies are likely responsible for the majority of inflammation in the normal K/BxN model. While expression of the inhibitory FcγRIIB receptor does not prevent development of arthritis in the K/BxN model, mice deficient in FcγRIIB develop arthritis earlier and exhibit more severe inflammation than controls. In addition to the effects of neutrophils and mast cells in initiating and promoting inflammation, monocytes/macrophages are also thought to play a part in the inflammatory process. Macrophages found in the synovial tissue express FcγRIII, and respond to stimulation with immune complexes by expressing large amounts of IL1, promoting increased inflammation and damage to the joint. These factors may all explain the transient nature of the K/BxN serum transfer model, whereby anti-GPI immune complexes activates resident synovial cells and promotes the production of C5a, TNF-α, and IL-
1β, but gradually decreasing levels of anti-GPI immune complexes eventually results in the loss of this model’s driving force, and an eventual a return to normalcy.

The final phase (perhaps concurrent with the second phase) results in osteoclast- and MMP-mediated degradation of the bone structure. Pettit et al note that swelling of the joint is independent of bone erosion, and that this erosion is caused by activated osteoclasts\textsuperscript{118}. Using an osteoclast deficient strain, they note that while these mice do develop inflammation at the joint, histopathology does not detect erosion of the bone. These observations support the dual roles of IL-1 and TNF-α in this model whereby these cytokines promote inflammation, but where possible also drive the differentiation of monocytes to osteoclasts, resulting in bone erosion\textsuperscript{7}. The erosions resulting from serum transfer are histologically significant, but the joints return to normal within 5 weeks, implying osteoblasts are capable of “reconstructing” the joint after the arthritic serum has run its course\textsuperscript{97}. Synovial fibroblasts comprise a large population in of cells bone erosions\textsuperscript{54}, and are thought to participate by production of MMPs\textsuperscript{55} and driving the formation of osteoclasts\textsuperscript{53}. 
Chapter 3

3 PLATELETS AND THEIR FUNCTION

3.1 Platelets and Hemostasis

Platelets are small (approximately 2-3μm) anucleated particles derived from megakaryocytes (large polyploidy precursor cells found in the bone marrow)\textsuperscript{119}. Platelets express various integral membrane proteins (integrins) key to the formation of a platelet “plug” at sites of endothelial injury. The GPIb-IX-V complex is present on resting platelets, and initiates platelet adherence through moderating platelet adhesion to von Willebrand factor (VWF) on activated vascular endothelial cells\textsuperscript{120}. GPVI and GPIa-IIa provide more stable adhesion to exposed collagen at the site of injury, allowing GPVI and GPIb-IX to activate the GPIIb-IIIa complex, allowing further networking of platelets mediated by fibrinogen and fibronectin\textsuperscript{121}. A more comprehensive review of platelet integrins in adhesion and aggregation can be found in the review by Ni and Freedman\textsuperscript{122}.

Platelets play a key role in angiogenesis, a process which we have already introduced as being important to the development of synovial inflammation in RA, and also in the mediation of tumor development\textsuperscript{123}. Platelets contain several important factors, including Vascular Endothelial Growth Factor (VEGF) for the permeabilization of the endothelial wall, MMPs to assist in the migration of endothelial cells, and Platelet Derived Growth Factor to promote maturation of the newly formed blood vessels\textsuperscript{121}. Based on this information alone it is conceivable that platelets play an role in inflammation and the immune response, a topic we will discuss below.
3.2 The Role of Platelets in Immune Response

While the traditional view of platelet function involves maintenance of hemostasis, a large body of evidence is arising to support their role in the inflammatory immune response (reviewed in REFs 124, 125). Of particular interest to this current study is the interplay between platelets and leukocytes in the circulation, especially neutrophils and monocytes (two key cells in the K/BxN model) which appear to bind platelets in whole blood samples, and bind even more avidly when platelets are activated126. Platelet-leukocyte complexes appear to have clinical relevance, as patients with Rheumatoid Arthritis have an elevated number of these complexes compared to controls127. The binding of platelets and leukocytes is thought to initially involve P-selectin on platelets (CD62P, GMP-140)128 binding to P-selectin Glycoprotein Ligand (PSGL) on leukocytes, with the activation of other leukocyte integrins for firm adhesion129. Platelets can also express soluble CD40L and IL-1β when activated, which can then mediate expression of adhesion molecules and pro-inflammatory chemokines on endothelial cells130,131. Platelets also serve as a circulating source of serotonin and histamine124, and can release vascular endothelial growth factor (VEGF) upon activation132. The activation of platelets through various pathways can induce the release of platelet microparticles, which appear to play a role in the promotion of inflammation (reviewed in REF 133). Taken together, these results all support an active role of platelets in inflammatory diseases, including Rheumatoid Arthritis, where their role in the disease is actively being investigated.

Platelets are known to contain two types of granules: dense granules containing serotonin and ADP, and alpha-granules containing PDGF, TGF-β, PF4, as well as P-selectin integral to the membrane121. The serotonin found in dense granules is present at an approximate concentration of 2-3 nmol per 10⁹ human platelets, with men having slightly higher concentrations than women, and a general decrease in serotonin content in older age groups134. Interestingly though, collagen or thrombin-activated platelets from older rats release much more serotonin relative to those taken from younger rats135. Platelet-derived serotonin is a factor in mouse models of arthritis, promoting vascular permeability136. Platelets also contain unspliced IL-1β RNA, which is rapidly spliced and translated following platelet activation by collagen or LPS, resulting in the release of platelet microparticles presenting IL-1β137, a pro-inflammatory mechanism we will discuss below.
3.3 The Role of Platelets in Inflammatory Arthritis

Inquiry into the connection between platelets and Rheumatoid Arthritis dates back to the 1950’s and 1960’s, where studies examined platelet counts as a result of RA treatment and general reduction in platelet counts in 7 patients suffering from RA. Later, in 1977, Ginsberg et al. noted the presence of platelets in the synovial space, though a functional role for platelets has only recently been demonstrated. Work by Boilard et al. recently identified a significant population of CD41+ (GPIIb) positive particles in the synovial fluid (SF) of patients with RA. These particles can be found free in the synovial fluid, or bound to CD45+ synovial leukocytes, a finding that has been demonstrated before in animal models of arthritis using IVM. Boilard et al. did not conduct a double stain to determine if any of these populations co-express both platelet and leukocyte markers, which has been previously demonstrated to occur, and claimed these particles to be derived from platelets.

Further experiments using both Fluorescence-Activated Cell Sorting (FACS) and cellular imaging confirmed that these CD41+ micro particles were much smaller than, and derived from, normal platelets, and could be found both free in the circulation or bound to synovial leukocytes obtained from RA patients. Boilard et al. used the K/BxN of arthritis to examine the role of platelets in inflammatory arthritis in mice, and found that depletion of platelets using a cocktail of anti-CD42b (GPIb) monoclonal antibodies (mAbs) significantly reduced the development of arthritis in this model. However, it is important to note that such a polyclonal mix of anti-CD42b antibodies might be capable of inhibiting primary adhesion of platelets through disrupting platelet-von Willebrand factor (VWF), complicating interpretation of this data, which we will discuss later in this report.

Inhibition of several standard pathways for MP formation (ADP, TxA2, GPIb) had very little effect on the progression of disease or MP formation. Boilard et al. then cultured platelets with primary mouse Fibroblast-Like Synoviocytes (FLS), and noted a significant increase in the number of platelet MPs. However, when platelets from mice deficient in either FcR-γ chain or GPVI were cultured with FLS, they formed significantly fewer platelet MPs in-vitro confirming that collagen induced activation of GPVI (likely from ECM components released by FLS) results in FcRγ-signaling, resulting in the release of MPs.
Human platelets were also cultured with human FLS or a GPVI ligand, and both conditions resulted in the formation of platelet MPs. The requirement for GPVI inducing platelet MP formation was then confirmed by passive transfer of KRN serum into Gp6/- mice, which developed less severe synovial inflammation/erosion of the joint than their wild-type controls. Co-incubation of collagen-stimulated human platelet MPs with FLS resulted in a significant release of IL-6 and IL-8, as did incubation of MPs from RA synovial fluid with FLS.

Platelet MPs from mice deficient in IL-1 α/β were unable to induce production of KC (ortholog of IL-8 in mice) in FLS, suggesting IL-1 on platelet MPs is required to stimulate KC/IL-8 production by FLS. A deficiency in the receptor for IL-1 on FLS also prevented normal KC production after platelet MP stimulation, as did incubation with IL-1 neutralizing antibodies. Similar experiments were conducted using human MPs and FLS, with nearly identical results. The outcome of these experiments presented an interesting link between platelets/MPs, cytokine mediated recruitment of immune cells, activation of resident cells, and corresponding inflammation. While disruption of the collagen-stimulation of MP formation reduced arthritis severity in GPVI-deficient mice, the reduction was only about 50% in all tests, while depletion of platelets almost eliminated arthritis. Boilard et al. interpret the difference between these two results to suggest that while collagen-activation of platelets to form MPs is an important step in arthritic inflammation, there are other mechanisms through which platelets and MPs modulate inflammation. A proposed model is shown in Figure 7 (below), whereby platelets may gain

**Figure 7:** Platelet microparticles form and infiltrate into synovial fluid. Platelets in the synovial vasculature are exposed to collagen of the synovial tissue, resulting in the production of platelet microparticles (MPs). These microparticles may then bind to synovial fibroblasts and neutrophils, activating them through IL-1 expression, promoting inflammation and leukocyte recruitment to the joint. From *REF(143)*
access to the synovial tissue through fenestrations in the endothelium. Once platelets enter the
tissue (possibly bound to leukocytes), they would become activated through the GPVI receptor,
and form platelet MPs. These MPs allow for IL-1-dependent stimulation of synovial cells (eg
fibroblasts), and up regulate the release of IL-8. Monocytes and neutrophils would then be
recruited to the joint, and once activated, further promote the inflammation of the joint.

Previous work has noted high levels of prostaglandins are found in the synovial fluid of RA
patients\textsuperscript{145}, and that pharmacological inhibition of Cox-1 (an obligate enzyme in the production
of prostaglandins) significantly reduced the production of PGI\textsubscript{2} and almost completely
ameliorated arthritic symptoms\textsuperscript{146}. Based on this rationale, Boilard \textit{et al.} set out to determine if
intact platelets might contribute to the production of PGI\textsubscript{2}\textsuperscript{147}. Using Cox-1 null mice, the authors
found that transfusion of platelet-rich plasma allowed for the development of arthritis, while
transfusion of platelet poor plasma resulted in no arthritic symptoms. However, further
examination showed that platelets were not themselves capable of synthesizing PGI\textsubscript{2}, leading the
authors to speculate that platelets might produce PGH\textsubscript{2}, which could in-turn stimulate FLS into
producing PGI\textsubscript{2}, an idea shown previously using endothelial cells\textsuperscript{148}. Indeed, culturing intact
platelets with FLS could induce secretion of PGI\textsubscript{2} \textit{in-vitro}, though no secretion was observed
when platelet MPs were used. Using platelets deficient in FcγR or GPVI, the author’s found
production of PGI\textsubscript{2} was not impaired in either case, pointing to some other source of platelet
activation to initiate this cascade. These findings were supported by similar findings in the CIA
model of arthritis where blockage of PGI\textsubscript{2}-PGI receptor signaling dramatically reduced vascular
leakage, the production of proinflammatory cytokines in the joint, and arthritic scores in
comparison to control mice\textsuperscript{149}. Taken together, this evidence indicates both platelets and platelet
microparticles appear to play an active role in promoting arthritis in the K/BxN model.
CD44 AND ITS ROLE IN INFLAMMATION

4.1 Molecular Characteristics of CD44

The inflammatory process involves homing and recruitment of cells to the site of inflammation, which is mediated by several different cellular receptors, including CD44. CD44 (Pgp-1, HCAM) is an integral single-chain glycoprotein expressed on almost every cell type in mice and humans\(^{150}\), with a conserved extracellular region for binding hyaluronan (HA), a stem region allowing for inclusion of up to 10 variant exons\(^{151}\), and a conserved transmembrane domain and cytoplasmic tail which is linked to the cytoskeleton through an adaptor protein\(^{152}\). The most common form of CD44 is the standard hematopoietic form containing no variable regions (CD44s), though many different isoforms can be created through alternative splicing. The DNA and amino acid sequences for CD44 in mice, rats, and humans is highly conserved, both in the standard and variant regions\(^{153}\). The only splice isoform implicated in RA is formed with the variable exons v3-v10 (CD44v3-v10) with an inserted trinucleotide, which is expressed on synovial fibroblasts of some RA patients, though the exact function of this isoform is unknown\(^{154}\). In addition to splice isoforms, post-translational modification can also influence the role of CD44, including N-linked glycosylation\(^{155,156}\), O-linked glycosylation\(^{156}\), the addition of Sialic Acid\(^{157}\), and the addition of chondroitin sulfate\(^{158}\). These post-translational modifications may be regulated by deglycosylating enzymes and neuraminidase, and the removal of these modifications often results in an increased affinity for HA\(^{159}\). Functionally, CD44 is thought to exist in one of three conformational states: non-binding, inducible-binding, and constitutionally binding HA\(^{160}\). Not all strains have identical expression of CD44 (Ly-24 or Pgp-1), with models used in antigen induced arthritis (DBA/1, BALB/C) having higher T-lymphocyte expression of CD44 (Ly-24.1) than the C57BL/6 strain used in the K/BxN model (Ly-24.2)\(^{161}\).
Complicating interpretation of experimental data is the fact that CD44 does not always remain as an integral protein, but can be released as soluble CD44 (sCD44) by cleavage with Membrane-Type 1 Matrix Metalloproteinase (MT1-MMP) and A Disintegrin and Metalloprotease 10 or 17 (ADAM10/17)\textsuperscript{162}, or by intracellular cleavage into a CD44 fragment that promotes transcription of several genes including that of CD44 itself\textsuperscript{163}. Soluble CD44 can also have a competitive effect, blocking cell-surface CD44 from binding HA in tumor formation and metastasis\textsuperscript{164}.
4.2 CD44 as a Homing and Adhesion Molecule

While CD44 may contribute to regulation of HA metabolism, CD44 can also function in normal tissues by acting as an adhesion molecule, presenting growth factors or cytokines, and as an “outside-in” signaling molecule for either apoptosis or cellular proliferation\(^\text{165}\). An early report noted the increased expression of CD44 on T-lymphocytes expressing the activated memory phenotype CD62L\(^\text{lo}\)/CD44\(^\text{hi}\)\(^\text{166}\). CD44 can also be found on CD34\(^+\) hematopoietic stem cells (HSCs), and has been implicated in assisting their homing to the bone marrow\(^\text{167}\), though CD44-null mice develop normally with no deficiency in thymocyte, lymphocyte, or HSC homing to their appropriate cellular compartments\(^\text{168}\). Other data suggests that CD44 is expressed on regulatory T-cells (T\(_\text{regs}\)), where the ability of T\(_\text{regs}\) to bind HA indicates a superior suppressor activity\(^\text{169}\). Interestingly, treatment with high molecular weight HA is able to further enhance the suppressor activity of activated T\(_\text{regs}\) \textit{in-vitro}\(^\text{170}\), possibly due to the up regulation of the FoxP3 transcription factor.

Given that both HA\(^\text{171}\) and CD44\(^\text{150}\) are ubiquitously expressed, it stands to reason that CD44 must be activated in order to promote this binding. Indeed, work has shown that inflamed capillaries promote “selectin-like” binding of CD44 for leukocytes rolling on endothelial cells \textit{in-vivo} and that there is a requirement for extra-cellular CD44 and HA expressed on endothelial cells (ECs) for extravasation of leukocytes into the site of inflammation\(^\text{172}\). TNF-\(\alpha\) appears to play a large role in this mechanism, increasing expression of HA on ECs derived from the microvasculature, though no such effect is found in ECs from large vessels\(^\text{173}\). TNF-\(\alpha\) is also able to induce HA binding in human peripheral blood monocytes\(^\text{174}\), possibly through the inhibition of post-translational modification of CD44\(^\text{175}\). Interestingly, CD44 is required on both rolling lymphocytes and endothelial cell, suggesting a “sandwich” model whereby CD44 on rolling cells

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<tr>
<th>CD44-associated functions</th>
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<tr>
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<td>Leukocytes, lymphocytes, ECs, metastatic tumor cells</td>
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<td>Adhesion</td>
<td>Leukocytes, lymphocytes, fibroblasts, ECs, platelets</td>
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<td>Proliferation</td>
<td>Tumor cells, ECs, fibroblasts, smooth muscle cells</td>
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<tr>
<td>Differentiation</td>
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<td>Phagocytosis</td>
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<td>ECM regulation</td>
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<td>Angiogenesis</td>
<td>ECs, smooth muscle cells</td>
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<td>Release of cytokines</td>
<td>Macrophages, T-cells, NK cells, PMNs</td>
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ECM: Extra Cellular Matrix, EC: Endothelial Cell, NK: Natural Killer cell, PMN: Polymorphonuclear Neutrophil. Adapted from \textit{REF(164)}
binds to HA presented by CD44 on activated endothelial cells\textsuperscript{176}. Similar requirements have been observed in neutrophil\textsuperscript{177} and macrophage recruitment\textsuperscript{178}. 
4.3 CD44 and Phagocytosis

In addition to the role for CD44 in leukocyte rolling and extravasation, early work has suggested that CD44 may have a co-stimulatory role in the clearance of microbial pathogens\(^\text{179}\) and apoptotic cells\(^\text{180}\), though the assumption was that CD44 acted purely as an adhesion molecule to HA. However, CD44 was later shown to mediate phagocytosis of molecules coated with CD44 ligands (HA or anti-CD44 antibodies), in a mechanism distinct from that mediated by the Fc\(\gamma\) receptors\(^\text{181}\). Further inquiry into the mechanism found that ligation of CD44 with soluble HA increased the ability of macrophages to phagocytose iC3b coated erythrocytes to a level comparable with PMA stimulation, and that CD44’s activation was not due to a physical interaction with Complement Receptor 3\(^\text{182}\), but was likely mediated by an “inside-out” activation mechanism. One theory suggests that some form of cytokine activation induces a conformational change in CD44 through a change in the conformation of its cytoplasmic tail and its interaction with the actin cytoskeleton\(^\text{183}\).

While the majority of HA is metabolized through the lymphatic system, regulation of HA metabolism also appears to be partially regulated through CD44-mediated degradation and synthesis in local tissue\(^\text{184}\). CD44 has previously been found to mediate the uptake of HA by macrophages expressing CD44\(^\text{185}\), and a lack of CD44 positive cells in the lung has been linked to persistent accumulation of low molecular weight HA (a product of local inflammation)\(^\text{180}\). In-vitro experiments in COS-7 cells (monkey fibroblasts) have also noted that CD44 may play a role in resident cells regulating the binding hyaluronan in the ECM, endocytosis and metabolism of HA, and the assembly of the pericellular matrix in the synovial tissue\(^\text{186}\).
4.4 CD44 in Mouse Models of Inflammatory Arthritis

The data regarding targeting CD44 as a therapy for inflammatory arthritis in mouse models varies, both in model, dosage, interval of treatment, and reported effectiveness. A study by Nedvetzki et al utilized the CIA model on the DBA/1 background, and reported that repeated injections of anti-CD44 mAbs IM7.8.1 (IM7), KM81, and IRAWB14.4 were “anti-arthritisogenic” in developing arthritis, though it is important to note that these mice maintained their pre-treatment levels of inflammation\(^{187}\). A similar study conducted in the same year induced arthritis in BALB/C mice using the PGIA model, and found that daily injections of KM201 and IM7 decreased inflammation in arthritic mice, while treatment with IRAWB14 slightly increased the severity of inflammation\(^{188}\).

The efficacy of anti-CD44 antibodies in mice models of arthritis so far has been attributed to an interference of CD44 function on leukocytes. Treatment with the IRAWB14.4 monoclonal antibody can significantly increase CD44 binding to HA \textit{in-vitro}\(^{189}\) and \textit{in-vivo}\(^{188}\), which may be attributed to CD44 clustering on the cell surface, though it remains possible that the antibody could induce a conformational change in CD44. Various monoclonal antibodies to CD44 are capable of blocking leukocyte binding to HA, including KM201\(^{188}\), KM114\(^{190}\), and KM81\(^{191}\). While IM7 does not appear to have a significant effect on leukocyte adhesion to endothelial cells\(^{142}\), treatment of mice in the PGIA model with both IRAWB14.4 and IM7 resulted in a significant increase in soluble CD44 in the serum\(^{188}\), most likely through cleavage by MT1-MMP. While genetic knock-out of CD44 would be expected to provide similar anti-inflammatory results, experiments have shown that CD44-deficient mice showed moderate\(^{192}\) or no resistance to CIA\(^{193}\), possibly explained by up regulation of the receptor for hyaluronan-mediated motility (RHAMM)\(^{193}\).

Intravital video microscopy (IVM) of arthritic synovial tissue showed that CD44 deficient mice exhibited a significant increase in the number leukocytes rolling on the endothelium, with a decreased number of firmly adherent cells\(^{142}\). The same group showed that, while anti-CD44 treatment with IM7 or KM81 reduced the number of rolling leukocytes, the number of adherent leukocytes remained essentially unchanged compared Rat IgG controls. Use of IM7 was capable of reducing leukocyte rolling, but IM7 Fab was not. The authors note that adherent leukocytes after IM7 treatment appear “dishevelled”, and further note that in-vivo labelling with anti-Gr-1
(granulocyte marker) and anti-CD41 (platelet marker) appear to show platelets bound to adherent granulocytes. Treatment with IM7 reduced circulating granulocyte and platelet counts to about 50% within 60 minutes of injection, which the authors suggest may indicate platelets and granulocytes form into complexes after IM7 treatment, are cleared together after injection with IM7. However, depletion of platelets prior to the administration of IM7 reduced the anti-inflammatory effects of IM compared to IM7 alone. Consequently, the authors concluded that IM7 works through several steps: preventing the rolling (but not adhesion) of leukocytes on inflamed endothelium, activation of platelets binding to leukocytes through CD44 cross linking, and clearance of these complexes from circulation.
4.5 CD44 and Thrombocytopenia

A link between CD44 and platelets was elaborated in the finding that CD44 expressed on murine platelets was capable of binding HA, which can be prevented by treatment with hyaluronidase or KM201. Previous work from our group has shown that CD44 may be an important target in treating immune thrombocytopenia (ITP), a condition where decreased circulating platelet counts present an increased risk of bleeding in patients. Our lab utilized an antibody-mediated passive transfer model where injection of an anti-CD41 mAb (MWReg30) caused thrombocytopenia independent of an adaptive immune response, a model that bears a resemblance to the passive nature of the K/BxN-serum induced RA model described above.

Using this model of ITP, 4 antibodies (KM201, KM114, KM81, and 5035-41.1D) were found to be partially effective in protecting mice against antibody-induced thrombocytopenia, while other antibodies were actually capable of inducing thrombocytopenia when administered alone. Most anti-CD44 antibodies in this study were found to react with both platelets and leukocytes, including two antibodies that were found to not prevent the induction of thrombocytopenia. Further data showed that treatment with KM114 was able to rescue normal platelets (CD44+) from clearance (as well as platelets from CD44-deficient mice which were injected into naïve mice), which was intriguing given the assumption that direct binding of platelets would provide the protection. Use of KM114 in SCID mice was also effective in rescuing platelet counts, showing that rescue is not dependant on T or B cells in our model of thrombocytopenia. While these results do not necessarily indicate efficacy in the K/BxN model of RA, they do provide points of interest for determining the role of CD44 in RA. The role of anti-CD44 treatment has been assumed to be related to disrupting the adhesive properties of CD44, so our data that some anti-CD44 antibodies induce thrombocytopenia suggests that they may be effective in the K/BxN model of arthritis by reducing platelet counts, a question we set out to address. Given that human platelets do not express CD44, human treatment with anti-CD44 antibodies poses no danger of inducing thrombocytopenia on their own.
Chapter 5

5 RATIONALE AND HYPOTHESIS

5.1 Rationale

Due to the wide range of possible mechanisms of anti-CD44 therapy, the current study was intended to serve several purposes. Firstly, we chose to examine the role of anti-CD44 treatment in the CIA active model of arthritis, hoping to determine if this treatment was working through a platelet-depleting mechanism. However, due to results we will discuss later, we then chose to transfer our attention to the efficacy of a panel of anti-CD44 antibodies in the treatment of the passive K/BxN serum-transfer model of RA, and generated what we believe is the first data regarding anti-CD44 treatment in the K/BxN model. The current study also provided novel data for two antibodies (5035-41.1D and T2-F4) in any mouse models of inflammatory arthritis, and a comparison for the largest panel of anti-CD44 antibodies within the same study (the largest number of anti-CD44 antibodies examined in a single study was 3187,188).

Based on our preliminary data, we isolated the role of platelet CD44 in the effector phase of inflammatory arthritis, compared to studies examining the role of CD44 in more complex models. We believe the passive transfer model may be the most appropriate animal model to examine our questions, as we will be able to eliminate the possibility that our anti-CD44 treatments block homing of T-cells required for the development of the CIA model. The passive transfer model allowed us to focus solely on the effector phase of joint inflammation and destruction, which may be more relevant to the clinical setting. Previous work has suggested anti-CD44 treatment works through disrupting HA binding172, or depletion of neutrophils142, while Boilard et al demonstrated that RA can be prevented simply by depleting platelet counts141. Given our data that indicate that some anti-CD44 antibodies can induce thrombocytopenia on their own, we believed it might be possible that these antibodies were working through their platelet depleting properties and not solely by disrupting HA binding. Finally, we proposed to examine whether these antibodies are inducing thrombocytopenia in an manner similar to that of MWRreg30197, or through some other mechanism, such as inducing platelets to bind neutrophils142.
5.2 Hypotheses

1) At high doses (150ug/mouse per injection), all anti-CD44 antibodies will induce significant thrombocytopenia relative to controls. These doses are similar to the use in previous models of arthritis where these antibodies have been shown to ameliorate arthritis.

2) Antibodies to CD44 which are capable of inducing thrombocytopenia will also be capable of preventing or ameliorating the development of arthritis. The therapeutic effect of anti-CD44 antibody will correlate to the severity of thrombocytopenia, with more severe thrombocytopenia resulting in a greater protection against arthritis.

3) Antibodies to CD44 will have the same abilities as anti-platelet treatment in protecting against the development of arthritis.
Chapter 6

6 MATERIALS AND METHODS

6.1 Materials

6.1.1 Mice

DBA/1 mice were obtained from the Jackson Laboratory (Bar Harbor, ME), and were used at approximately 6-8 weeks of age. C57BL/6 mice were obtained from Charles River (St-Constant, QC), and were also used at approximately 6-8 weeks of age. Male NOD/ShiLtJ mice were obtained from Jackson Laboratory (Bar Harbor, ME), and used for breeding with female heterozygous KRN +/- females. Mice deficient in the Fc Receptor \(\gamma\)-chain (B6.129P2-Fcer1g\(^{tm1Rav}\) N12 from Taconic Farms Inc, NY) were bred in the St. Michael’s Hospital Vivarium, and used at 16 weeks of age.

6.1.2 Breeding

All breeding was conducted by Donna Lyons in the St. Micheal’s Hospital vivarium. Heterozygous male KRN+/- transgenic mice were paired with C57BL/6 females in order to ensure the maintenance of the KRN +/- line in our laboratory for continued serum production. Female heterozygous KRN +/- mice were bred with NOD/ShiLtJ males (2 or 3 females per male, per cage), producing the F1 K/BxN progeny. NOD/ShiLtJ mice were removed from the breeding pool at the discretion of the St. Michael’s Hospital vivarium staff (once they started developing diabetes), and were replaced with new males. Mice were not bred to be homozygous for the KRN transgene, due to a decrease in the breeding efficiency of mice homozygous for KRN\(^{198}\). KRN-negative mice were removed from the breeding pool, and F1 K/BxN mice that did not develop visible inflammation were sacrificed without bleeding for serum.
6.1.3 KRN maintenance and production of K/BxN serum

KRN +/- mice were typed using flow cytometry to assess the population of cells that are double positive for Vβ6 (KRN) and CD4\textsuperscript{198}. In short, 50uL of whole blood was suspended in 100uL of PBS/EDTA (1%), and then treated with ACK lysis buffer for 5 minutes at room temperature to eliminate red blood cells from the sample. White blood cells were obtained by centrifugation (850x g, 10 minutes, 4°C), and incubated at a concentration of 10\textsuperscript{6}/mL in staining buffer containing 1:100 Vβ6-FITC and 1:20 CD4-PE. Stained cells were washed once with 10 volumes of PBS, and re-suspended in 300uL PBS for testing. In KRN-positive mice, the majority of CD4+ cells were also Vβ6+, compared to ~10% in negative mice\textsuperscript{198}. Strongly arthritic K/BxN mice (clinical score greater than 9) were anesthetized using 300uL Avertin (2.5%), then exsanguinated via cardiac puncture at approximately 9-10 weeks of age to obtain the maximum volume of anti-GPI containing serum. The whole blood was allowed to clot for approximately 30 minutes (room temperature), then centrifuged (10 minutes at 10,000 rpm, 4°C) to pellet non-serum components. The serum was removed, placed in microfuged tubes, and stored at -80°C. The K/BxN serum used in these experiments had previously been pooled from approximately 60 mice, and stored at -80°C prior to use, according to a standard protocol\textsuperscript{198}. To prepare a batch of pooled serum, individual serum samples from a large group of mice (atleast 25-30 mice) were combined, and separated into small fractions (0.5-1.0mL). Fractions were not be re-frozen once thawed. A dosage experiment was conducted to determine the dose of serum required to induce a consistent arthritis across experiments and batches of serum.
6.1.4 Antibodies and other reagents

Anti-CD44 antibodies were obtained as follows: IM7.8.1 (BD Biosciences, Cat No. 553131), KM201 (Fitzgerald Industries International, Cat No. 10R-CD44gMS), KM114 (BD Biosciences, Cat No.), KM81 (Cedarlane Labs, Cat No.), T2-F4 (Accurate Chemical & Scientific, Cat No.), and 5035-41.1D (Lifespan Biosciences, Cat No. 558739). IRAWB14.4 was obtained as a kind gift from Dr Katalin Mikecz (Rush Medical Centre, Chicago). A batch of IM7.8.1 (anti-CD44, used for Figures 21-24) was from BioXcell (West Lebanon, NH. Cat No. BE0039). Normal rat IgG was obtained from Caltag Laboratories (Burlingame, CA. Cat No. 10700). Rat anti-mouse antibody (MWReg30, anti-platelet) was obtained from BD Biosciences (Mississauga, ON. Cat No. 553847). Anti-CD4 (L3/T4 clone) conjugated to R-phycoerythrin (CD4-PE) was obtained from Cedarlane Labs (Burlington, ON. Cat No. CL012PE), and used per manufacturer’s instructions. Anti-Vβ6 (RR4-7 clone) conjugated to Fluorescein isothiocyanate (Vβ6-FITC) was obtained from BD Biosciences (Mississauga, ON. Cat No. 553193), and used per manufacturer’s instructions.

Functional Grade lipopolysaccharide (LPS) isolated from E.coli 0111:B4 was obtained from Sigma-Aldrich (Oakville, ON. Cat No. L5293), while Complete and Incomplete Freund’s Adjuvant (CFA, Cat No. 7008/IFA, Cat No. 7002) and Bovine CII Collagen (Cat No.20022) were obtained from Chondrex (Redmond, WA). Human IVIG (Gamunex, 10%) was obtained from Talecris Biotherapeutics (Triangle Park, NC). Isoton II (Cat No. NC9343512) and the Z2 Particle Count and Size Analyzer were from Beckman Coulter (Mississauga, ON). Gibco ACK Lysis buffer (Red blood cell lysis buffer) was obtained from Invitrogen (Burlington, ON. Cat No A10492-01). Avertin (2,2,2-Tribromoethanol, Cat No. T48402) was obtained from Sigma-Aldrich (Oakville, ON), and a 2.5% stock solution of Avertin was prepared using 1% saline.
6.2 Methods

6.2.1 Collagen Induced Arthritis Protocol

CIA was induced according to a standard protocol previously used in our lab, distributed by Chondrex (Redmond WA). DBA/1 mice were immunized by subcutaneous injection at the base of the tail with 100µL of a stable 1:1 emulsion of bovine CII Collagen and CFA. 28 days later (day 0) the ankle width of the mice’s hind paws were recorded to determine a baseline, as well as the platelet counts, as previously described\textsuperscript{199}. Ankle width (in millimetres) of the hind paws were measured at the widest point (the malleoli) with the legs fully extended, using a digital caliper. The mice were then injected intraperitoneally (IP) with the appropriate treatment (150µg anti-CD44 or 2g/kg IVIG), and one hour later arthritis was induced by injecting 50µg LPS (dissolved in PBS), also IP. From this time, mice were monitored every 24 hours for paw swelling and platelet counts were performed where indicated. In our next experiment we attempted to create a dose-response curve by inducing arthritis with varying amounts of LPS on day 0 (5, 10, 20, 30, 40µg), again with the same initial injection of CII in CFA, and monitored for platelet counts and ankle width every 24 hours.
6.2.2 **K/BxN Serum Transfer Protocol**

For the K/BxN passive transfer model, pre-treatment backgrounds of ankle measurements and platelet counts were obtained (day 0) as described above for all experiments. Average ankle width in C57BL/6 mice pre-treatment was approximately 2.7mm before arthritis induction. In addition, clinical scoring of inflammation and swelling was also recorded on a scale of 0-3 (per paw, reported as the sum of all four paws). A clinical score of 1 indicated mild swelling that did not noticeably change the shape of the paw, 2 indicated moderate swelling which modified the foot and joint to be of approximately equal width throughout, and 3 indicated severe swelling which reversed the normal “v-shape” of the foot when examined from below the foot\(^{198}\).

Antibody treatments were administered 2 hours prior to arthritis induction, which was accomplished by injection of 160μL of K/BxN serum (diluted 1:4 in PBS) intraperitoneally (Figures 10-20). A second batch of serum was assessed for potency, and a dose of 200μL was chosen for its similarity to our previous dose (Appendix 2), and was used at a 1:3 dilution in PBS (Figures 21-24). All measurements (platelets and arthritis) were recorded every 24 hours from day 0 (induction) until day 10 (or day 15 where indicated).

For our initial experiments involving IVIG, anti-platelet treatment (MWReg30), or anti-CD44 treatment (panel of antibodies), each injection was prepared in a total volume of 200μL PBS. IVIG was administered at a dose of 2g/kg, and MWReg30 was administered at a dose of 2ug per injection, intraperitoneally. For all anti-CD44 antibodies, an initial dose of 150ug was administered to approximate doses administered in previous studies, and a second injection of 150ug was administered in the indicated groups on day 2 (after measurements for the day had been recorded). To ensure our anti-CD44 was not inducing a non-specific response, normal rat IgG was injected in the same dosage and timing as anti-CD44 treatment. Mice receiving anti-platelet treatment were given two additional injections at 24 and 48 hours, for a total dose of 6ug over three days to approximate the thrombocytopenia observed in our initial IM7-treated groups. A separate experiment was conducted to examine whether continuous depletion of platelet counts affected arthritis. Mice were injected with 2ug of MWReg30 IP on day 0, arthritis was induced with K/BxN serum 2 hours later, and MWReg30 administered every 24 hours following induction for a total of 10 days. Clinical scores, ankle widths, and platelet counts were monitored as normal, with measurements being taken prior to that day’s injection of MWReg30.
To induce a thrombophilic response, mice were pre-treated with either a single dose of IM7 (150ug, day 0) or three daily doses of MWReg30 (3x 2ug, days 0-2). Arthritis was induced on day 5 (after daily measurements had been obtained) in order to capture the majority of the thrombophilic response in both groups. Both groups were then monitored for 10 days as in our normal experiments.

To examine the therapeutic window where IM7 and MWReg30 might be effective, arthritis was induced at day 0 as per normal. Mice were then monitored as normal for platelet counts and arthritis scores for 4 days to ensure arthritis has begun developing. After measurements for day 4 had been obtained, mice were then injected with either IM7 (150ug, IP) or MWReg30 (2ug, IP). To ensure a similar level of thrombocytopenia, a second and third injection of MWReg30 was administered on days 5 and 6. Mice were then monitored per our normal experiments until day 10 after a significant thrombophilic rebound was observed.

6.2.3 Miscellaneous

Binding of the anti-CD44 monoclonal antibodies T2-F4 and IM7 (from BioXcell) to platelets (using platelet rich plasma) and splenic leukocytes at relevant doses was assessed by a technician in our laboratory (see Acknowledgements) using FACS, all other data regarding CD44 binding to platelet and leukocytes has been previously been published\textsuperscript{195}, and was not reproduced in this study. All figures were produced using GraphPad Prism, error calculated by SEM, and statistical analyses performed using two-tailed, unpaired Student’s t-test.
Chapter 7

7 RESULTS

7.1 Collagen Induced Arthritis Model

7.1.1 IM7 and IVIG ameliorate the severity of CIA

The CIA model of arthritis was used due to this model’s similarity to the normal development of autoimmunity in RA. The positive arthritic control group (CIA) demonstrated a noticeable increase in ankle width by 48 hours, and a statistically significant increase from 72 hours onward, relative to untreated controls (Figure 9A). The first anti-CD44 mAb examined was IM7.8.1 (IM7) given its ability to induce significant thrombocytopenia\(^{195}\), even at a lower dose than has been used in previous studies of RA in mouse models. A single injection of IM7 (150µg) was chosen as a dose similar to that used as in previous studies\(^{187,188}\), and was found to effectively ameliorate arthritis, relative to the CIA group, through a 7-day experiment (as indicated by ankle width). However, a single injection of 2g/kg IVIG was not as effective as IM7. IVIG only slightly reduced the development or arthritis in the first 4 days (not statistically significant), though in the latter part of the experiment it did significantly reduce arthritis (Figure 9A).

All groups where CIA was induced also developed a significant thrombocytopenia (Figure 9B), likely due to the use of LPS to induce arthritis\(^{200}\). 24 hours after the induction of arthritis, the free circulating platelet count in the CIA positive control group had dropped to approximately 50% of untreated controls, and the group treated with IVIG had almost identical platelet counts. IM7 further exacerbated the thrombocytopenia, reducing platelet counts to less than 20% compared to untreated mice, and a further 30% compared to CIA controls and IVIG-treated mice. Platelet counts in all groups gradually increased from days 2 to 4, followed by a period of increased platelet counts in all treated groups (the thrombophilic period). While the IM7-treated group experienced the more severe drop in platelet counts, the same group had the lowest rebound in the thrombophilic period. Conversely, the CIA and CIA+IVIG groups experienced a milder drop in platelet counts at 24 and 48 hours, but higher platelet counts in the thrombophilic period (approximately 10-30% increase relative to the IM7-treated group) (Figure 9B).
Based on the observation that thrombocytopenia was induced in LPS-induced arthritic groups relative to untreated controls (Figure 9B), a dosage-response experiment was carried out in the CIA model (Appendix), whereby varying amounts of LPS were administered to induce arthritis. Of the doses tested (5, 10, 20, 30, 40ug), only 30 and 40ug of LPS resulted in a noticeable increase in ankle width measurements (Appendix). However, the increase in ankle width was much smaller and took longer to develop than the arthritis measured in mice receiving a 50ug dose of LPS (CIA group, Figure 9A).

The standard dose of 50ug LPS resulted in a drop of ~50% (Figure 9B), as did doses of 20, 30, and 40ug of LPS (Appendix). Doses below 20ug of LPS appeared to have little effect on circulating platelet counts, though all groups treated with LPS showed a large increase in circulating platelet counts by day 5. Based on this data, it did not appear possible to separate the LPS-induced thrombocytopenia from the requirement of LPS to induce arthritis in this model. As a result, we chose to change our arthritis model to the K/BxN serum-induced arthritis model.
Figure 9: IM7 and IVIG treatment of CIA. DBA/1 mice were immunized with CII collagen 28 days prior to day 0, and baseline ankle width and platelet counts were established on day 0. Mice were pre-treated with IVIG (2g/kg, green) or IM7 (150ug, blue), and 2 hours later arthritis was induced by injecting 50ug of LPS (3 mice per group, 1 experiment). A) Mice induced with CIA (red) develop the most severe arthritis, IVIG reduced ankle swelling in late stage arthritis, and IM7 reduces arthritis throughout the experiment. B) Thrombocytopenia was observed in all groups relative to untreated controls (black circles), with IM7 causing the most severe thrombocytopenia. *P<.05, **P<0.01, ***P<0.001
7.2 K/BxN Serum Transfer Model

7.2.1 Dosage of K/BxN serum for arthritis induction

Observing that thrombocytopenia occurred in all LPS-treated groups (even those that did not develop CIA), we adopted the K/BxN passive transfer model of arthritis for subsequent experiments. A dose-response experiment was conducted to determine our future dosage of serum to induce arthritis, and 160µL of K/BxN serum was chosen for its slightly more severe arthritis as measured by clinical scoring and ankle width measurements (Figure 10A, Supplementary Figure 1). Clinical scoring was adopted as the “arthritic unit” of choice to maximize the reproducibility of our data, given that it took into account all four paws, as opposed to ankle width which only examines the rear paws. Significant inflammation was apparent by 48 hours in the mice treated with K/BxN serum, which reached at plateau at a clinical score of approximately 8-9. Our standard experiment was extended to 10 consecutive days to include the plateau of arthritis, in comparison to the 7 day experiment used in the CIA model (Figure 9). The arthritis after day 10 gradually decreases, though mild inflammation was still apparent in one or more joints at day 21 (data not shown).

The K/BxN serum itself did not induce thrombocytopenia, with a slight trend toward increased platelet counts near the mid-point of the experiment. However, platelet counts on day 10 were almost identical to the counts determined on day 0 as the experimental baseline. While the increase in platelet counts near the mid-point of the experiment was not be significant, platelet counts in all treatment groups in later experiments were compared to the counts from positive arthritic controls to eliminate any possible effect of the serum. Untreated mice were also monitored to ensure consistent platelet counting from day to day.
Figure 10: Dosage of K/BxN serum in C57BL/6 mice. C57BL/6 mice were injected with the indicated volumes of K/BxN serum on Day 0, after a baseline for platelet counts was determined. Clinical scores were noted every 24 hours, and platelet counts were monitored where indicated (3 mice, one experiment). A) Arthritis was apparent 2-3 days post-injection, as confirmed by clinical scoring as well as ankle measurements (Supplementary Figure 1). 160uL of K/BxN serum inducing a slightly more severe arthritis. B) Platelet counts trended slightly higher towards day 5 after injection of the K/BxN serum.
7.2.2 IM7 and IVIG ameliorate serum-induced arthritis

Figure 11 shows the data gathered from our next experiment, where we repeated the initial experiment from the CIA model, adding IM7 alone as an additional control to determine the thrombocytopenia caused by IM7. Similar to the curve found in Figure 10, K/BxN serum induced arthritis that reached a plateau at clinical score of 8-9. IVIG treatment (2 g/kg) significantly reduced the severity of arthritis from day 5 until the end of the experiment on day 10 (Figure 11A, Supplementary Figure 2), though mice did develop moderate inflammation and swelling of the joints, which is expected in this model\textsuperscript{201}. IVIG alone did not induce inflammation, nor did it significantly affect platelet counts (data not shown). In contrast to IVIG, the group treated with a single dose of IM7 (150ug) showed minimal inflammation for the first 5 days of the experiment, but a moderate level of inflammation was apparent in the latter half of the experiment. IM7 alone did not induce any inflammation in the absence of K/BxN serum (Figure 11A).

As in Figure 9, IVIG treatment did not significantly affect platelet counts relative to positive arthritic controls. As expected, IM7 (alone, and as a treatment for arthritis) did induce significant thrombocytopenia over the first 72 hours of the experiment, with IM7 in K/BxN-treated mice almost identical to IM7 alone. As in the CIA model, IM7-induced thrombocytopenia resolved by 96 hours (day 4). Following the return to baseline, IM7-treated groups underwent a thrombophilic period of 3-4 days, finally returning again to baseline at day 10. The development of arthritis in IM7-treated mice (day 5) followed the return of platelets to pre-treatment levels (day 4)(Figure 11B).
Figure 11: IM7 and IVIG treatment in serum induced arthritis. C57BL/6 mice were injected with K/BxN serum (+ 2g/kg IVIG or 150ug IM7) at day 0, except for a group which was treated with IM7 alone. Clinical scoring and platelet counts were taken every 24 hours for 10 days. A) IVIG reduces arthritis after day 4 (green), while a single injection of IM7 (dashed blue) delays and reduces the severity of arthritis compared to arthritic controls (red). B) IM7 (alone and in arthritic mice) causes a severe thrombocytopenia compared to the arthritic control group. (3 mice, one experiment) *P<.05, **P<0.01, ***P<0.001
7.2.3 MWReg30 partially protects against serum-induced arthritis

Platelet-depletion has been shown to almost completely ameliorate arthritis in the K/BxN model\(^{141}\), therefore we conducted a similar experiment using multiple injections of an anti-platelet antibody (MWReg30, anti-GPIIb). Three injections were administered to attempt to mimic the thrombocytopenia observed when mice were treated with 150µg IM7 (Figure 12B). As with previous experiments, arthritic controls (K/BxN serum only) developed arthritis and reached a plateau at a clinical score of approximately 8-9. Mice treatment with MWReg30 (3x, 2µg/day) developed an attenuated arthritis that was less than positive arthritic controls from day 5 onward (Figure 12A, Supplementary Figure 3). However, the MWReg30-treated mice did develop significant inflammation (relative to naive mice) from day 3 onwards. The progression of arthritis in this group was constant and linear (to a maximum clinical score of ~5), in contrast to arthritic controls, which developed arthritis quickly over the first 5-6 days, then reached a plateau near a clinical score of 8-9. Our results are similar, but quantitatively different, from the work of Boilard et al\(^{141}\), who showed that a polyclonal anti-platelet treatment (anti-GPIIβ) reduces inflammation ~85% relative to arthritic controls.

Treatment with MWReg30 induced significant thrombocytopenia from 24 to 96 hours (Figure 12B), and platelets eventually returned to baseline at approximately day 5. The thrombocytopenia was quite similar to that caused by a single injection of IM7 (Figure 12B), though the decrease in platelet counts in the anti-platelet group lasted approximately 24 hours longer than the IM7-treated group. The anti-platelet group underwent a thrombophilic period from days 6-9, with an eventual return to baseline on day 10. There did not appear to be any change in the progression of arthritis in MWReg30-treated mice, regardless of whether the mice were thrombocytopenic or thrombophilic. As well, despite the similarities in their platelet depletion, IM7 and MWReg30 had significantly different curves describing their development of arthritis (Figure 11A, 12A, and Supplementary Figure 12).
Figure 12: MWReg30 partially protects against serum-induced arthritis. C57BL/6 mice were assessed for baseline measurements on day 0. The group receiving MWReg30 (blue) was injected with 2ug on days 0, 1, and 2 (every 24 hours). Arthritis was induced by injecting mice with 160uL of K/BxN serum. Clinical scoring and platelet counts were taken every 24 hours for 10 days. A) Platelet depletion reduced the severity of arthritis relative to positive controls (red). B) MWReg30 induced significant thrombocytopenia. (9 mice, 3 experiments) *P<.05, **P<0.01, ***P<0.001
7.2.4 Panel of anti-CD44 antibodies in serum-induced arthritis

To determine which anti-CD44 antibodies were able to ameliorate arthritis and induce thrombocytopenia, we assessed 8 different anti-CD44 antibodies in the K/BxN model, monitoring arthritis indicators and platelet counts every 24 hours for 10 days. Two groups were tested for each antibody in our panel, one receiving a single injection (150ug) and the second receiving a second injection on day 2 (300ug total), as we anticipated a second dose would prolong any thrombocytopenia after treatment. Almost all antibodies induced statistically significant thrombocytopenia in the group receiving one injection, regardless of antibody isotype (Table 2), and a second injection generally prolonged the thrombocytopenia. All anti-CD44 antibodies that induced thrombocytopenia also reduced arthritis as measured by clinical scoring and ankle measurements (Table 2, Supplementary Figures 4-9).

Treatment with one injection of IM7 essentially eliminated inflammation over the first 4 days of the experiment (Figure 13), though a moderate level of inflammation was observed in the latter half, similar to Figure 11A. A second injection of IM7 on day 2 further delayed the development of arthritis, and reduced the overall level of inflammation in this group up to, and including, day 10 (Figure 13A, Supplementary Figure 4). As in Figure 12B, a single injection of IM7 induced thrombocytopenia, which resolved by approximately day 4, followed by a thrombophilic period. Two injections of IM7 extended the thrombocytopenic period by an additional 2 days, though the severity of the thrombocytopenia did not appear to be impacted by the second injection. In both IM7-treated groups there was a thrombophilic period, though a second injection did not appear to significantly increase the length or net increase in the thrombophilic period (Figure 13B).

IRAWB14.4-treated mice were also protected against arthritis, and also underwent anti-CD44 induced thrombocytopenia. A single injection reduced the level of inflammation relative to arthritic controls, though the difference did not reach statistical significance. However, a second injection of IRAWB14.4 resulted in a statistically significant reduction in inflammation until day 8 (Figure 14A, Supplementary Figure 5). The over-all anti-inflammatory effect of IM7 and IRAWB14.4 appeared to be quite similar, though slightly different in the first 5 days of the experiment. A single injection of IRAWB14.4 induced significant thrombocytopenia until day 4, though platelet counts were only depleted to approximately 40% of arthritic controls. A second injection appeared to have an additive effect, both extending the length and severity of the
thrombocytopenia. However, no significant thrombophilic rebound was observed in either group of IRAWB14.4-treated mice (Figure 14B).

5035-41.1D significantly protected against arthritis until day 7 (at a dose of 150µg/mouse), with a slow and consistent increase in inflammation over the entire experiment (Figure 15A, Supplementary Figure 6). A second injection further reduced the development of inflammation, as well as the maximum clinical score. The group receiving two injections of 5035-41.1D, reached a maximum clinical score was 2 (day 10), a decrease of 75% relative to arthritic controls (Figure 15A). Mice treated with a single injection of 5035-41.1D experienced a 40-50% drop in platelet counts by 24 hours, with average platelet counts remaining slightly depressed over the course of the experiment. A second injection did not significantly affect the length or severity of the thrombocytopenia (Figure 15B). Similar to IRAWB14.4, 5035-41.1D-treated mice did not develop a thrombophilic period over the 10 days tested. While IM7 induced the most severe thrombocytopenia, 5035-41.1D appeared to be the most effective anti-CD44 treatment in preventing the development of inflammatory arthritis in this model.

Treatment with a single injection of KM201 protected against any significant inflammation until day 5, after which inflammation rapidly developed, albeit not as severely as the arthritic controls (Figure 16A, Supplementary Figure 7). A second injection had a significant additive effect, with minimal inflammation until days 9 and 10 (Figure 16A). The thrombocytopenia induced by KM201 was similar to that observed in 5035-41.1D-treated mice, reaching a maximum drop of 40-50%. However, unlike 5035-41.1D, a second dose of KM201 had a slight additive effect, decreasing platelet counts significantly until day 4 (Figure 16B). In both groups, platelet counts returned to baseline gradually, with no apparent thrombophilic period.

Treatment with KM114 and KM81 was effective in preventing the early development of arthritis, but significant inflammation was apparent by day 6 in both the 150µg and 300µg treatment groups (Figures 17A,18A, Supplementary Figures 8,9). Both KM114 and KM81 induced significant thrombocytopenia in all groups, though none reach the severity seen in IM7 or MWRex30-treated mice. Platelet counts in KM114 and KM81-treated mice underwent a gradual return to baseline counts, again with no significant thrombophilic period. There did not appear to be any noticeable difference between counts in mice treated with either 150µg or 300µg of
KM114, though a second injection of KM81 may have had a slightly additive effect (Figures 17B, 18B).

T2-F4 did not induce thrombocytopenia, at either dose, when used as a treatment for arthritis (Figure 19B), and was our only anti-CD44 antibody to not do so. Unfortunately, it was only capable of binding to platelets at a concentration of 100ug/ml (Appendix 3). This antibody was the only anti-CD44 antibody we tested that did not affect the progression of arthritis (Figure 19A, Supplementary Figure 10). Similar results were obtained using normal Rat IgG as a control, where neither group reduced arthritis (Figure 20A) or significantly affected platelet counts (Figure 20B).

These results allow us to group our panel anti-CD44 antibodies by effect on platelet counts: those where a second dose of antibody aggravated the thrombocytopenia (either in severity or length), and those where a second dose appeared to have no significant effect of the thrombocytopenia. In the first group we list IM7, IRAWB14.4, KM201, and KM81, while in the second group we list 5035-41.1D, and KM114. Regarding our first hypothesis, we found that all anti-CD44 antibodies induced significant thrombocytopenia (as predicted), with the exception of T2-F4, which did not induce thrombocytopenia even though it did bind platelets (Supplementary Figure 12A). Regarding our second hypothesis, we found it partially true, in that all antibodies that induced significant thrombocytopenia were able to significantly reduce inflammation relative to arthritic controls (Table 2). However, the length or severity of the thrombocytopenia alone did not appear to have a specific correlation to any therapeutic effect. As a result, we next decided to test if there is a difference between the thrombocytopenic effect of anti-platelet (MWRreg30) and anti-CD44 (IM7, most similar to MWRreg30), and whether IM7 does work through its thrombocytopenic effect alone.
Figure 13: IM7 induces robust thrombocytopenia while protecting against the development of serum-induced arthritis. C57BL/6 mice were assessed for baseline measurements on day 0. Mice were then pre-treated with a 150ug dose of IM7 on day 0 (solid blue), then injected with K/BxN serum. Mice receiving a total dose of 300ug (dotted blue) were injected with a second 150ug dose on day 2. Clinical scoring and platelet counts were taken every 24 hours for 10 days. A) A single injection of IM7 (3 mice, 3 experiments) was effective at ameliorating arthritis relative to arthritic controls (red squares, 6 mice from 3 experiments). A second dose (3 mice, 3 experiments) further protected against the development of arthritis. B) IM7 induced robust thrombocytopenia in both groups. *P<.05, **P<0.01, ***P<0.001
Figure 14: IRAWB14.4 induces robust thrombocytopenia while protecting against the development of serum-induced arthritis. C57BL/6 mice were assessed for baseline measurements on day 0. Mice were then pre-treated with a 150ug dose of IRAWB14.4 on day 0 (solid yellow), then injected with K/BxN serum. Mice receiving a total dose of 300ug (dotted yellow) were injected with a second 150ug dose on day 2. Clinical scoring and platelet counts were taken every 24 hours for 10 days. A) A single injection of IRAWB14.4 (3 mice, 3 experiments) reduced arthritis relative to arthritic controls (red squares, 6 mice from 3 experiments). A second dose of IRAWB14.4 (3 mice, 3 experiments) further protected against the development of arthritis. B) IRAWB14.4 induced robust thrombocytopenia in both groups. *P<.05, **P<.01, ***P<.001
Figure 15: 5035-31.1D induces intermediate thrombocytopenia while protecting against the development of serum-induced arthritis. C57BL/6 mice were assessed for baseline measurements on day 0. Mice were then pre-treated with a 150ug dose of 5035-41.1D on day 0 (solid orange), then injected with K/BxN serum. Mice receiving a total dose of 300ug (dotted orange) were injected with a second 150ug dose on day 2. Clinical scoring and platelet counts were taken every 24 hours for 10 days. A) A single injection of 5035-41.1D (3 mice, 3 experiments) was effective at ameliorating arthritis relative to arthritic controls (red squares, 6 mice from 3 experiments). A second dose (3 mice, 3 experiments) further protected against the development of arthritis. B) 5035-41.1D induced significant thrombocytopenia in both groups. *P<.05, **P<.001, ***P<.0001
Figure 16: KM201 induces intermediate thrombocytopenia while protecting against the development of serum-induced arthritis. C57BL/6 mice were assessed for baseline measurements on day 0. Mice were then pre-treated with a 150ug dose of KM201 on day 0 (solid blue), then injected with K/BxN serum. Mice receiving a total dose of 300ug (dotted blue) were injected with a second 150ug dose on day 2. Clinical scoring and platelet counts were taken every 24 hours for 10 days. A) A single injection of KM201 (3 mice, 3 experiments) was effective at ameliorating arthritis relative to arthritic controls (red squares, 6 mice from 3 experiments). A second dose (3 mice, 3 experiments) further protected against the development of arthritis. B) KM201 induced significant thrombocytopenia in both groups. *P<.05, **P<0.01, ***P<0.001
Figure 17: KM114 induces intermediate thrombocytopenia while protecting against the development of serum-induced arthritis. C57BL/6 mice were assessed for baseline measurements on day 0. Mice were then pre-treated with a 150ug dose of KM114 on day 0 (solid blue), then injected with K/BxN serum. Mice receiving a total dose of 300ug (dotted blue) were injected with a second 150ug dose on day 2. Clinical scoring and platelet counts were taken every 24 hours for 10 days. A) A single injection of KM114 (3 mice, 3 experiments) was effective at ameliorating arthritis relative to arthritic controls (red squares, 6 mice from 3 experiments). A second dose (3 mice, 3 experiments) further protected against the development of arthritis. B) KM114 induced significant thrombocytopenia in both groups. *P<.05, **P<0.01, ***P<0.001
Figure 18: KM81 induces significant thrombocytopenia while protecting against the development of serum-induced arthritis. C57BL/6 mice were assessed for baseline measurements on day 0. Mice were then pre-treated with a 150ug dose of KM81 on day 0 (solid brown), then injected with K/BxN serum. Mice receiving a total dose of 300ug (dotted brown) were injected with a second 150ug dose on day 2. Clinical scoring and platelet counts were taken every 24 hours for 10 days. A) A single injection of KM81 (3 mice, 3 experiments) was effective at ameliorating arthritis relative to arthritic controls (red squares, 6 mice from 3 experiments). A second dose (3 mice, 3 experiments) further protected against the development of arthritis. B) KM81 induced significant thrombocytopenia in both groups. *P<.05, **P<0.01, ***P<0.001
Figure 19: T2-F4 does not induce significant thrombocytopenia and does not protect against the development of serum-induced arthritis. C57BL/6 mice were assessed for baseline measurements on day 0. Mice were then pre-treated with a 150ug dose of T2-F4 on day 0 (maroon circles), then injected with K/BxN serum. Mice receiving a total dose of 300ug (dotted maroon) were injected with a second 150ug dose on day 2. Clinical scoring and platelet counts were taken every 24 hours for 10 days. A) A single injection of T2-F4 (3 mice, 3 experiments) did not affect the development of arthritis relative to arthritic controls (red squares, 6 mice from 3 experiments). A second dose (3 mice, 3 experiments) did not affect the development of arthritis. B) T2-F4 did not induce significant thrombocytopenia in either group. *P<.05, **P<0.01, ***P<0.001
Figure 20: Normal Rat IgG does not induce significant thrombocytopenia and does not protect against the development of serum-induced arthritis. C57BL/6 mice were assessed for baseline measurements on day 0. Mice were then pre-treated with a 150ug dose of Rat IgG on day 0 (solid grey), then injected with K/BxN serum. Mice receiving a total dose of 300ug (dotted grey) were injected with a second 150ug dose on day 2. Clinical scoring and platelet counts were taken every 24 hours for 10 days. A) A single injection of Rat IgG (3 mice, 3 experiments) did not reduce the development of arthritis relative to arthritic controls (red squares, 6 mice from 3 experiments). A second dose (3 mice, 3 experiments) also did not significantly affect the development of arthritis. B) Rat IgG did not induce significant thrombocytopenia in either group. *P<.05, **P<0.01, ***P<0.001
### Table 2: Summary of Results

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*a-As stated by the manufacturer
b-As described by Crow AR et al. (2011) Blood. 117(3):971 and this study
c-Yes; statistically significant on Day 4 at 300ug (anti-CD44) or 6ug (anti-platelet) relative to controls
d-Mean clinical score of group treated with 300ug (anti-CD44) or 6ug (anti-platelet).

* P<.05, ** P<.01, *** P<.001

### Table 3: Antibody Information

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<td>n/a</td>
<td>n/a</td>
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<td>Polyclonal IgG</td>
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<td>Rat IgG2b</td>
<td>yes</td>
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<td>Rat IgG2a</td>
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<tr>
<td>KM201</td>
<td>Rat IgG1</td>
<td>yes</td>
<td>decrease ^1^</td>
</tr>
<tr>
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<td>Rat IgG1</td>
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<td>decrease ^1^</td>
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<td>Rat IgG2b</td>
<td>yes</td>
<td>decrease ^1^</td>
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<tr>
<td>T2-F4</td>
<td>Rat IgG2</td>
<td>minimal</td>
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</table>

*a-As stated by the manufacturer
b-As described by Crow AR et al. (2011) Blood. 117(3):971 and this study
c-Yes; statistically significant on Day 1 at 150ug (anti-CD44) or 2ug (anti-platelet) relative to controls
d-References indicated, IM7.8.1 induces MT1-MMP mediated CD44 shedding

^1^ Increase or decrease from baseline.
7.2.5 Increased platelet counts do not increase the development or severity of serum-induced arthritis

Being unable to determine a relationship between the length or severity of antibody-induced thrombocytopenia and amelioration of arthritis, we set out to determine if the opposite scenario might be true. Using MWReg30 as our anti-platelet treatment and IM7 as our anti-CD44 treatment (given that it most resembles the effect of MWReg30 on platelet counts), we took advantage of our previous observation that these antibodies induced a significant thrombophilic period. Both MWReg30 and IM7-treated groups developed thrombocytopenia normally. Five days later (once the thrombocytopenic period had resolved and the thrombophilic period was beginning), we induced arthritis in the normal manner. Day 5 was chosen for induction of arthritis in order to capture the thrombophilic rebound in both groups (groups returned to baseline between days 4 and 5), with elevated platelet counts lasting approximately 4 days before returning again to baseline (Figure 21B).

Arthritis developed normally in the positive arthritic controls, reaching a plateau at approximately 7-8 days after induction. The arthritis in the IM7 pre-treated group was almost identical to the positive controls, demonstrating that IM7 was unable to treat arthritis in this model when administered four days prior to arthritis induction. As well, pre-treatment with MWReg30 did not significantly affect average clinical score in this group compared to arthritic controls. By day 15 (10 days after induction), all groups had reached nearly identical average clinical scores. The increase in platelet counts observed in the groups pre-treated with MWReg30 and IM7 did not have any significant effect on the development or severity of arthritis in this model (Figure 21A, Supplementary Figure 13). Our results indicate what appears to be a window of opportunity for these treatments (MWReg30 and IM7) to have a significant therapeutic effect, and that treatment five days prior to arthritis induction sits outside this window.
Figure 21: Increased platelet counts are not sufficient to increase the severity or rate of development of arthritis. C57BL/6 mice were assessed for baseline measurements on day 0. Mice were then pre-treated either with a single 150ug dose of IM7 (dotted blue) on day 0 or with 3 daily doses of 2ug MWReg30 (light blue) on days 0-2. Arthritis was induced in by injecting mice with K/BxN serum on day 5 after daily measurements had been taken. Clinical scoring and platelet counts were taken every 24 hours for 15 days. A) Neither treatment significantly influenced the progression or severity of arthritis. B) Both IM7 and MWReg30 induced a similar thrombocytopenia. (6 mice from 3 different experiments). *P<.05, **P<0.01, ***P<0.001
7.2.6 Anti-CD44, but not anti-platelet treatment, is capable of reversing the ongoing development of arthritis

There appears to be a window of opportunity for anti-platelet and anti-CD44 treatment to have an effect in this model, therefore we next questioned whether the window of treatment might extend past arthritis induction and into the development stage of this model. Arthritis was induced in all groups on day 0, and allowed to progress normally (Figure 22). Once arthritis had reached a clinical score of approximately 5 on day 4 (midway to the plateau stage in most experiments), groups were treated with either a single injection of IM7 or our usual regimen of MWReg30, while the positive arthritic controls were allowed to continue developing arthritis untreated. Both IM7 and MWReg30 induced significant thrombocytopenia by 24 hours, returning to baseline four and five days later respectively. Both groups also began to develop the normal thrombophilic rebound prior to the end of the experiment.

IM7 treatment on day 4 rapidly reversed the developing arthritis, with a 50% decrease in apparent inflammation 24 hours after IM7 treatment (Figure 22A, Supplementary Figure 14). After 72 hours there was no significant inflammation relative to untreated mice. Six days after the injection of IM7 inflammation had been completely resolved in all mice, while positive arthritic controls remained at their plateau level of a clinical score of approximately 8-9. We observed that MWReg30-treated mice continued to develop arthritis at the same rate as positive arthritic controls, reaching the same approximate plateau in clinical scores. The platelet-depleting effect of IM7 did not appear to explain its ability to reverse ongoing inflammation, as MWReg30-treated mice undergo a thrombocytopenia that lasts 24 hours longer than that induced by IM7. We also observed that the increase in platelet counts from days 6-10 in IM7-treated mice was in contrast to the continuous decrease in the clinical scores over the same time period.
Figure 22: Anti-CD44, but not anti-platelet treatment, is capable of reversing the ongoing development of arthritis. C57BL/6 mice were assessed for baseline measurements on day 0, then arthritis was induced by injecting mice with 160uL of K/BxN serum. Arthritis was allowed to develop for 4 days, then mice were treated with either a single 150ug dose of IM7 (dotted blue) or 3 daily doses of 2ug MWReg30 (light blue) on days 4-6. Clinical scoring and platelet counts were taken every 24 hours for 10 days. A) MWReg30 had no significant effect on the progression of arthritis, while IM7 quickly reversed the developing arthritis. B) Both IM7 and MWReg30 induced a similar thrombocytopenia. (6 mice from 3 different experiments) *P<0.05, **P<0.01, ***P<0.001
Anti-CD44 treatment induces mild thrombocytopenia in the absence of the Fc receptor γ-chain

IM7 and MWReg30 induced a similar degree of thrombocytopenia, therefore we decided to examine whether the mechanism of action is similar between these two antibodies. Previous work had shown that MWReg30 required the presence of the Fc receptor γ-chain and FcγRIII to induce thrombocytopenia, so we tested the effect of IM7 in γ-chain knockout mice. Age-matched C57BL/6 and γ-chain KO’s were pre-bled to determine background platelet counts, and then injected intraperitoneally with 150µg of IM7. Twenty four hours later mice were bled to determine platelet counts, and a significant drop in platelet counts was observed in both the C57BL/6 and γ-chain KO mice (Figure 23). While the thrombocytopenia in C57BL/6 mice was consistent with our previous experiments (~90%), the decrease in platelet counts in γ-chain KO mice was much less (~30%). Consequently, we concluded that the majority of the thrombocytopenia from IM7 was through a γ-chain dependant mechanism, though a significant drop in platelet counts may also be induced in a γ-chain independent mechanism.
Figure 23: Anti-CD44 treatment induces a significant decrease in platelet counts in the absence of the Fc Receptor γ-chain. 16 week old C57BL/6 (female) and γ-chain KO mice (male) were bled to determine pre-treatment platelet counts. Each group was then injected with 150ug of IM7, and bled 24 hours later. Platelet counts were significantly decreased in both groups, though the severity of the thrombocytopenia was markedly reduced in the γ-chain KO group. (4 mice from 2 separate experiments). *P<.05, **P<0.01, ***P<0.001
7.2.8 Continuous treatment with MWReg30 does not significantly change its protective effect against inflammation

Anti-platelet treatment that ameliorated arthritis has been shown to induce thrombocytopenia for 6 days\(^{141}\), therefore we next questioned whether the difference in our results might be a result of different lengths of thrombocytopenia. Consequently, we chose to carry out a standard serum-transfer experiment, but with daily treatments of 2u of MWReg30. While this treatment regimen did reduce the development of arthritis (Figure 24A, Supplementary Figure 15), we did not observe any enhancement of the therapeutic effect observed in the group treated with 3 injections of MWReg30 (Figure 13). The group receiving daily injections of MWReg30 experienced a normal drop in platelet counts over the first 72 hours, but platelet counts remained significantly reduced compared to controls for the entire experiment (Figure 24B). There did appear to be a slight increase in the platelet counts from days 4-7, analogous to the thrombophylic rebound in our normal MWReg30-treated groups. However, the absolute increase in platelet counts for our normal MWReg30-treatments rebounds from \(~100\) to \(~1500\times10^6\) platelets/mL, as compared to an absolute increase from \(~100\) to \(~200\times10^6\) platelets/mL in mice treated continually with MWReg30.
Figure 24: Continuous treatment with MWReg30 does not significantly increase the protection against development of serum-induced arthritis. C57BL/6 mice were assessed for baseline measurements on day 0. MWReg30-treated mice were then injection with 2ug of MWReg30 (light blue), and arthritis induced normally 2 hours later. Clinical scoring and platelet counts were taken every 24 hours for 10 days, and mice were treated with 2ug of MWReg30 every 24 hours (following arthritis and platelet measurements). A) Platelet depletion reduced the severity of arthritis relative to positive controls (red squares). B) MWReg30 induced significant thrombocytopenia throughout the entire experiment. (7 mice from 3 separate experiments) *P<.05, **P<0.01, ***P<0.001
Chapter 8

8 DISCUSSION

8.1 Collagen Induced Arthritis Model

The relationship between anti-CD44 treatment of arthritis and thrombocytopenia was initially performed in the Collagen Induced Arthritis active model. The CIA model relies on the development of an autoimmune response to immunization with CII collagen, which induces joint-specific inflammation (the only site where CII is present)\(^80\). The immunization with CII collagen results in the maturation of dendritic cells (DCs) and presentation of a specific epitope via the MHC II 1-Aq molecule (in DBA/1 mice)\(^73\). While the mice will spontaneously develop arthritis over the course of weeks, we used lipopolysaccharide (LPS) to simultaneously trigger the inflammatory response, which likely acts by further driving maturation and activation of DCs through TLR4, and magnifying the immune response\(^80\).

IM7 has been investigated in the CIA model before, and was shown to be effective in treating arthritis in these mice\(^187\). However, the authors utilized the model in a different manner, whereby they induced arthritis and monitored the mice until they had reached a pre-determined level of ankle swelling, and then treated every second day with IM7. Based on the findings of Boilard et al that platelet depletion prevents the development of arthritis in the K/BxN model\(^141\), and our findings that anti-CD44 treatment can induce significant thrombocytopenia\(^195\), we chose to examine the effect of IM7 in preventing the development of CIA, hoping to elaborate the possible role of platelets in this model. Here we pre-treated mice with a single dose of IM7 prior to synchronizing the onset of arthritis with LPS, and found that arthritis was almost completely eliminated. Unfortunately, we also found that groups treated with LPS resulted in significant thrombocytopenia, and while IM7-treatment further aggravated the thrombocytopenia, we were unable to determine a dose of LPS capable of inducing arthritis without also causing thrombocytopenia.

Our finding that doses of LPS capable of initiating CIA also induced thrombocytopenia is unsurprising in light of the findings of Aslam et al. The authors confirmed that the receptor for LPS (Toll-like Receptor 4, TLR4) can be found on a large population of resting platelets, and
that treatment of mice with as little as 1ug of LPS results in a significant decrease in circulating platelet counts in as little as 1 hour\textsuperscript{200}. As well, treatment with LPS induces platelet-dependant release of TNF\textgreek{a}, a critical mediator of CIA\textsuperscript{85}. Combined with our results, this information would seem to indicate that our CIA model was not suitable for examining whether anti-CD44 mediated thrombocytopenia accounts for its therapeutic effect. In addition, CD44 is a known memory T-cell marker\textsuperscript{166}, and antibody blockage of CD44 binding to HA prevents T-cell homing to sites of inflammation\textsuperscript{172,176}, suggesting that pre-treatment with anti-CD44 may prevent T-cell homing, a critical step in the development of this model\textsuperscript{74}. The exact mechanism of how this occurs is unclear, as some antibodies (KM201 and KM81) are able to simply block CD44 binding to hyaluronan\textsuperscript{188}, while IM7 induces MT1-MMP to cleave and release extracellular CD44\textsuperscript{202} as a method for inhibiting cellular adhesion. While we are unable to provide a definitive link to any platelet-mediated effects, anti-CD44 effects on T-cells may explain why our pre-treatment with IM7 is effective in almost completely preventing the development of any significant inflammation, while treatment after arthritis is established only prevents a worsening of the inflammation\textsuperscript{187}. We are aware that IM7 is capable of depleting circulating neutrophils\textsuperscript{142} (a key effector phase cell), as well as reducing platelet counts\textsuperscript{195} (proposed as another key component of the innate immune response in arthritis). Perhaps IM7 as treatment in progressing arthritis is only capable of preventing the innate response, while pre-treatment with IM7 is also able to interfere with the development of the adaptive immune response in addition to the innate response.

The timing of our treatment may also explain the fact that in our study a single treatment of IM7 was sufficient, in contrast to the recurring treatments required by Nedvetzki \textit{et al}\textsuperscript{187}. The authors found that both IM7 and IRAWB14.4 were capable of preventing any further inflammation, but did not ameliorate the inflammation that had developed prior to anti-CD44 treatment. As well, the authors demonstrated that while KM81 was effective at reducing inflammation, it’s Fab’ fragment only partially protected against arthritis. Our pre-treatment with IM7 could interrupt the homing of CD44+ memory T-cells, preventing them from becoming activated by DCs in the draining lymph nodes and spleen\textsuperscript{80}. In contrast, once the adaptive immune response has been initiated, treatment with anti-CD44 may not be able to block the production of anti-CII antibodies and the various Fc\textgamma R-mediated effects shown in Figure 5. The critical requirement for the Fc receptor \textgamma-chain\textsuperscript{78} in the development of CIA supports this concept, as well as the fact that an Fab’ fragment of KM81 was not as effective as the intact antibody at suppressing arthritis\textsuperscript{187}.
Treatment using IVIG in animal models of arthritis has been shown to be partially effective, in agreement with our data using the CIA model. One group administered IVIG as a treatment to CIA in a rat model, and found that at 200mg/kg IVIG, arthritis was only about 30% decreased throughout the course of the study\textsuperscript{203}. A second group utilized the K/BxN serum transfer model, and found that IVIG at 1g/kg was able to effectively suppress arthritis development\textsuperscript{201}. Here we showed that at higher dose of IVIG (2g/kg), we observed a significant decrease in arthritis by day 5, though IVIG was much less effective than IM7 when used as a pre-treatment against CIA. The varied efficacy of IVIG can likely be explained by the different doses and the different immune responses mounted in the CIA and K/BxN serum transfer models. The FcγRIIB inhibitory receptor appears to provide an important regulatory mechanism in CIA, as mice deficient for this receptor developed a more severe arthritis compare to wild-type controls, and produced much higher titres of anti-CII IgG\textsuperscript{79}. In the K/BxN model, FcγRIIB on macrophages invading the synovial tissue was triggered by IVIG, protecting against the development of arthritis\textsuperscript{204}, raising the possibility that IVIG engagement of the inhibitory receptor on synovial macrophages prevented IgG mediated arthritis through Fc receptors. In the CIA model, macrophages from γ-chain KO mice were unable to produce an effector response when stimulated with immune complexes containing CII and anti-CII antibodies\textsuperscript{78}. Taken together, this data would explain why IVIG is less effective than IM7 in our CIA model: IM7 would be capable of blocking the adaptive immune response through inhibition of T-cell homing, and cellular depletion, completely eliminating arthritis. In contrast, IVIG would be unable to prevent the induction of the adaptive immune response and could only block anti-CII IgG pathways, resulting in less protection overall.

While our results expand on the work previously done by Nedvetzki et al\textsuperscript{187} by showing that IM7 was more effective as a preventative treatment, we ultimately were unable to avoid the confounding factor that LPS-induction of CIA resulted in thrombocytopenia, the key parameter we wished to examine.
8.2 K/BxN Serum Transfer Model

8.2.1 IM7 and MWRReg30 have similar effects, but are functionally different

The relationship between anti-CD44 amelioration of arthritis and thrombocytopenia was then examined using the K/BxN serum transfer model. The CIA model is complex in both the types of cells involved, and the participation of the innate and adaptive immune responses, confounding our ability to determine any contribution of anti-CD44 mediated thrombocytopenia. The K/BxN serum transfer model induces arthritis independent of lymphocytes\(^97\), allowing us to focus on the effect of anti-CD44 treatment outside of the adaptive immune response. The K/BxN model relies on the innate immune response to GPI:anti-GPI immune complexes\(^106\), and requires platelets for full arthritis development\(^141\), making it an excellent model for examining our hypothesis that anti-CD44 treatment prevents arthritis through a platelet-depleting mechanism.

Repeating our first experiment in the CIA model, we utilized the K/BxN model to test the ability of IVIG and IM7 to prevent the development of inflammation. The ability of IVIG to ameliorate arthritis in this model is well established\(^201,204-205\), though the degree of protection varies slightly from publication to publication. A simple dosage experiment comparing two batches would eliminate most of the variation, ensuring that a similar level of arthritis is induced independent of the “potency” of any given batch. Our results compared favorably with several studies, with approximately a 50% reduction in joint inflammation by day 4 or 5\(^204-205\). While most of this work has centered on a mechanism whereby IVIG activates FcyRIIB, raising the activation threshold of macrophages and neutrophils, IVIG is capable of inducing a decrease in circulating neutrophil counts in the peripheral blood\(^206\). Teeling et al determined that IVIG induced sequestration of neutrophils to the lungs of rats, with a corresponding drop in neutrophils in the peripheral circulation. Given the essential requirement for neutrophils in the K/BxN model\(^108\), cellular depletion of any kind (platelets and leukocytes) caused by any treatment (IVIG, anti-platelet, anti-CD44, etc) may have a previously unconsidered impact on the results in this model. For our uses, the IVIG-treated group served as a control to show that our serum induced arthritis model functioned as expected over the course of a 10 day experiment, and can be ameliorated, as has been previously demonstrated\(^204-205\).
The return of platelets to pre-treatment levels in IM7-treated mice coincided with the development of arthritis in the K/BxN model. Given the findings of Boilard et al\textsuperscript{141} that platelets are necessary for full arthritis in this model, we chose to continue examining the role of platelets using an anti-platelet treatment regimen (3x 2ug of MWReg30) to approximate the thrombocytopenia induced by 150ug of IM7. The thrombocytopenia and thrombophilic periods were quite similar to those induced by IM7, though a dramatic difference appeared when the arthritis scores in each group are examined. We find that depletion of platelets using a monoclonal antibody did indeed provide protection, but the treated mice still developed significant inflammation, albeit at a reduced rate and overall severity relative to controls. The resulting arthritis in anti-platelet treated mice in the previous study appeared at approximately day 6, in comparison to our results finding arthritis apparent at day 3. We note that while Boilard \textit{et al.’s} arthritis was reported in ankle width (examining only the hind paws), we reported clinical scoring in all paws, including the front paws (which are necessarily excluded from ankle width measurements). However, our clinical scoring and ankle width measurements were consistent with each other (Supplementary Figure 12), both in rate of development and absolute value relative to arthritic controls, therefore it is unlikely that any differences in results was due to differing units of arthritis severity. We also note the very recent publication by Cloutier \textit{et al}\textsuperscript{136} where platelet-depleted mice in the same serum-transfer model are protected in a fashion more similar to our data than to that of Boilard \textit{et al}\textsuperscript{141}.

The differences between our results and that of Boilard \textit{et al}\textsuperscript{141} are likely due to differences in anti-platelet treatments: we utilized a monoclonal antibody (Rat IgG1) to GPIIb, while their treatment employed a polyclonal antibody preparation against GPIb (Emfret Analytics). Our results confirm their conclusion that platelet depletion was likely a complicated mechanism in ameliorating arthritis, possibly explained by the molecular target of anti-platelet treatment. GPIIb is the most abundant protein in the platelet membrane, whose main role appears to involve platelet aggregation\textsuperscript{121}, which has not been associated with arthritis in this model. In comparison, GPIb is associated with GPVI\textsuperscript{207}, an important signalling molecule in the K/BxN model\textsuperscript{141} and they may act as mutual accessory signalling proteins\textsuperscript{121}, possibly explaining why anti-GPIb mediated thrombocytopenia is more effective than simply disrupting the proposed GPVI-dependant pathway for platelets to amplify inflammation in arthritis\textsuperscript{141}. However, the clinical scores of our platelet depletion regimen very closely mirrored the reduction of arthritis seen in
GPVI knock-out mice, pointing to an interpretation whereby platelet depletion was effective largely through removing the GPVI-collagen activation pathway for platelets in this model.

One alternative explanation for our results is that MWReg30 induced an Fc-dependant thrombocytopenia\textsuperscript{197}, while anti-GPIb antibodies are thought to induce thrombocytopenia in an Fc-independent manner\textsuperscript{208}. Our data regarding anti-CD44 treatment might also support this interpretation, as IM7 induced both Fc $\gamma$-chain-dependant and independent platelet clearance, as well as completely ameliorating arthritis over the initial parts of our experiments. Such an interpretation would suggest that Fc $\gamma$-chain-dependant platelet clearance was only partially effective, and that some $\gamma$-chain independent mechanism is more effective in ameliorating arthritis. An Fc-dependant mechanism for platelet destruction would likely occur in the liver or spleen, while a $\gamma$-chain independent mechanism might occur through inducing platelet aggregation\textsuperscript{208}. Interestingly, IM7 has been shown to induce the formation of platelet-leukocyte complexes in vessels of arthritic joints\textsuperscript{142}, supporting the idea of antibody-induced platelet-leukocyte aggregation as a $\gamma$-chain independent mechanism for amelioration of arthritis.

Another tempting interpretation for different efficiency of different platelet-depletion regimens is that our treatment did not induce as prolonged thrombocytopenia as the 6-day thrombocytopenia described the manufacturer of the polyclonal preparation. However, we note that continued treatment with MWReg30 over a 10 day period induced a thrombocytopenia of an even greater length than the polyclonal treatment, but still did not significantly affect the protection against arthritis. Consequently, the mere occurrence of thrombocytopenia might only play a partial role in the amelioration of arthritis in the K/BxN, with the mechanism for platelet clearance or blockade providing a significant portion of the therapeutic effect. Our results would appear to indicate that absolute platelet counts may only play a significant role in the initial stage of the K/BxN model (no later than 4 days), while platelet depletion after day 4 of a standard experiment does not affect arthritis.

A significant thrombophilic period of several days was apparent in anti-platelet treated mice, which has been previously observed\textsuperscript{209} after antibody-induced thrombocytopenia. However, manipulation of the model to induce arthritis after an antibody-induced thrombophilic rebound did not significantly enhance the progression or severity of arthritis. Given the varied mechanisms for antibody induced platelet clearance, it might be possible that the mechanism of
platelet clearance (Fc dependant/independent), rather than the absolute number of circulating platelets, contributed to the apparent role in the role of platelets in the K/BxN model. As a result, the importance of platelets in the K/BxN model should be examined carefully, as the therapeutic effect of anti-platelet treatment might be the result of a peculiarity of this particular model. The discovery of the presence of microparticles containing platelet markers in the synovial fluid by Boilard et al\textsuperscript{141} appeared to support the role of platelets in arthritis, though our results suggest care should be taken in interpreting their data. If platelet microparticle IL-1β is indeed responsible for driving inflammation in this model, one might expect that more platelets in the circulation would promote even more inflammation, though we found that increased platelet counts did not significantly increase inflammation. To conclusively determine the role of platelets in arthritis, it would be interesting to examine the effect of anti-platelet treatment on a more active model of arthritis (eg CIA or PGIA) to determine if the role of platelets are somehow magnified in the K/BxN model. Examining the adhesion and entrance of platelets into the joint using intravital microscopy similar to that used by Hutas et al\textsuperscript{142} might also contribute to answering this particular question.

The efficacy of IM7 in this model was unsurprising, given that IM7 has also been shown to effective in both PGIA\textsuperscript{142} and CIA\textsuperscript{187}, as well as Experimental Autoimmune Encephalomyelitis\textsuperscript{210}, and insulin-dependent diabetes mellitus\textsuperscript{211}. IM7 induced significant thrombocytopenia, which has been described at a dose 3 times lower than ours (50ug)\textsuperscript{195}, and the severity and duration of the thrombocytopenia were also quite similar. The IM7-induced thrombocytopenia appeared to occur mainly through an Fc γ-chain dependant mechanism, though an Fc γ-chain mechanism could also occur. The γ-chain independent mechanism may be explained by the fact that IM7 has been demonstrated to induce the formation of platelet-neutrophil complexes in PGIA\textsuperscript{142}. However neutrophil counts return to 50% of pre-treatment levels within 24 hours in this model, and Hutas et al only examined decreased platelet counts at 1 hour, in contrast to the thrombocytopenia observed in our study which lasted for over 72 hours. We find it unlikely that the drop in circulating platelet counts could be attributed exclusively to formation and removal of platelet-neutrophil complexes from circulation, as the increase in neutrophil counts at 24 hours would then be expected to cause a corresponding increase in platelet counts, which we did not observe.
Though we observed a reduction in the development of arthritis in mice treated anti-platelet antibody, the therapeutic effect was not completely effective, while IM7 treatments essentially eliminated any visible inflammation for 5-6 days. Both treatments induced a similar level of thrombocytopenia, and after this period both the IM7 and MWReg30-treated groups reached a similar level of inflammation. These results suggest that while IM7 may target platelets, additional mechanisms are needed to explain why it was so effective in preventing arthritis over the first 5-6 days. Further support can be found in the fact that while IM7 resolved developing arthritis, treatment with anti-platelet antibody induced a similar thrombocytopenia, but was unable to reverse the same developing arthritis.

IM7 has been shown to be effective in many different models of autoimmunity, though all these models are dependent on a strong T-cell response, while the K/BxN serum-induced model proceeds independent of either T or B-cells. We can clearly discount IM7 blocking the homing of T-cells as accounting for its ability to treat serum-induced arthritis, though IM7 might have other targets in this model. For example, neutrophils are required for the progression of serum-induced arthritis, and CD44 has been demonstrated to mediate binding of neutrophils to inflamed vessels. IM7 is correlated with increased serum levels of soluble CD44 fragments in treated mice, which is mediated by Membrane Type 1 MMP (MT1-MMP), though the exact mechanism is unclear. Perhaps binding of IM7, which has an epitope distinct from other anti-CD44 antibodies (outside the HA binding pocket), induced a conformational change in CD44 that renders it more accessible to MT1-MMP. Combined with the previously demonstrated ability of IM7 to reduce leukocyte rolling on the endothelium and deplete neutrophils in the circulation, we theorize that IM7 also targets neutrophils, complementing its thrombocytopenic effect. A combination of thrombocytopenia, neutropenia, and blockage of neutrophil binding might explain why IM7 was even more effective than anti-platelet over the first half of our serum-induced arthritis experiments.

If the ability of IM7 to resolve developing inflammation did indeed depend on its effect on neutrophils, this would explain why anti-platelet treatment was unable to resolve the same inflammation, despite inducing thrombocytopenia similar to that caused by IM7. While IM7 and anti-platelet both induced significant thrombocytopenia, IM7 clearly relies on more than just a decrease in circulating platelet counts for its therapeutic effects in this model. Our results with IM7 were quite similar to those achieved through platelet depletion by Boilard et al, with
almost complete protection against arthritis over the first half of the arthritis experiment, and gradual development of attenuated inflammation in the latter half of those experiments. We found this data was consistent with our discovery that simply depleting platelets was not enough to eliminate arthritis in this model, and that the target cell, antigen, and/or mechanism of antibody mediated clearance are an important factor. Perhaps IM7 depleted several different cell types, resulting in the need for the immune system to “reset” prior to the development of arthritis that we saw 5-6 days after treatment with IM7.
8.2.2 Other anti-CD44 antibodies: IRAWB14.4 and 5035-41.1D

The results from our panel of antibodies found an interesting correlation where all antibodies that induced thrombocytopenia were also effective in ameliorating arthritis. Given that we had hypothesized that anti-CD44 works through its ability to induce thrombocytopenia, we initially assumed this data supported our hypothesis. For example, IM7 induced the most significant thrombocytopenia and IRAWB14.4 induced a slightly less severe drop in platelet count, in agreement with Crow et al\textsuperscript{195}, while a second dose of these antibodies prolonged both thrombocytopenia and arthritis protection. However, our finding that absolute platelet counts had a lesser effect that we had initially believed, required that we re-examine the data from our anti-CD44 panel experiment.

Unlike IM7, IRAWB14.4 has been shown to significantly enhance CD44 binding to HA, most likely through linking of adjacent CD44 molecules in an Fab’\textsubscript{2} manner\textsuperscript{189}. Previous work has shown it to be ineffective in treating arthritis in the PGIA model\textsuperscript{188}, likely due to the fact that it actually increases the binding of lymphocytes to HA and supporting cellular extravasation into the joint\textsuperscript{189}. Our finding that IRAWB14.4 ameliorated arthritis in the K/BxN serum transfer model was interesting, as lymphocytes have no known role in our serum transfer model. In addition, IRAWB14.4 has been shown to increase neutrophil binding to HA\textsuperscript{212}, though increasing the affinity of neutrophils for HA (up regulated at sites of inflammation) would seem to indicate IRAWB14.4 should aggravate inflammation, not reduce it. The fact that IRAWB14.4 did induce significant thrombocytopenia may provide a partial explanation for why it is effective in this model. However, while two doses of IRAWB14.4 induced less thrombocytopenia (both in severity and length) than our anti-platelet treatment, two doses of IRAWB14.4 was much more effective than our anti-platelet treatment in preventing inflammation. We are unaware of any data indicating that IRAWB14.4 may deplete neutrophils in mice, though it is tempting to speculate that a neutropenic effect might contribute to this antibody’s effectiveness, similar to IM7.

While IM7 and IRAWB14.4 both induced a relatively severe thrombocytopenia, 5035-41.1D induced a much more moderate drop in platelet counts. As well, two doses of 5035-41.1D did not significantly increase the length of the thrombocytopenic period, in contrast to both IM7 and IRAWB14.4, where two injections significantly prolonged the drop in platelet counts. Of all the
antibodies examined in this study, 5035-41.1D was the most effective in preventing arthritis, dramatically reducing the rate of development and severity of inflammation in this model.

A previous study found that 5035-41.1D did not induce significant thrombocytopenia at a dose of 50ug, though it bound both platelets and leukocytes, and was actually able to reverse thrombocytopenia induced by our anti-platelet antibody$^{195}$. The same study found that 5035-41.1D required the presence of the Fc$\gamma$RIIB to raise platelet counts, raising the possibility that 5035-41.1D prevents arthritis by interacting with this receptor, in an IVIG-like manner. However, such a mechanism relies on IVIG (or possibly 5035-41.1D) interacting with dendritic cells to prime them for regulatory action$^{199}$, a mechanism which to our knowledge has never been examined in the serum transfer model. There is no published data regarding 5035-41.1D affecting binding of CD44 to HA, though the epitope for this antibody appears to lie well outside of HA binding domain when sequences are compared. The KM-group of anti-CD44 antibodies (discussed below) bind to a very small section of the CD44 molecule to block HA binding, making it unlikely that 5035-41.1D worked through a mechanism involving blocking CD44-HA binding. The fact that two doses of 5035-41.1D increased the protection against arthritis, but did not increase thrombocytopenia, supports our interpretation that anti-CD44 likely did not act solely through inducing thrombocytopenia. We also note that this was the first description of the ability of 5035-41.1D to ameliorate arthritis in any model.
8.2.3 Other anti-CD44 antibodies: KM201, KM114, KM81, and T2-F4

The KM-group of antibodies all appear to have the same function, in contrast to the other anti-CD44 antibodies we examined here. Instead of modulating the surface expression of CD44, or aggregating CD44 molecules, they all bind to epitopes on CD44 in the hyaluronan binding pocket\textsuperscript{190}. All three antibodies significantly delayed arthritis development in the K/BxN model, though a single injection only provided a moderate degree of protection. A second injection of KM201 significantly added to the protection against arthritis, and while a second injection of KM114 also increased the degree of protection, the difference between one and two injection of KM114 did not reach significance. We find it interesting that although a second injection of KM81 increased both the severity and length of the thrombocytopenia, it did not appreciably affect the progression of arthritis.

KM201 has been used before in the PGIA-model of arthritis, and was found to be slightly effective at reducing ongoing inflammation\textsuperscript{188}. The dose used in that study was twice as large as in our study (6x 100ug doses compared to 2x 150ug doses), though our results indicated that this antibody is significantly more effective in the K/BxN model than in the PGIA model. While this antibody did induce significant thrombocytopenia, the severity and length were similar to 5035-41.1D, suggesting that if this antibody was working through a platelet-dependant mechanism, the absolute drop in platelet counts was not a factor. Perhaps part of the mechanism might be explained by the fact that KM201 has been shown to block platelet binding to hyaluronan\textsuperscript{194}, preventing them from accessing the joint to promote inflammation. We are unable to rule out the possibility that KM201-induced thrombocytopenia contributes to its therapeutic effect, though it seems likely that there is a thrombocytopenia-independent mechanism at work to explain the very effective protection in the two-dose group.

Assuming a blocking function of CD44-HA binding, this would imply that KM201 targeted a non-lymphoid cell to prevent the development of serum-induced arthritis. 300ug of KM201 given to mice in an allergic lung inflammation model increased the number of circulating eosinophil in the peripheral blood, while decreasing the number of cells infiltrating into the lungs\textsuperscript{213}. IM7 was unable to produce a similar result, and while IM7 increased the level free CD44, KM201-treated mice had no such increase. While the authors did not examine any effect on neutrophils or other granulocytes, we suspect that a similar mechanism might be at play in our
model whereby KM201 blocked neutrophil binding and migration into the joint, lowering the number of cells recruited to the joint which are capable of promoting inflammation in this model.

KM114 has been previously shown to slightly reduce circulating platelet counts (at a dose of 50ug/mouse)\textsuperscript{195}, so our results that it caused significant thrombocytopenia are unremarkable. However, it is quite interesting that an increase in dosage from 50ug to 150ug significantly decreases platelet counts, but an addition injection (300ug total) did not further affect platelet counts. KM114 was effective at reducing arthritis over the first five to six days of our experiment, after which arthritis developed quite rapidly, similar to KM201. Given that both antibodies block CD44-HA binding\textsuperscript{188, 191}, we are tempted to speculate both antibodies may act by blocking neutrophil binding/extravasation, as well as through their thrombocytopenic effects, and possibly by blocking platelet binding at the inflamed joint. A second dose of KM114 increased protection against arthritis (albeit not significantly) without increasing the resulting thrombocytopenia, supporting our theory that there must be some non-thrombocytopenic mechanism at play.

The last of the blocking antibodies, KM81, has also been shown to prevent CD44-HA binding and cellular extravasation, albeit in a mouse model of T-cells homing to peritoneal inflammation\textsuperscript{172}. As with KM114, there is a lack of evidence of for KM81 blocking granulocyte binding, though we note that KM81 induced the worst thrombocytopenia of the KM-group, with no noticeable increase in its therapeutic effect. Interestingly, a second injection of KM81 significantly increased both the severity and length of platelet depletion relative to a single injection, but both groups were virtually identical when their arthritic clinical scores were examined.

Similar to IM7, KM81 has been shown to reduce the number of granulocytes circulating in the periphery\textsuperscript{142}, providing further support for the concept that anti-CD44 antibodies may be treating arthritis in a mechanism that does not rely solely on platelet depletion. However, without the ability to distinguish between their thrombocytopenic and their blocking effects, complete interpretation of the data from these antibodies is not possible. We postulate that both thrombocytopenic and blocking effects may contribute to the efficacy of these antibodies, but would require some method to separate these two effects to reach a conclusion. We had initially speculated that an F(ab’)\textsubscript{2} fragment would help separate the two effects, but the ability of IM7 to
induce thrombocytopenia in a Fc \( \gamma \)-chain independent manner would further complicate any information gained from an F(ab’)\(_2\) experiment.

Finally, we provided novel data for the T2-F4 anti-CD44 antibody, which has not been characterized to our knowledge. The antibody did not protect against arthritis in doses of 150ug or 300ug, and did not induce thrombocytopenia. Interestingly, we note that while this antibody was not able to induce thrombocytopenia, it did bind to platelets (albeit very weakly, at best). We are unable to explain why T2-F4 was able to bind weakly to platelets but not splenic leukocytes, as the standard form of CD44 (CD44s) is thought to be expressed on all hematopoietic cells, arguing that if an antibody is capable of binding platelets, it should also be capable of binding leukocytes. A lack of leukocyte binding could possibly explain T2-F4’s lack of therapeutic effect in the serum-transfer model, though this interpretation is purely conjecture based on the incomplete data at hand. At this present time we are unable to provide an satisfactory explanation for why T2-F4 neither ameliorated arthritis, nor induced thrombocytopenia. However, this antibody might prove useful as a negative control for future experiments to determine if anti-CD44 binding to platelets is indeed part of the therapeutic mechanism in mouse models of arthritis.
Chapter 9

9 Conclusion

In conclusion, we note the fact that at our high dose all our anti-CD44 antibodies induced a significant thrombocytopenia relative to controls, in agreement with our first hypothesis. However, we also note that there appears to be a limit to the ability of some antibodies to induce thrombocytopenia, as a second dose of 5035-41.1D and KM114 did not extend the thrombocytopenia. Further study is warranted to determine the exact mechanism for how these antibodies induce thrombocytopenia, as at least one antibody (IM7) is capable of inducing thrombocytopenia independent of the Fc-γ chain.

Our second hypothesis appears partially correct, as all antibodies that induced thrombocytopenia also had a therapeutic effect in our serum-induced model of arthritis. However, we had hypothesized that the severity of thrombocytopenia would be linked to the level of protection against arthritis, a theory that we find is now without support. We conclude that the absolute number of circulating platelets was not the only factor affecting the severity of arthritis, given the fact that mice with a 50% increase in circulating platelets did not develop an aggravated arthritis. We suggest that both the antigen targeted, and mechanism for platelet depletion, may be determining factors in the efficacy of anti-platelet treatment in this model. Though we note a correlation between anti-CD44 amelioration of arthritis and thrombocytopenia, it was likely not the sole mechanism for anti-CD44 amelioration of arthritis in this model. As well, our data represents the first study of anti-CD44 treatment in the K/BxN serum transfer model, as well as the first description of 5035-41.1D and T2-F4 in any animal model of arthritis that we are aware of.

Finally, we had hypothesized that anti-CD44 treatment would function similarly to anti-platelet treatment, which did not appear to be the case. IM7 was the anti-CD44 treatment most similar to anti-platelet treatment in its ability to induce thrombocytopenia, but was clearly more effective than specifically targeting platelets for depletion in treating arthritis. As well, IM7 was capable of protecting against developing arthritis, an effect we were unable to replicate with anti-platelet treatment. Several of our anti-CD44 antibodies have been described as affecting neutrophil...
counts or adhesion, and we propose that anti-CD44 targeting these cells may provide an additional therapeutic mechanism of action, in addition to platelet depletion. We have shown that anti-CD44 is not effective as a pre-treatment several days before the induction of arthritis, though it can be quite useful as a treatment immediately before, or several days after, arthritis induction. Conversely, anti-platelet treatment was only useful when administered immediately prior to arthritis induction, raising the question as to exactly how critical platelets are in the development of the K/BxN model of arthritis, and RA in general.
Chapter 10

10 Future Directions

Our data conclusively showed that anti-CD44 treatment has at least two properties which are significantly different than our anti-platelet treatment in the serum induced model, namely the ability to reverse ongoing inflammation and the ability to induce a significant decrease in platelet counts independent of the Fc receptor γ-chain. However, exactly which anti-CD44 treatments have these properties, and how they are being achieved, is of considerable interest. As such, we propose the following future experiments to help address these questions:

1) Examine the various other antibodies in our panel to determine if they also are able to reverse ongoing inflammation. Due to time restraints, we were unable to address this question, but believe that this data will likely provide a starting point to answering the mechanistic “how?” Though it is speculative, it might be possible that any other antibodies (KM81 is of special interest in this hypothetical experiment) which cause neutropenia are capable of reversing ongoing inflammation.

2) Examine the effects of anti-CD44 treatment on various hematopoietic populations (e.g. lymphocytes, monocytes, neutrophils, platelets). Any depleting effect would be of utmost interest, as well as any ability to reduce leukocyte rolling on the synovial vessels and/or homing to the joints. Intra-vital microscopy techniques for examining this have previously been described\(^\text{142}\), and would likely be key for developing experiments to examine this topic.

3) Determine the mechanism of clearance and/or blocking CD44-HA binding. We find that at least part of the therapeutic effect of IM7 may be independent of the Fc receptor γ-chain. We were recently provided with a small amount of deglycosylated IM7, and believe the use of this form of IM7 would provide useful data in determining the mechanism of how anti-CD44 functions. If Fc-dependant clearance of targeted hematopoietic cells is the mechanism for anti-CD44 therapy, we would expect this antibody to exhibit a diminished therapeutic effect in this model.
4) Examination of platelet-depletion in an active model of arthritis would also provide interesting data, given the more complex system and involvement of the adaptive immune response in addition to the innate immune response. We question whether anti-platelet treatment is in fact as effective as initially described. Given our results and the results of Cloutier et al\textsuperscript{136}, we theorize that platelets may simply play a role in aiding the initiation of this specific passive model of arthritis. Inducing arthritis after a larger pre-treatment phase (e.g. 24 hours) might provide interesting insight as to how anti-platelet depletion is affecting this model. As well, we note that our anti-platelet regimen had a distinctly different effect than that of Boilard et al, and suggest that testing other anti-platelet antibodies might be important to answering this question. Using several antibodies to major platelet antigens may begin to shed light on this question. We also suggest determining the minimum anti-platelet treatment period required for therapeutic effect, given that we did not observe any significant additive protection in extending the thrombocytopenia from 3 days to 10 days.
Supplementary Figure 1: Dosage of K/BxN serum in C57BL/6 mice. C57BL/6 mice were injected with the indicated volumes of K/BxN serum on Day 0. Arthritis was apparent 2 days post-injection, as confirmed by ankle measurements. (3 mice, 1 experiment)
Supplementary Figure 2: IM7 and IVIG treatment in serum-induced arthritis. C57BL/6 mice were injected with K/BxN serum (+ 2g/kg IVIG or 150ug IM7) at day 0, except for a group which was treated with IM7 alone. Ankle widths were taken every 24 hours for 10 days. IVIG reduced arthritis after day 4 (green), while a single injection of IM7 (dotted blue) delayed and reduced the severity of arthritis compared to arthritic controls (red squares). (3 mice, one experiment) *P<.05, **P<0.01, ***P<0.001
**Supplementary Figure 3: MWReg30 partially protects against serum-induced arthritis.**

C57BL/6 mice were assessed for baseline measurements on day 0. The group receiving MWReg30 (blue) was injected with 2ug on days 0, 1, and 2 (every 24 hours). Arthritis was induced by injecting mice with K/BxN serum. Ankle width measurements were recorded every 24 hours for 10 days. Platelet depletion reduced the severity of arthritis relative to positive controls (red squares). (9 mice, 3 experiments) *P<.05, **P<0.01, ***P<0.001
Supplementary Figure 4: IM7 protects against the development of serum-induced arthritis. C57BL/6 mice were assessed for baseline measurements on day 0. Mice were then pre-treated with a single 150ug dose of IM7 on day 0 (solid blue). Arthritis was induced in by injecting mice with K/BxN serum. For mice receiving a 300ug total dose (dotted blue), a second 150ug dose of IM7 was administered on day 2. Ankle width measurements were taken every 24 hours for 10 days. A single injection of IM7 (3 mice, 3 experiments) was protected against arthritis over the first 5 days relative to arthritic controls (ed squares, 6 mice, 3 experiments). A second dose of IM7 (3 mice, 3 experiments) further protected against the development of arthritis. *P<.05, **P<0.01, ***P<0.001
Supplementary Figure 5: IRAWB14.4 protects against the development of serum-induced arthritis. C57BL/6 mice were assessed for baseline measurements on day 0. Mice were then pretreated with a 150ug dose of IRAWB14.4 on day 0 (solid yellow), then injected with K/BxN serum. Mice receiving a total dose of 300ug (dotted yellow) were injected with a second 150ug dose on day 2. Ankle width measurements were taken every 24 hours for 10 days. A single injection of IRAWB14.4 (3 mice, 3 experiments) slightly reduced arthritis relative to arthritic controls (red squares, 6 mice from 3 experiments). A second dose of IRAWB14.4 (3 mice, 3 experiments) further protected against the development of arthritis. *P<.05, **P<0.01, ***P<0.001
Supplementary Figure 6: 5035-31.1D protects against the development of serum-induced arthritis. C57BL/6 mice were assessed for baseline measurements on day 0. Mice were then pre-treated with a 150ug dose of 5035-41.1D on day 0 (solid orange), then injected with K/BxN serum. Mice receiving a total dose of 300ug (dotted orange) were injected with a second 150ug dose on day 2. Ankle width measurements were taken every 24 hours for 10 days. A single injection of 5035-41.1D (3 mice, 3 experiments) was effective at ameliorating arthritis relative to arthritic controls (red squares, 6 mice from 3 experiments). A second dose (3 mice, 3 experiments) further protected against the development of arthritis. *P<.05, **P<0.01, ***P<0.001
**Supplementary Figure 7**: KM201 protects against the development of serum-induced arthritis. C57BL/6 mice were assessed for baseline measurements on day 0. Mice were then pretreated with a 150ug dose of KM201 on day 0 (solid blue), then injected with K/BxN serum. Mice receiving a total dose of 300ug (dotted blue) were injected with a second 150ug dose on day 2. Ankle width measurements were taken every 24 hours for 10 days. A single injection of KM201 (3 mice, 3 experiments) protected against the development of arthritis relative to arthritic controls (red squares, 6 mice from 3 experiments). A second dose (3 mice, 3 experiments) further protected against the development of arthritis. *P<.05, **P<0.01, ***P<0.001
Supplementary Figure 8: KM114 protects against the development of serum-induced arthritis. C57BL/6 mice were assessed for baseline measurements on day 0. Mice were then pretreated with a 150ug dose of KM114 on day 0 (solid blue), then injected with K/BxN serum. Mice receiving a total dose of 300ug (dotted blue) were injected with a second 150ug dose on day 2. Ankle width measurements were taken every 24 hours for 10 days. A single injection of KM114 (3 mice, 3 experiments) was effective at ameliorating arthritis relative to arthritic controls (red squares, 6 mice from 3 experiments). A second dose (3 mice, 3 experiments) further protected against the development of arthritis. \*P<.05, \**P<0.01, \***P<0.001
Supplementary Figure 9: KM81 protects against the development of serum-induced arthritis. C57BL/6 mice were assessed for baseline measurements on day 0. Mice were then pretreated with a 150ug dose of KM81 on day 0 (solid brown), then injected with K/BxN serum. Mice receiving a total dose of 300ug (dotted brown) were injected with a second 150ug dose on day 2. Ankle width measurements were taken every 24 hours for 10 days. A) A single injection of KM81 (3 mice, 3 experiments) protected against the development of arthritis relative to arthritic controls (red squares, 6 mice from 3 experiments). A second dose (3 mice, 3 experiments) further protected against the development of arthritis. *P<.05, **P<0.01, ***P<0.001
Supplementary Figure 10: T2-F4 does not protect against the development of serum-induced arthritis. C57BL/6 mice were assessed for baseline measurements on day 0. Mice were then pre-treated with a 150ug dose of T2-F4 on day 0 (solid maroon), then injected with K/BxN serum. Mice receiving a total dose of 300ug (dotted maroon) were injected with a second 150ug dose on day 2. Ankle width measurements were taken every 24 hours for 10 days. A single injection of T2-F4 (3 mice, 3 experiments) did not affect the development of arthritis relative to arthritic controls (red squares, 6 mice from 3 experiments). A second dose (3 mice, 3 experiments) also did not affect the development of arthritis.
Supplementary Figure 11: Normal Rat IgG does not protect against the development of serum-induced arthritis. C57BL/6 mice were assessed for baseline measurements on day 0. Mice were then pre-treated with a 150ug dose of Rat IgG on day 0 (solid grey), then injected with K/BxN serum. Mice receiving a total dose of 300ug (dotted grey) were injected with a second 150ug dose on day 2. Ankle width measurements were taken every 24 hours for 10 days. A single injection of Rat IgG (3 mice, 3 experiments) did not significantly affect the development of arthritis relative to arthritic controls (red squares, 6 mice from 3 experiments). A second dose (3 mice, 3 experiments) also did not significantly affect the development of arthritis.
Supplementary Figure 12: Compiled data from all K/BxN, IM7, and MWReg30-treated mice. C57BL/6 mice were assessed for baseline measurements on day 0. The group receiving MWReg30 (light blue) was injected with 2ug on days 0, 1, and 2 (every 24 hours, 9 mice), while the group receiving IM7 (dark blue) were treated once with 150ug on day 0 (16 mice). Arthritis was induced by injecting mice with K/BxN serum on day 0, 2 hours after pre-treatment (where applicable). Clinical scoring and ankle width measurements were taken every 24 hours for 10 days. IM7 was most effective at reducing arthritis as measured by A) clinical scoring or B) ankle width measurements.
Supplementary Figure 13: Increased platelet counts are not sufficient to increase the severity or rate of development of arthritis. C57BL/6 mice were assessed for baseline measurements on day 0. Mice were then pre-treated either with a single 150ug dose of IM7 (dotted blue) on day 0 or with 3 daily doses of 2ug MWReg30 (light blue) on days 0-2. Arthritis was induced in by injecting mice with K/BxN serum on day 4 after daily measurements had been taken. Ankle width measurements were taken every 24 hours for 15 days. Neither treatment significantly influenced the progression or severity of arthritis.
Supplementary Figure 14: Anti-CD44, but not anti-platelet treatment, is capable of reversing the ongoing development of arthritis. C57BL/6 mice were assessed for baseline measurements on day 0, then arthritis was induced by injecting mice with K/BxN serum. Arthritis was allowed to develop for 4 days, then mice were treated with either a single 150ug dose of IM7 (dotted blue) or 3 daily doses of 2ug MWReg30 (light blue) on days 4-6. Ankle width measurements were taken every 24 hours for 10 days. MWReg30 had no significant effect on the progression of arthritis, while IM7 quickly reversed the developing arthritis. (6 mice from 3 different experiments) *P<.05, **P<0.01, ***P<0.001
Supplementary Figure 15: Continuous treatment with MWReg30 does not significantly increase the protection against development of serum-induced arthritis. C57BL/6 mice were assessed for baseline measurements on day 0. MWReg30-treated mice were then injected with 2ug of MWReg30 (light blue), and arthritis induced normally 2 hours later. Ankle width measurements were taken every 24 hours for 10 days, and mice were treated with 2ug of MWReg30 every 24 hours (following arthritis and platelet measurements). Platelet depletion reduced the severity of arthritis relative to positive controls (red squares). (7 mice from 3 separate experiments) *P<.05, **P<0.01, ***P<0.001
LPS induces thrombocytopenia at doses which are unable to induce arthritis. DBA/1 mice were immunized with CII collagen in CFA 28 days prior to the induction of arthritis with LPS on Day 0. A baseline measurement for both ankle width and platelet counts was taken on Day 0, and mice were then injected with indicated dosage of LPS to induce arthritis (one mouse). A) Mice treated with 30 and 40ug of LPS developed mild arthritis (increased paw width), lower doses were unable to induce arthritis. B) Mice treated with 20, 30, and 40ug of LPS showed a noticeable drop in platelet counts 24 hours after injection of LPS.
Dosage for second batch of serum. C57BL/6 mice were injected with the indicated volumes and dilutions of serum to determine the dose required for similar degrees of arthritis between batches. Clinical scores (A) and ankle widths (B) were monitored for 8 days to quantify arthritis severity, and 200uL (1:3 dilution in PBS) was chosen as most similar to our previous batch (3 mice, one experiment).
T2-F4 only binds platelets at high dose in-vitro. IM7 (red) and T2-F4 (black) were assessed for platelet and leukocyte reactivity by FACS at concentrations approximating a single injection (100ug antibody per mL whole blood). A) Both IM7 and T2-F4 were found to react with platelets isolated from naive C57BL/6 mice, though high amounts of T2-F4 were required to detect binding. B) Only IM7 was found to react with splenic leukocytes. (4 mice from two different experiments)
REFERENCES


