UNIVERSITY OF CALIFORNIA SAN DIEGO

The Role of Type I Interferons in Mechanical Allodynia and Swelling in a K/BxN Arthritis Mouse Model

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

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The Thesis of Erin Leigh Harryman is approved, and is acceptable in quality and form for publication on microfilm and electronically:

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LIST OF ABBREVIATIONS

ACPA = Anti-Citrullinated Protein Antibodies

ACR = American College of Rheumatology

ADAR = Adenosine Deaminase Acting on RNA

ALCAM = Activated Leukocyte Cell Adhesion Molecule

APOBEC = Apolipoprotein B mRNA-Editing Enzyme, Catalytic Polypeptide

BAK1 = BCL2 Antagonist/ Killer 1

BMDC = Bone Marrow-Derived Dendritic Cells

CASP = Caspase

CD = Cluster of Differentiation

CCP = Cyclic Citrullinated Peptide

CDC = Centers for Disease Control and Prevention

cGAS = Cyclic GMP-AMP Synthase

CTGF = Connective Tissue Growth Factor

COX = Cyclooxygenase

DAP = Death-Associated Protein

DMARD = Disease Modifying Anti-Rheumatic Drugs

DMSO = Dimethyl Sulfoxide

DMXAA = Xanthenone Analog a.k.a Vadimezan or ASA404

DRG = Dorsal Root Ganglion

ECM = Extracellular Matrix

ELISA = Enzyme-Linked Immunosorbent Assay

ER = Endoplasmic Reticulum

EULAR = European League Against Rheumatism (EULAR)

FCL = Familial Chilblain Lupus (FCL)

FGF = Fibroblast Growth Factors

FLS = Fibroblast-Like Synoviocytes

G6PI = Glucose-6-Phosphate Isomerase

GC = Glucocorticoids

GM-CSF = Granulocyte Monocyte-Colony Stimulating Factor

GPI = Glucose-6 Phosphate Isomerase

GTP = Guanosine Triphosphate

HLA = Human Leukocyte Antigens

HMGB1 = High Mobility Group Box 1

HSP = Heat Shock Proteins

HTS = High-Throughput Screening

ICAM1 = Intercellular Adhesion Molecule 1

IFI6 = Interferon Alpha-inducible Protein 6

IFITM = Interferon-Induced Transmembrane

IFN = Interferon

IFNAR = Interferon Receptor

Ig = Immunoglobins

IL = Interleukin

IL-1Ra = IL-1 Receptor Antagonist

IP = Intraperitoneal

IP10 = Interferon Gamma-Induced Protein 10

IRF = Interferon Regulatory Factor

ISG = Interferon Stimulated Gene

JAK = Janus Kinase

JAK/STAT = Janus Kinase–Signal Transducers and Activators of Transcription

LPS = Lipopolysaccharide

MAL = MyD88 Adaptor-Like

MHC = Major Histocompatibility Complex

MyD88 = Myeloid Differentiation Primary Response 88

Mx = Myxovirus Resistance Protein

N.D. = Not Detected

NF-κB = Nuclear Factor Kappa B

NLM = National Library of Medicine

NOD = Non-Obese Diabetic

NSAID = Non-Steroidal Anti-Inflammatory Drugs

OAS1 = Oligoadenylate Synthetase 1

PADI = Peptidyl Arginine Deiminase

PAMP = Pathogen-Associated Molecular Pattern

PBS = Phosphate-Buffered Saline

pDC = Plasmacytoid Dendritic Cells

PDGFRL = Platelet Derived Growth Factor Receptor-Like

PLSCR1 = Phospholipid Scramblase 1

PRR = Pattern Recognition Receptors

R-INH = Repurchased Inhibitor

RA = Rheumatoid Arthritis

RANKL = Receptor Activator of Nuclear Factor κB Ligand

RF = Rheumatoid Factor

ROS = Reactive Oxygen Species

SAVI = STING Associated Vasculopathy in Infancy

SD = Standard Deviation

SELL = Selectin L

SEM = Standard Error of the Mean

SLE = Systemic Lupus Erythematosus

SOCS = Suppressor of Cytokine Signaling

ssRNA = Single Stranded RNA

STAT = Signal Transducers and Activators of Transcription

STING = Stimulator of Interferon Genes

TIRAP = Toll-interleukin 1 Receptor Adaptor Protein

TLR = Toll-Like Receptor

TNF = Tumor Necrosis Factor

TRAM = TRIF-Related Adaptor Molecule

TRIF = TIR-Domain-Containing Adapter-Inducing Interferon-β

TYMP = Thymidine Phosphorylase

VEGF = Vascular Endothelial Growth Factor

VRP = Vascular Endothelial Growth Factor-Related Protein

USP = Ubiquitin Specific Peptidase

WT = Wild Type

XAF1 = XIAP-associated Factor 1

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ABSTRACT OF THE THESIS

The Role of Type I Interferons in Mechanical Allodynia and Swelling in a K/BxN Arthritis Mouse Model

by

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Master of Science in Biology

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Rheumatoid arthritis (RA) is a chronic inflammatory disease involving synovial inflammation, autoantibody production and cartilage and bone destruction. It's a progressive disease that if left untreated can cause irreversible damage. Current treatments have numerous mechanisms of action,

deleterious side effects and are not always effective as monotherapies. Rheumatoid arthritis is a consequence of self-reactivity; however, it's cause is not fully understood. Non-specific targeting of immunosuppressants can leave RA patients at risk for other diseases and infections. To better understand the molecular pathway involved in pain-like behavior and inflammation characteristic of this disease, the experiments performed focused on the innate immune system, specifically type I interferons, in a K/BxN-induced arthritis mouse model. It was found that interferon receptors expressed by microglia may be involved in the onset and persistence of allodynia. Several other interferon pathways studied had only slight attenuation or no impact on allodynia. Previous studies identified a compound, R-INH#5, that had immunosuppressant characteristics and attenuated allodynia in K/BxN arthritis potentially by inhibiting STING, a predominant type I interferon pathway. It was confirmed the compound significantly inhibited cytokine production and attenuated allodynia in a K/BxN arthritic mouse; however, the attenuation was transient and only observed if administered at the onset of arthritis.

1. Introduction

1.1 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a systemic, chronic disease that was estimated by the Centers for Disease Control and Prevention (CDC) to impact 22.7% of the U.S. population between 2013 and 2015 (CDC, 2017) with a higher prevalence in females averaging a 3:1 ratio (Favalli et al., 2019). It is a progressive disease characterized by inflammation, autoantibody production, and bone and cartilage destruction (Guo et al., 2018). Although the epidemiology has been well studied, the causal agents of this disease are not completely understood but are believed to include both genetic and environmental factors (Bluml and Smolen, 2020). Arthritis can occur at any age, however, its onset is typically between the ages of 30 and 50 years old, and it can have significant economic impact on those afflicted. In 2013, the CDC estimated the medical costs associated with this disease to be $140 billion in the U.S. Potentially due to its effect on overall mobility and function, rheumatoid arthritis was also listed in 2013 as the third highest cause of workforce disability for workers between the ages of 18-64 years old. Disability associated with loss of employment from RA is estimated to equal $614 billion dollars in wages nationally (CDC, 2013).

Clinical classification, as determined by the American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR) for research and diagnosis purposes, is based off the presence of at least one joint with synovitis (Aletaha et al., 2010). A predominant feature of this disease is the aberrant hypertrophy and invasiveness of the lining of the

synovial joints (Guo et al., 2018). Bone erosion sets RA apart from most other inflammatory diseases (Bluml and Smolen, 2020); it's progressive and irreversible nature can lead to disability and early mortality (Köhler et al., 2019). In conjunction with poor quality of life, RA increases the probability of developing other inflammatory diseases. Studies have shown RA patients have a 1.5-2.0-fold higher likelihood to develop coronary artery disease than the general population (Crowson et al., 2013).

Early detection of RA is paramount to successful treatment as joint damage is irreversible and present in some patients as early as the initial presentation (Nielen et al., 2004). Rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA) are involved in early detection and can be found in the blood of patients' years before the manifestation of the disease (Nielen et al., 2004). RF consists of several isotypes, but IgM-RF followed by IgG-RF and/or IgA-RF, are all found in RA patients. High levels of IgM-RF can be a predictor of RA as it precedes the clinical onset of the disease (Bluml and Smolen, 2020); however, despite 80% of diagnosed RA patients testing RF positive, it's presence or lack thereof is not a guarantee of disease development (Firestein, 2003). High levels of IgM-RF suggest the involvement of B1-B cells in the pathology of RA (Bluml and Smolen, 2020). B1-B cells are non-adaptive immune cells found mainly in peripheral areas that can produce antibodies and present antigens but have no memory (Cunningham et al., 2014). The high levels of IgM antibodies secreted by B1 cells are polyreactive, binding to multiple antigens with low affinity, and can display self-reactivity (Kantor and Herzenberg, 1993). B1 cell activation can also be independent of T helper cells

(Cunningham et al., 2014); however, the presence of IgG-RF and IgA-RF suggests T cell involvement is still a factor in the progression of the disease. In fact, IgA-RF is associated with increased pathology of RA in some patients (Bluml and Smolen, 2020).

Anti-citrullinated protein antibodies (ACPAs) can also be integral in the early detection of RA (Nielen et al., 2004). Citrulline is formed by the deamination of an arginine by peptidyl arginine deiminase (PADI). This amino acid modification can be found in a multitude of proteins and, although was originally thought to be a more efficient RA predictor, studies have shown its presence in an increasing amount of diseases. Theoretically, the dual presence of RF and ACPAs might have higher efficacy as a predictor as it is seen in 90% of RA patients and may be a marker of RA severity (Bluml and Smolen, 2020).

Genetic predisposition of RA has been observed through familial studies between siblings. Compared to an upwards of 1% occurrence in the general population, the occurrence of RA increases to 2-4% in siblings and 15% in twins (Bluml and Smolen, 2020). A potential genetic link to this disease is contained in the HLA Class II locus (Feldmann et al., 1996). HLA, or Human Leukocyte Antigen, is a group of about 200 histocompatibility encoded genes located in proximity on chromosome 6. HLA's are essential components of the adaptive immune system and assist with T and B cells recognition of foreign agents known as epitopes. HLA class II proteins are predominantly expressed on dendritic cells and macrophages and, in humans, this expression is mainly through six loci: HLA-DGA1, HLA-DPB1, HLA-DRA, HLA-DQB1, HLA-DPA1 and HLA-DRB1. There are several variations within each HLA allele that

enables it to recognize a wide range of epitopes to potentially initiate an immune response (National Library of Medicine [NLM], 2019). A pooled 80% of patients affected with RA contain a version of an HLA-DRB1 allele that encodes for a similar amino acid sequence in positions 70-74. The 70-74 position is in the variable region of the class II HLA beta chain important for peptide (epitope) and T cell binding (Bluml and Smolen, 2020). Theoretically, if this variation led to the identification of “self” components as foreign entities, the resultant immune response could lead to a disease phenotype. In addition, the change of arginine to citrulline in a protein digested for peptides could lead to a recognition of self as foreign. The association with HLA class II highlights the involvement of the adaptive immune system (B and T cells) in RA (Feldmann et al., 1996).

Cytokine production is also a hallmark of RA. Neutrophils, macrophages, dendritic cells and fibroblasts are enriched in the synovial fluid and membrane of RA joints. The cytokines they secrete can have pleiotropic effects, including pro-inflammatory and/or anti-inflammatory properties. Pro-inflammatory cytokines, such as tumor necrosis factor (TNF) alpha and Interleukin (IL)-1, are present in high concentrations and may direct the destruction of cartilage by stimulating macrophages and fibroblasts to produce matrix metalloproteinase enzymes (Feldmann et al., 1996). Type I Interferons (IFN) are peripherally detectible in the sera of RA patients and have been investigated as biomarkers of RA. Type I interferons can have both pro and anti-inflammatory effects in RA (Firestein, 2003).

There are several treatments on the market for RA; however, typical pharmaceutical treatments, such as, glucocorticoids (GC) and Disease Modifying Anti-Rheumatic Drugs (DMARD), target the clinical symptoms of RA and not the underlining cause (Köhler et al., 2019) and can have a wide range of side effects. Glucocorticoids, a class of steroid hormone with structural and pharmacological similarity to cortisol, have anti-inflammatory and immunosuppressive properties to help manage pain and slow joint damage (Yasir et al., 2020), but can be associated with increased risk of osteoporosis, hyperglycemia, heart failure, gastrointestinal ulceration and behavioral changes (Moghadam-Kia and Werth, 2010). Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) help to manage pain, inflammation and swelling associated with RA, but do not stop or slow down the disease (Bally et al., 2017). NSAID's inhibit cyclooxygenase enzymes and are characterized as non-selective (inhibit COX-1 and COX-2) and selective (COX-2 inhibition only) (Day and Graham, 2004). NSAID's are used in combination with RA treatment with potential side effects including reduced blood clotting, gastrointestinal ulcers/ bleeding and kidney disease (Brater et al., 2001). Well known examples of NSAIDs are aspirin and ibuprofen.

Oral DMARD's remain the first line of treatment for RA (Virtanen et al., 2019). The immunosuppressive and immunomodulatory properties of conventional DMARDs and biologics help to manage pain and slow the progression of RA (Onecia et al., 2020) by targeting the immune system in a more general (conventional) or specific (biological) manner. Methotrexate, a common treatment of RA since the 1980's, has several modes of action

including cytokine profile alterations, adenosine signaling, folate antagonism and generation of reactive oxygen species (ROS). Antagonizing folic acid reduces lymphocyte proliferation and the production of proinflammatory cytokines, lending to the effectiveness of methotrexate (Virtanen et al., 2019); however, increased extracellular adenosine signaling has also been hypothesized to be a main mode of action in RA. A high affinity adenosine transmembrane receptor, A2A, associated among other things with T cell/ neutrophil inhibition and the differentiation of macrophages to an anti-inflammatory phenotype is believed to be the primary mechanism associated with the benefits of methotrexate. Methotrexate directed increase in adenosine can have opposing effects as not all adenosine receptors have an anti-inflammatory response (Friedman and Cronstein, 2019).

Biological DMARD's have higher specificity than their conventional counterparts, targeting cytokines and the pathways they induce. Janus kinase (JAK) inhibitors are small molecule inhibitors that block the Janus kinase–Signal Transducers and Activators of Transcription (JAK/STAT) pathway. Tofacitinib, one of the first JAK inhibitors approved in the U.S., inhibits the induction of the JAK/STAT pathway from multiple cytokine receptors (Virtanen et al., 2019).

Most treatments start as monotherapies utilizing either conventional or biologic DMARDs. Combination therapies are utilized depending on the effectiveness of the initial treatment (Virtanen et al., 2019). The dampening of the immune system can have unintended effects. Gastrointestinal distress, increased risk of infections, bone marrow suppression, liver, kidney or lung

problems and cancer are a few seen with long term treatment using DMARDs (Onecia et al., 2020).

1.2 Type I Interferons

Interferons are small proteins classified as families of cytokines associated with the innate immune system and thought to play an essential role in viral infection and other immunoregulatory responses. Interferons are divided into 3 families: Type I, Type II and Type III. Type I interferons are pleiotropic cytokines, with anti- and pro-inflammatory characteristics that have been used in therapies ranging from chronic viral infection to tumor suppression. In humans, there are 13 structurally similar type I interferons clustered on the same chromosome (chromosome 9). Two subsets of type I interferons are IFN-α and IFN-β (Kalliolias and Ivashkiv, 2010).

The expression of type I interferons can be stimulated through several pathways involving different receptors and adaptor proteins. Dendritic cells, among others, have high levels of type I interferon expression that may play a role in innate and the adaptive immune responses. Interferon regulatory factors (IRF) are transcriptional factors that induce transcription of type I interferon genes. Interferon regulatory factors 3 and 7 have been linked to the regulation of IFN expression in RA (Sweeney et al., 2010).

The expressed IFN-α and IFN-β cytokines induce paracrine and autocrine signaling with their common receptor, IFNAR (consists of two subunits IFNAR1 and IFNAR2); leading to the expression of several interferon stimulated genes (ISGs) (Barrat et al., 2019). Although they interact with the

same interferon receptor (IFNAR), IFN-α and IFN-β induce distinct conformation changes on the cytosolic side of the transmembrane receptor, initiating differential signaling outcomes mediated by STAT proteins (Muskardin and Niewold, 2018). Upon receptor binding, JAK is recruited to IFNAR, which leads to the phosphorylation of designated tyrosine residues on the receptor. It is the recognition of these phosphorylated residues by STAT (and other molecules) that results in the phosphorylation and dimerization of STAT proteins. The dimerized STAT forms a complex with IRF 9, IFN-stimulated gene factor 3, before translocating into the nucleus (Walker and Smith, 2005). Once inside the nucleus, this complex acts as a transcription factor and regulates the transcription of interferon stimulated genes (Figure 1 and Table 1). The functionality of ISGs are diverse and some have been linked to autoimmunity and inflammation (Barrat et al., 2019).

Type I interferons can self-amplify through ISGs creating a positive feedback loop. Factors that enhance interferon expression, such as interferon regulatory factors and the stimulator of interferon genes (STING), are among the ISGs stimulated by interferons (Table 1). The interferon stimulated autocrine and paracrine signaling can amplify their expression on all localized cells (Choubey and Moudgil, 2011).

Another ISG expressed is the tumor necrosis factor (TNF) (Li et al., 2017). TNF has been associated with a variety of diseases, including autoimmune diseases. In RA patients, TNF-α has been shown to increase the proliferation of macrophages, activate T and B cells and synovial lining cells (Vasanthi et al., 2007). Synovial lining thickens in RA and the activation of these

proliferative synovial fibroblasts invade/ destroy adjacent cartilage (Nygaard and Firestein, 2020). TNF-α is also thought to induce several inflammatory cytokines including granulocyte monocyte - colony stimulating factor (GM-CSF), IL-1, IL-6 and IL-8. Reduced levels of these proinflammatory cytokines have been seen in the synovial cells of RA patients after treatment with anti-TNF-α antibodies (Vasanthi et al., 2007).

IFN-α has been detected in the synovial fluid and tissue of RA patients (Conigliaro et al., 2010) and studies have suggested IFN-α can act as a bridge between the innate and adaptive immune system (Muskardin and Niewold, 2018). IFN-α activates and upregulates CD86, MHC I and MHC II (Table 1) on antigen presenting cells, such as, dendritic cells, macrophages and B cells (Muskardin and Niewold, 2018). In the synovial lining of RA patients, an excess of IFN-α/β excreting plasmacytoid dendritic cells (pDC), correlating with serum levels of RF and anti-cyclic citrullinated peptides, has been detected (Conigliaro et al., 2010) and primarily excretes IFN-α which promotes pDC maturity (Rodríguez-Carrio et al., 2015). Increasing pDC maturity can lead to increased dendritic arthritogenic antigen presentation to T cells and the promotion and progression of RA (Rodríguez-Carrio et al., 2015). IFN-α has also been shown to upregulate the expression of Toll-like receptors (TLRs) and other cytosolic nucleic acid receptors (Choubey and Moudgil, 2011), increasing a cell's responsiveness to these innate receptors. Through these mechanisms IFN-α can play into the regulation and threshold of self-activity and, therefore, autoimmune diseases (Muskardin and Niewold, 2018).

In clinical studies, IFN-α was hypothesized to be an RA trigger, especially in patients that are predisposed to the disease. The occurrence of RF was associated in 34% of therapeutic IFN-α induction of RA and the persistence of RA after treatment was predominately seen in patients who exhibited anti-CCP (cyclic citrullinated peptide) antibodies as well as other markers (Rodríguez-Carrio et al., 2015). These findings suggest that although IFN-α can trigger the manifestations of RA it is not the only mechanism involved in the disease.

IFN-β is thought to have anti-inflammatory and anti-proliferative properties working in opposition of IFN-α. IFN-β has shown to have inhibitory effects on IL-1β and secreted metalloproteinase induced production of IL-6, IL-8, and GM-CSF in RA fibroblast-like synoviocytes (FLS). It has also been shown to inhibit TNF-α in macrophages, monocytes, lymphocytes and FLS, while simultaneously enhancing the production of anti-inflammatory mediators like IL-1 receptor antagonist (IL-1Ra) and IL-10 by fibroblast-like synoviocytes (Conigliaro et al., 2010). TNF-α, IL-1β, and IL-6 have all been associated in the pathogenesis of RA (Tak, 2004).

Clinically, the protective qualities of IFN-β in RA patients can include reducing inflammation by inhibiting adhesion molecule expression (regulates cell migration), reducing the development of osteoclasts and cartilage destruction through inhibition of c-Fos activation by receptor activator of nuclear factor κB (NF-κB) ligand (RANKL) and inhibiting tissue remodeling by regulating levels of metalloproteases and protease inhibitors (Rodríguez-Carrio et al., 2015). IFN-β can be produced from a variety of cells (Rodríguez-Carrio

et al., 2015) and synovial tissue isolated from RA patients have higher levels of IFN-β, especially the intimal lining layer and vascular endothelium. These levels were markedly higher compared to other arthritic diseases, such as osteoarthritis (Conigliaro et al., 2010).

The detection of type I interferons and their mRNA signatures in RA patients have made them prominent biomarkers in RA (Rodríguez-Carrio et al., 2015); however, their pleiotropic nature as immunostimulatory and immunosuppressive cytokines has led to controversy in determining their exact role (Conigliaro et al., 2010). Concentration, co-cytokine signaling, and cell type can all impact the role and consequence of IFN expression in RA. Further insights into these factors could potentially lead to a better understanding on how to treat the disease (Muskardin and Niewold, 2018).

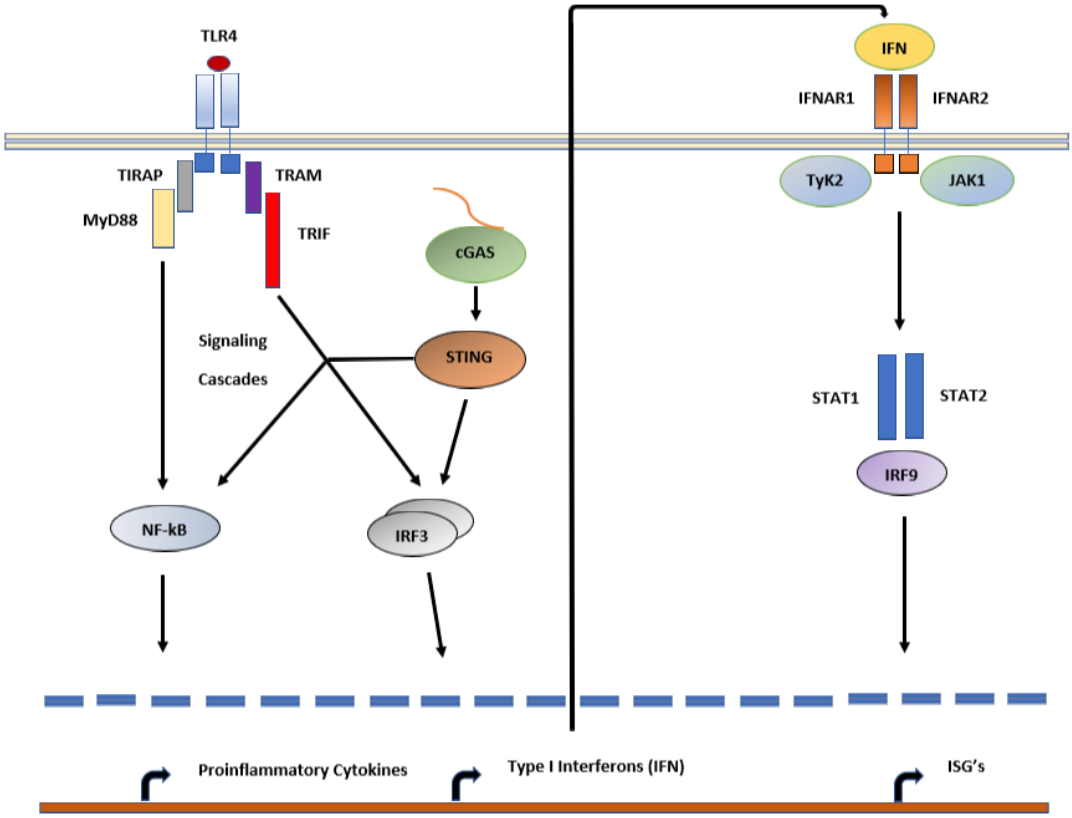


Figure 1: Type I interferon production and subsequent ISG expression through the Janus kinase pathway. Production of type I interferons is accomplished through multiple pathways, examples of which are the TLR4 and STING pathways. Interferon activation of IFNAR leads to the expression of ISGs. Ligands of cGAS and TLR4 not are not specified in the above figure.

Table 1: Examples of Interferon Stimulated Genes**.** Summary of genes stimulated by type I interferons and associated cellular activity. ISGs are involved in a range of cellular functions from pathogen protection to cellular movement; however, the table below predominantly indicates singular associations (Li et al., 2017; Wan et al., 2020).

Cellular Activity	**Interferon Stimulated Genes (ISGs)**
Immune Modulators	MxA, MxB, ICAM1, SELL, CD47, ALCAM, IRF1–5 & 7, LGALS3B, IFN-γ, IL-12, TNF-α, SOCS, USP18
Induce Apoptosis	CASP4, CASP8, BAK1, Fas/ CD95, PLSCR1, XAF-1, DAP kinase, RID
Cellular Attraction/ Adhesion	VEGF, FGF, VRP, PDGFRL, ECGF1, EREG, MHC I&II, CTGF
Detection of Virus/Pathogens	STING, c-GAS, OAS1, Protein Kinase R, Viperin, Tetherin, IFI6, IFITM2, IFITM3, IRF1, IRF7, MHC I&II, Ribonuclease L, GTPase Mx1, ISG15, ISG20, ADAR1, APOBEC

1.3 Toll-like Receptors (TLRs)

The innate immune system is a non-specific, fast-acting defense against foreign invaders that relies on the recognition of PAMPs (pathogen-associated molecular patterns) for activation. The recognition of PAMPs is reliant on PRRs (pattern recognition receptors), which a notable example are Toll-like receptors (TLRs). (Huang and Pope, 2009). Named for their similarity to the *Toll* gene found in *Drosophila* (Hansson and Edfeldt, 2005), Toll-like receptors mediate immune responses through signal transduction pathways, stimulating the expression of inflammatory mediators and cytokines. Intracellular domain adaptor proteins, such as Myeloid differentiation primary response gene 88 (MyD88) and TIR-domain-containing adaptor-inducing interferon- beta (TRIF), further modulate TLR signaling pathways and their resultant molecule expression (Yuk and Jo, 2011). The location of specific TLRs generally correspond to the PAMPs they recognize and fall into two general categories: the outer cell membrane and intracellular endosomes (Beutler, 2019). TLRs can be further separated out by cellular expression and the response they produce (Table 2).

Toll-like receptor signaling has been implicated in the pathology of RA. In addition to the defined PAMPs associated with each receptor, several endogenous ligands have been found in the synovial tissue of RA patients, which are theorized to increase the expression and activation of TLRs. Different heat shock proteins (HSP) have been implicated in the activation of TLR2 and TLR4 in macrophages, fibroblasts and non-specifically in synovial tissue in RA. Studies have shown that the expression of inflammatory cytokines

in synovial tissue membranes decrease after inhibition of MyD88 and Mal/TIRAP; known modulators of TLR2 and TLR4 signaling. Ex vivo experiments have also shown inhibition of IL-1β and TNF expression in synovial tissue in the presence of a TLR4 antagonist (Huang and Pope, 2009).

The expression and activation of other TLRs have also been identified and associated with the RA phenotype. Toll-like receptor 3 in necrotic synovial fluid cells is a defined source of RA. Toll-like receptor 3 and 7 are also co-expressed with of type I interferons in RA synovium. These toll-like receptors are hypothesized to have mediating effects on TLR4 potentially enhancing its responses (Huang and Pope, 2009).

A Toll-like receptor believed to be of importance in the persistence of arthritic allodynia (pain) is TLR4. Stimulation of TLR4 by its agonist, Lipopolysaccharide (LPS), induces two main pathways: MyD88 dependent and independent. The MyD88 dependent pathway leads to the expression TNF via the transcription factor NF-κB quickly but can also induce type I interferon production (Christianson et al., 2011). The MyD88 independent pathway which utilizes TRIF strongly induces interferon transcription through the interferon regulatory factors (IRF) (Kawasaki and Kawai, 2014). The pleiotropic characteristics of type I interferons could play opposing roles in RA (Conigliaro et al., 2010); however, TNF-α is a potent proinflammatory cytokine and its high levels in RA synovial fluid plays a pivotal role in inflammation and joint destruction seen in this disease (Vasanthi et al., 2007). In additional to this localized response, TNF-α has also been implicated in the development of

neuropathic pain (Leung and Cahill, 2010) and, hypothetically, TNF-α directed neuropathic pain could play a role in the chronic pain associated with RA.

Table 2: Toll-like receptor ligands, immune cell and cytokine expression. Toll-like receptors recognize various PAMPs associated with viral and bacterial infection and endogenous ligands that can lead to self-reactivity. The recognition induces various immune responses mediated through adaptor proteins (Hosseini et al., 2015 & Shah et al., 2013).

Receptor	Location; Cellular Expression	Endogenous Ligands	Adapter Protein(s)	Cytokine Expression
TLR 1 (w TLR 2)	Plasma Membrane; Neutrophils, Macrophages/Monocytes, Dendritic Cell Subsets, B Cells	N.D.	MyD88/ TIRAP	Proinflammatory Cytokines
TLR 2 (w TLR 1 or TLR 6)	Plasma Membrane; Neutrophils, Macrophages/Monocytes, Myeloid Dendritic Cells, T Cells	HSP-60, -70,-gp96, Hyaluronic Acid, ECM HMGB1, Versican	MyD88/ TIRAP	Proinflammatory Cytokines
TLR 3	Endosome; Myeloid Dendritic Cells, B Cells, T cells	self-RNA (damaged cells)	TRIFF	Proinflammatory Cytokines, Type I IFNs
TLR 4	Plasma Membrane; Neutrophils, Macrophages/Monocytes, Myeloid Dendritic Cells	HSP-22,-60, -70, and -72,-gp96, HMGB1, Oxidized Phospholipids, Heparin Sulfate, Hyaluronic Acid, Fibronectin, Tensascin-C, B-Defensin 2, Versican	MyD88/ TIRAP TRIF/TRAM	Proinflammatory Cytokines, Type I IFNs
TLR 5	Plasma Membrane; Neutrophils, Macrophages/Monocytes, Myeloid Dendritic Cells, T Cells	N.D.	MyD88	Proinflammatory Cytokines
TLR 6 (w TLR 2)	Plasma Membrane; Neutrophils, Macrophages/Monocytes, Dendritic Cell Subsets, B Cells	See TLR 2	MyD88/TIRAP	Proinflammatory Cytokines
TLR 7	Endosome; Neutrophils, Macrophages/Monocytes, Plasmacytoid Dendritic Cells, B Cells	ssRNA	MyD88	Proinflammatory Cytokines, Type I IFNs
TLR 8	Endosome; Neutrophils, Macrophages/Monocytes, Myeloid Dendritic Cells, T Cells	ssRNA	MyD88	Proinflammatory Cytokines, Type I IFNs
TLR 9	Endosome; Neutrophils, Plasmacytoid Dendritic Cells, B Cells, T Cells	Chromatin IgG complex	MyD88	Proinflammatory Cytokines, Type I IFNs

1.4 Stimulator of Interferon Genes (STING)

An important signaling pathway leading to the expression of type I interferons involves the stimulator of interferon genes, STING. STING is a cytoplasmic endoplasmic reticulum (ER) receptor involved in innate immunity and found in various endothelial and epithelial cells, T cells, macrophages and dendritic cells (Barber, 2015). STING is an adaptor protein in the cytosolic dsDNA sensing pathway initiated by cyclic GMP-AMP synthase (cGAS). It binds to cyclic dinucleotides produced by cGAS and initiates a cascade that leads to the production of interferons and other cytokines (Li et al., 2017). The STING molecular pathway is an important antiviral response, but also can sense cellular stress if an excess of dsDNA is released into the cytosol. Although STING is a part of the dsDNA sensing pathway it's actions may not be limited to it. Gain-of-function mutations in STING have been linked to STING-associated vasculopathy in infancy (SAVI) and Familial Chilblain lupus (FCL) based on excess type I interferon responsiveness. The elevated levels of type I interferons found in these patients leads to sustained signaling and over production of ISGs. In SAVI patients, the excess responsiveness in fibroblasts, keratinocytes and immune cells amasses a large proinflammatory response leading to lesions in capillaries and tissues (Li et al., 2017).

Several autoimmune diseases are believed to be associated with type I interferonpathologies, including early onset rheumatic diseases (Volpi et al., 2016). Developing STING antagonists could have therapeutic benefits for patients suffering from RA and other autoimmune diseases. In vitro experiments of SAVI patients revealed that type I interferon production was

reduced in biopsy samples after the administration of a JAK adaptor inhibitor associated with the STING pathway (Li et al., 2017).

1.5 Allodynia

Allodynia, or centralized pain, is defined as neuropathic pain or a pain response to a non-painful stimulus (He and Kim, 2019). Chronic inflammation, as seen in RA, can lead to nerve damage and neuropathic pain, but the mechanisms are yet unknown. Cytokine recruitment of innate cells, notably macrophages, enhance further cytokine production- a positive feedback cycle (Norikazu et al., 2017). High concentrations of macrophages are found in inflamed synovial fluid and play key roles in the severity of RA (Kinne et al., 2000). Nociceptor cytokine sensitization can alter the processing of non-painful stimuli by sensory neurons. Centralized pain is the consequence of injury induced prolonged peripheral signaling into the spinal dorsal horn and the overactivation of pain neurons and glial cells. Notably cytokines that enhance nociceptor activity and are overly expressed in the dorsal horn after nerve injury including TNF, IL-1β and IL-6 (Norikazu et al., 2017).

1.6 The K/BxN Murine Model of Arthritis

Previous models used to study inflammatory diseases had limited capacity to investigate pain in relation to chronic infection. Clinical signs were short lived, had no B or T cell activation and could not explain the persistence of pain despite the resolution of inflammation. The K/BxN mouse model is a KRN mouse bred on a C57BL/6 background and crossed with a nonobese

diabetic (NOD) mouse (Kouskoff et al., 1996). KRN mice are transgenic for a T cell receptor that has specificity for bovine pancreatic RNase. However, the K/BxN mouse produces antibodies against Glucose-6-Phosphate Isomerase (G6PI). G6PI is ubiquitously expressed and arrayed around joint cartilage. The passive transfer of anti-G6PI antibodies to a recipient mouse results in the predictable onset of clinical arthritic symptoms reminiscent of the inflammatory disease. The inflammatory phase also reliably resolves upon clearing of the injected antibodies, allowing the study of persistent or chronic pain (Christianson et al., 2010).

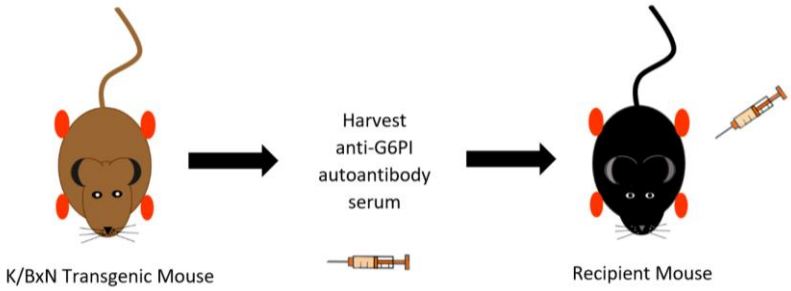


Figure 2: K/BxN model of passive serum transferred arthritis. K/BxN transgenic mice are bled and serum containing anti-G6PI autoantibodies is injected into a recipient mouse intraperitoneally to induce an arthritic phenotype.

1.7 The Role of Toll-Like Receptor 4 in Pain using a K/BxN Murine Model of Arthritis

In a K/BxN-induced mouse model of transient arthritis mechanical hypersensitivity (assessed by pain-like behavior), persists even after the resolution of inflammation in a male wild type (WT) mouse. The persistence in this model is reminiscent of the chronic pain state in an RA patient. Recent studies have indicated that TLR4 plays an important role in the transition from acute to chronic pain in arthritis. *Tlr4* $^{-/-}$ mice with K/BxN serum transferred arthritis had a significant recovery in mechanical hypersensitivity congruent with the recovery in peripheral inflammation compared to the WT mice. Furthermore, early intrathecal treatment with a TLR4 receptor antagonist appeared to prevent the transition to persistent mechanical hypersensitivity in K/BxN serum induced arthritic WT mice; however, the transition was still observed if treatment was performed later in the progression of disease (Christianson et al., 2011). These studies suggested that TLR4 is essential in the initial transition from acute to chronic pain, but not in the reversal of this transition.

1.8 Anti-TNF-alpha and IFN-beta Treatments

Previous experiments investigating mechanical pain in rheumatoid arthritis have shown post-inflammatory joint tactile allodynia in a serum transfer K/BxN murine model similar to what is observed in RA. The post-inflammatory tactile allodynia in WT female murine models was less severe when compared to the male WT murine models. Toll-like receptor 4 signaling coupled with the

expression of TNF and IFN-β were found to mediate the transition to post-inflammatory tactile allodynia. In WT males, the co-administration of anti-TNF-α antibodies and IFN-β intrathecally permanently absolved tactile allodynia. Independent administration of anti-TNF-α antibodies and IFN-β, however, was unable to resolve persistent allodynia in WT males (Woller et al., 2019).

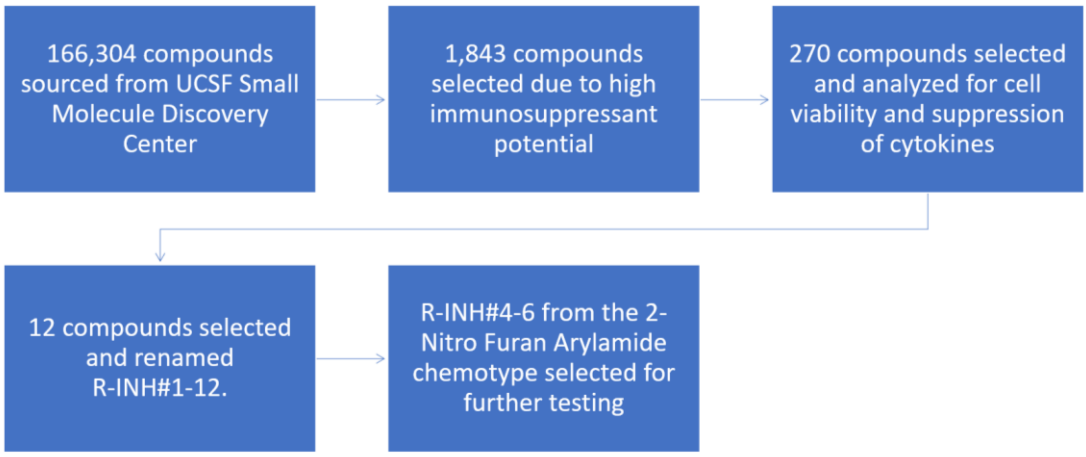
1.9 Failure of IFN-beta to Induce Remission in RA Clinical Trials

Although IFN-β has been clinically effective in multiple sclerosis (van Holten et al., 2002) and is an FDA approved therapeutic it did not show significant efficacy in RA clinical trials (Genovese et al, 2004). Alternative administration locally in the joint may have circumvented this toxicity.

1.10 2-Nitrofuran Arylamide Compounds as Potential Therapeutics

In an effort to identify novel compounds that could yield complementary approaches to the current treatments of RA the data from two existing high throughput screens were mined and candidate compounds were tested to reduce cytokine production and cellular toxicity (Fujita, 2019).

Table 3: R-INH#4-6 identified in prior High-Throughput Screening (HTS) (Fujita, 2019).



2. Methods

2.1 Mice

Wild type C57BL/6 mice, NOD/Lt, Ifnar1$^{flox/flox}$, Itgax Cre, LysM Cre and *Tmem173*$^{-/-}$ mice, originally purchased from The Jackson Laboratory in Bar Harbor, Maine, USA, were housed, maintained and bred in the American Association for Accreditation of Laboratory Animal Care, University of California San Diego Animal Facility. All animal experiments followed protocols approved by the Institutional Animal Care and Use Committee of the University of California San Diego. Maximum occupancy did not exceed 4 per standard cage and they were housed at room temperature and on a 12 hour light-dark cycle. All testing was conducted during the light cycle and food and water was provided *ad libitum*.

The KRN T cell receptor (TCR) mice were gifted from Drs. D Mathis and C. Benoist at the Harvard Medical School, Boston, Massachusetts, USA and the Institut de Génénetique et de Biologie Moléculaire et Cellulaire in Strasbourg, France. The reference mice were bred on a C57BL/6 background (K/B). The arthritic mice (K/BxN) were produced by crossing of K/B mice with NOD/Lt (N).

2.2 Passive Serum Transfer of Arthritis

Blood collected through retro-orbital bleeding of anesthetized K/BxN mice 6-8 weeks old was centrifuged in a Beckmann centrifuge at 1400 rpm for 10 minutes. The serum was pooled and frozen at -20 °C until use. 100µl of serum was administered to each test mouse on day 0 and 2 via intraperitoneal

(IP) injection. On the day of sacrifice, the experimental mice were bled, and the serum extracted and stored at -20°C.

2.3 Assessment of K/BxN Induced Inflammation

K/BxN-induced inflammation was assessed by measuring the ankle width in each mouse using a caliper. Measurements took place days 0-6 and then every third day afterwards until the last measurement on day 28. The change from the baseline was calculated and compared between each strain and sex. The averages were analyzed using a two-way ANOVA with a Bonferroni post hoc test (PRISM V.6.). At the end of the experiment, the hind paws were removed and fixed in a 10% formaldehyde solution.

2.4 von Frey Behavioral Testing

To evaluate tactile allodynia in K/BxN serum induced arthritis a minimal withdrawal threshold was assessed through the employment of the up-down von Frey method (Christianson et al, 2012). The test mice were isolated in clear, plastic containers on a wire-mesh surface, which allowed ventral paw access. Based on the Standard Operating Procedure sourced from The Jackson Laboratory, von Frey filaments ranging from 2.44 to 4.31 (0.03 g to 2.00 g) were evenly applied to the ventral side of the paw to determine the minimal force (g) needed for withdrawal (The Jackson Laboratory, 2019). Mice were acclimated on day -2 and day -1 for 45 minutes prior to the experimental date (day 0). On day -1, each mouse was acclimated with von Frey 4.17 filament 10 times on the ventral side of each hind paw. The up-down von Frey method was

administered day 0-6 of the experiment and every third day from day 6-28. The von Frey filament, or mechanical force, that elicited a response was recorded and the 50% response threshold analyzed using the following equation (Deuis et al., 2017):

50% threshold (g) = 10(X+kd)/104,

X = value of final von Frey filament (log units)

k = response pattern (tabular value)

d = average increment between von Frey filaments (log units)

The final values were averaged and analyzed using a two-way ANOVA with a Bonferroni post hoc test (PRISM V.6.).

Figure 3: Diagram of the von Frey testing apparatus and measurement of tactile allodynia. Clear plastic containers to acclimate and isolate each mouse (left), wire mesh bottom of the apparatus and application of von Frey filament (middle), target placement of filament to measure withdrawal threshold (right).

2.5 Murine Bone Marrow Dendritic Cell Harvesting

Primary bone marrow dendritic cells (BMDC) were flushed from the femurs and tibia of WT C57BL/6 and *Tmem173*$^{-/-}$ mice (Datta et al. 2003) with 30 ml of RP10 medium. The cells were centrifuged, resuspended with RP10 and 5 ml plated on 150mm petri dishes with 5µl of GM-CSF (0.1 mg/ml; ebioscence 14-9331-80). Cell plates were incubated at 37 °C and RP10 media was added on day 3 and 6 with 5µl of 0.1 mg/ml GM-CSF. On day 8, BMDC cells were harvested from each plate, centrifuged at 100 rpm for 5 minutes and resuspended in RP10. They were transferred to 96 well round bottom well plates for a concentration of 10^5 cells per well in 100µl.

2.6 Treatment of Cells with Compounds and Cytokine Evaluation through ELISA

Murine WT and *Tmem173*$^{-/-}$ BMDCs were plated at concentrations of 10^5 cells/mL per well in a 96-well round bottom plates. Cells were treated with R-INH compounds at discrete concentrations or a DSMO control (considered no treatment) in the presence of 10 µg/ml DMXAA, 100 ng/ml LPS (catalog# LPS-EB) or 1 µM of 1V270, agonists for STING (Weiss et al., 2017), TLR4 (Pålsson-McDermott and O'Neill, 2004) and TLR7 (Sato-Kaneko et al., 2017) respectively, or media (no cytokine production) as the control overnight. The supernatant of each well was isolated and the production of TNF-α, IFN-β and IP10 was analyzed through an ELISA using the manufacturer's protocols (ebioscience).

2.7 Preparation and Administration of Compound R-INH#5

The R-INH#5 compound was prepared from a 200mM concentrated stock in DMSO. The administered compound consisted of a 750 nmol suspended in 300 µL of PBS. It was delivered to the recipient mice twice daily via IP injection. A 1.25% DMSO solution in PBS was used in the vehicle treated mice.

3. Results

3.1 K/BxN Induced Arthritis in Immune Deficient Murine Models

Studies have shown that the innate immune system plays an important role in the pathology of RA. The consequential cytokine production of an activated innate pathway can contribute to synovial inflammation, a signature of RA, and factor into the pain response (Sweeney et al., 2010). Understanding molecular pathways that contribute to the progression of the disease can lead to therapeutic targets; however, signaling pathways are typically cascade events involving novelty and redundant factors whose expression can be spatially and temporally specific (Kholodenko, 2006). Attenuating or modifying immune responses can also have deleterious effects and increase susceptibility to pathogens and other diseases (Nicholson, 2016). Reduction of these deleterious effects may be accomplished by increasing specificity not only to specific pathways, but also to specific cells shown to mediate pain associated with RA and it's progression. In these experiments, the focus was on the role of type I interferon associated pathways and their modulating effects in an arthritic mouse model in a cell specific and non-specific capacity.

To better understand the role of type I interferons in the progression of rheumatoid arthritis, genetically altered immune deficient mice were induced with K/BxN serum arthritis and tested for pain-like withdrawal and swelling over a 28 day period. Through IP injections, 100µl of K/BxN serum was administered to each mouse on day 0 and day 2 of the experiment. Von Frey meaurements for allodynia and caliper measurements of ankle swelling were taken on days 0-6 and then every third day afterwards. Comparisons between sexes within each immune deficient strain and comparisons to the WT phenotype were made.

The onset of the arthtitic swelling in the male and female WT C57BL/6 mice was observed around days 2-3, with its peak for both sexes between days 9 and 12 (Figure 4A). This onset was expected based on what was observed in previous experiments. Ankle swelling steadily resolved from days 15 to 28; however, it did not reach baseline levels (Figure 4A). The overall averaged female swelling appeared to be more severe than their male counterparts (Figure 4B). Mechanical withdrawal, or allodynia, a pain-like behavior in mice was immediately evident on day 1 of the experiment in both males and females. Baseline measurements (day 0) did indicate that females had a slightly higher initial pain-like response than males (Figure 4C). The pain-like behavior steadily increased until the mechanical withdrawal reached minimum thresholds on days 9 and 12 in both males and females (Figure 4C), congruent with the ankle swelling peak (Figure 4A). Female WT mice appeared to begin to resolve this pain-like behavior around day 18 of the experiment, while the WT male pain-like behavior persisted at or around their minimum threshold

throughout day 28; however, there was no significant difference (Figure 4C). The persistence in pain-like behavior despite the resolution of swelling in the WT males has been noted in prior experiments studying this mouse model and has the pharmacological features of neuropathic pain (Christianson et al., 2010).

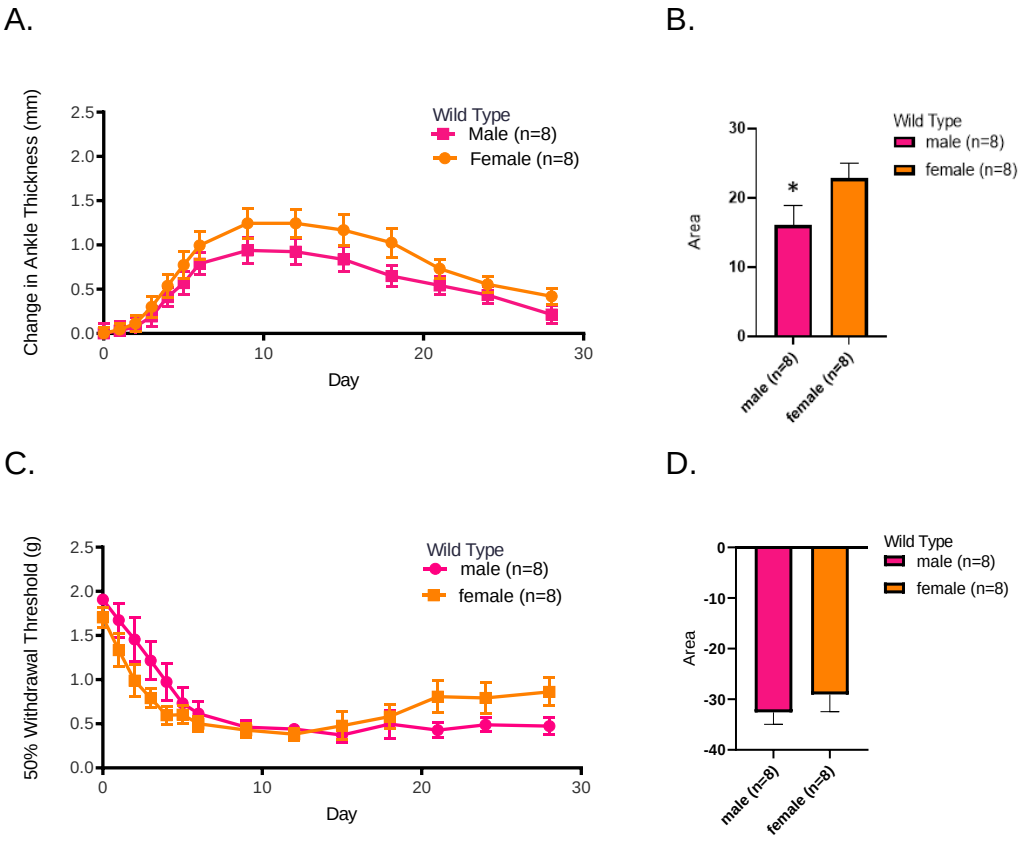


Figure 4: K/BxN serum induced ankle swelling and allodynia in wild type mice. K/BxN serum was administered to male and female mice. Ankle swelling (A and B) and mechanical withdrawal thresholds (C and D) were assessed on the indicated days. Means of the area under the curves ± SEM were calculated (B and D). Females had greater swelling [Two-way ANOVA; $F(1, 194) = 23.51$, $P<0.001$]. Male pain-like behavior persisted [$F(1, 191) = 1.284$, $P=0.2585$].

Mice deficient in the Ifnar1 gene coding for a subunit of the interferon alpha/beta (IFN) receptor (IFNAR1) were used to analyze IFNAR1 directed type I interferon signaling in multiple cells. Ankle swelling was observed in both males and females on day 1; 1-2 days earlier than the WT mice (Figure 4A and 5A). Consistent with the WT, maximum swelling was also achieved around days 9-12, but Ifnar1 deficient females had significantly more swelling than the Ifnar1 males on those days (Figure 5A). On average the females appeared to have more swelling than their male counterparts, but it resolved to similar levels by the end of the experiment (Figure 5A and 5B). Like the WT, the swelling did not resolve itself down to baseline levels (Figure 4A and 5A). Allodynia measured in the Ifnar1 deficient male and female mice had a linear graphical representation indicative of limited mechanical allodynia. The pain-like response for both males and females was consistent throughout the experiment with no significant or major observable difference (Figure 5C and 5D). A similar relationship was not observed between the concurrence of maximum ankle swelling and pain-like behavior that was seen in the WT mice and, in fact, the *Ifnar1*$^{-/-}$ mice appeared to have increased swelling compared to the WT mice, but less pain-like behavior (Figure 4 and 5).

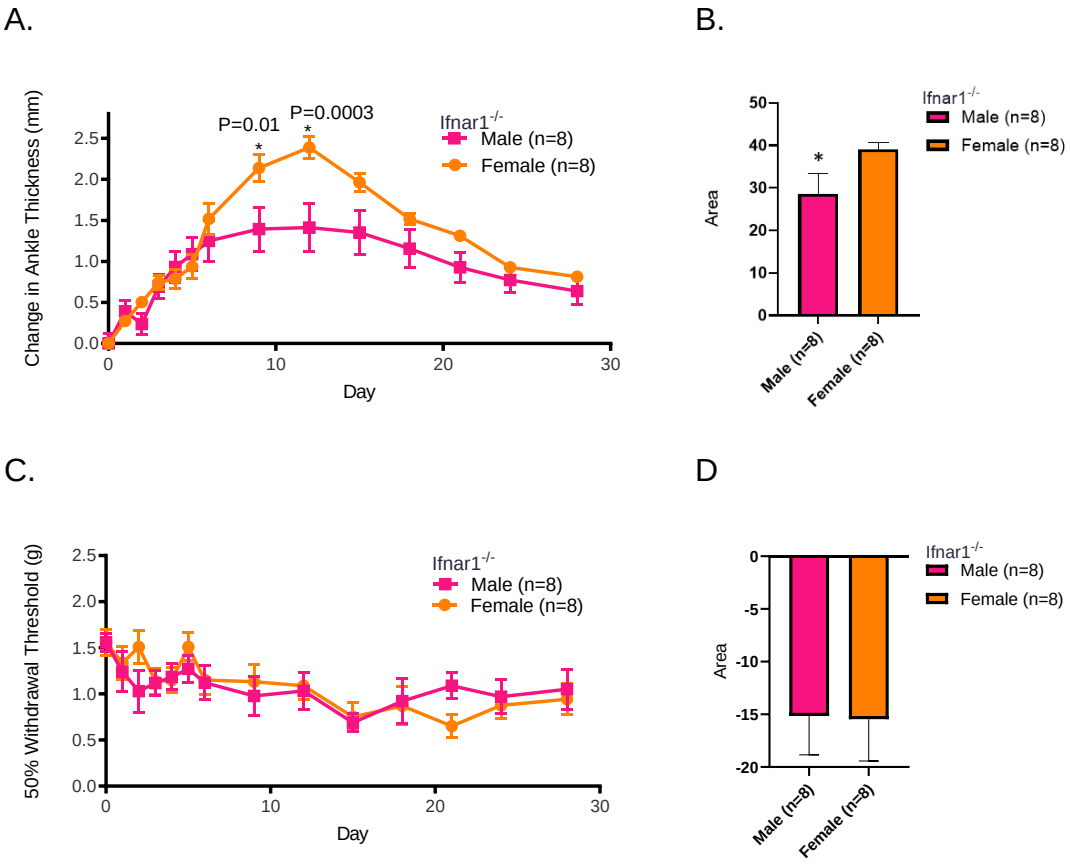


Figure 5: K/BxN serum induced ankle swelling and allodynia in *Ifnar1*$^{-/-}$ mice. K/BxN serum was administered to male and female mice. Ankle swelling (A and B) and mechanical withdrawal thresholds (C and D) were assessed on the indicated days. Means of the area under the curves ± SEM were calculated (B and D). *Ifnar1*$^{-/-}$ female mice had more severe swelling compared to the *Ifnar1*$^{-/-}$ males [Two way ANOVA; F(1, 196) = 18.04, P<0.0001]. The *ifnar1*$^{-/-}$ males and females both developed little tactile allodynia [F(1, 196) = 0.1958, P=0.6586].

To investigate specific cell types that may be associated with this lack of pain-like behavior we crossed Ifnar1$^{f/f}$ mice to mice with the cre recombinase driven by the promotors for Itgax (encodes CD11c) (Ley et al., 2016) and LysM (encodes lysozyme) (Clausen et al., 1999). In immune cells, expression of Itgax or LysM controlled genes is believed to be primarily in dendritic cells and macrophages (Ley et al., 2016) or macrophages (Clausen et al., 1999) and less so in microglia, respectively (Greenhalgh et al., 2018). The LysM conditional Ifnar1 deletion had a later onset of ankle swelling compared to both the WT and Ifnar1 deficient mice (around day 3 and 4). The pattern of swelling was similar between males and females with maximum swelling on day 9, similar to WT and *Ifnar1*$^{-/-}$ mice maximum swelling (Figure 4A, 5A and 6A), but the females on average appeared to have increased swelling compared to the males (Figure 6B) with a significant difference on day 12 (Figure 6A). The measured tactile allodynia in the *Ifnar1*$^{\Delta LysM}$ mice was reminiscent of the Ifnar1 deficient males and females. The linear trend indicates a reduced pain-like behavior compared to the WT mice that is not congruent with the ankle swelling; however, the *Ifnar1*$^{\Delta LysM}$ females averaged a more minimal threshold of mechanical withdrawal compared to the males (Figure 6C and 6D). The Itgax directed Ifnar1 deletion followed the basic trend with a higher overall ankle swelling in the females and a peak around day 9 (Figure 7A). A significant difference between the males and females was observed day 6 (Figure 7A). Tactile allodynia did not follow the linear trend observed in both the *Ifnar1*$^{-/-}$ and *Ifnar1*$^{\Delta LysM}$ mice (Figure 5C, 6C and 7C). In the *Ifnar1*$^{\Delta Itgax}$ mice, pain-like behavior did progressively increase with the lowest threshold of mechanical

withdrawal measured at maximum ankle swelling (Figure 7A and 7C). This trend was more reminiscent of the WT mice. Unlike, the WT mice however, resolution of the pain-like behavior was observed in congruence with the resolution of ankle swelling in both males and females towards the end of the experiment (Figure 7A and 7C). The averaged tactile allodynia was very similar for males and females (Figure 7D).

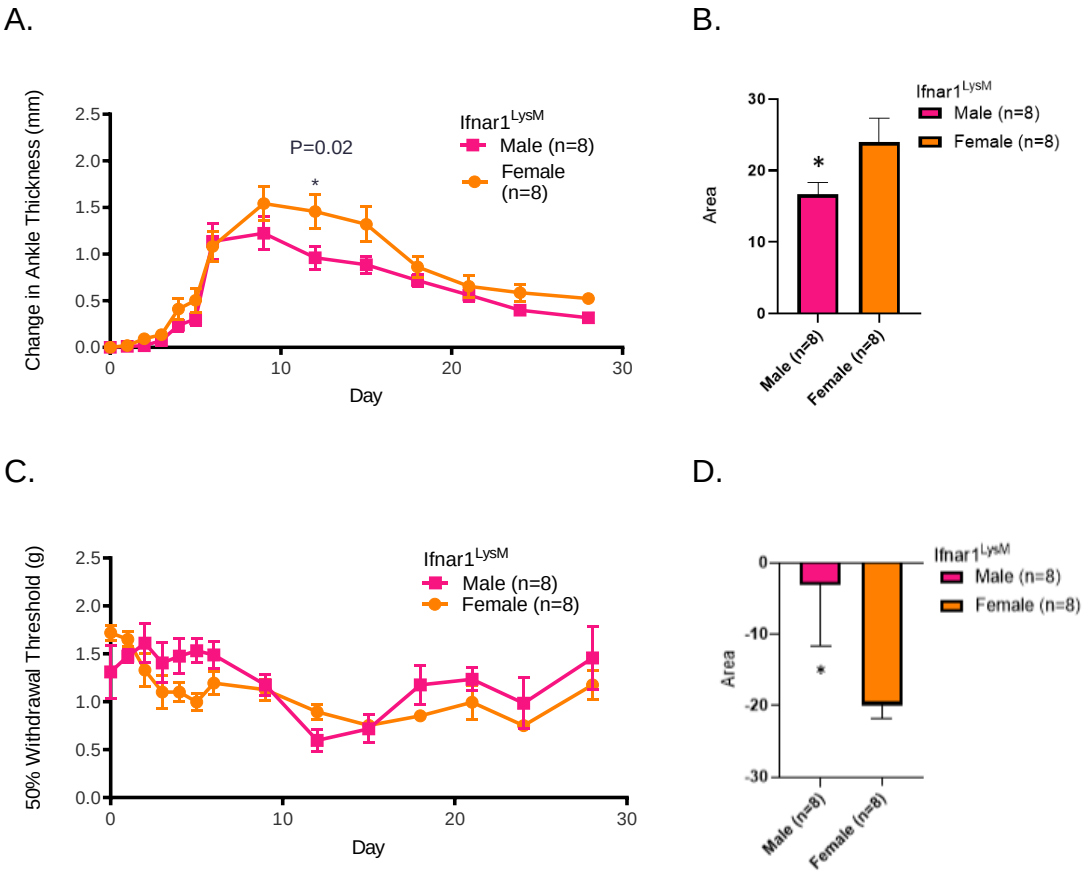


Figure 6: K/BxN serum induced ankle swelling and allodynia in *Ifnar1*$^{\Delta LysM}$ to monitor pain and swelling in LysM conditional Ifnar1 gene deletion. K/BxN serum was administered to male and female mice. Ankle swelling (A and B) and mechanical withdrawal thresholds (C and D) were assessed on the indicated days. Means of the area under the curves $\pm$ SEM were calculated (B and D). Ankle swelling pattern similar between males and females; however, females developed higher swelling [Two-way ANOVA; F(1, 190) = 16.41, P<0.0001]. Females had a greater pain-like behavior [F(1, 190) = 6.419, P=0.0121].

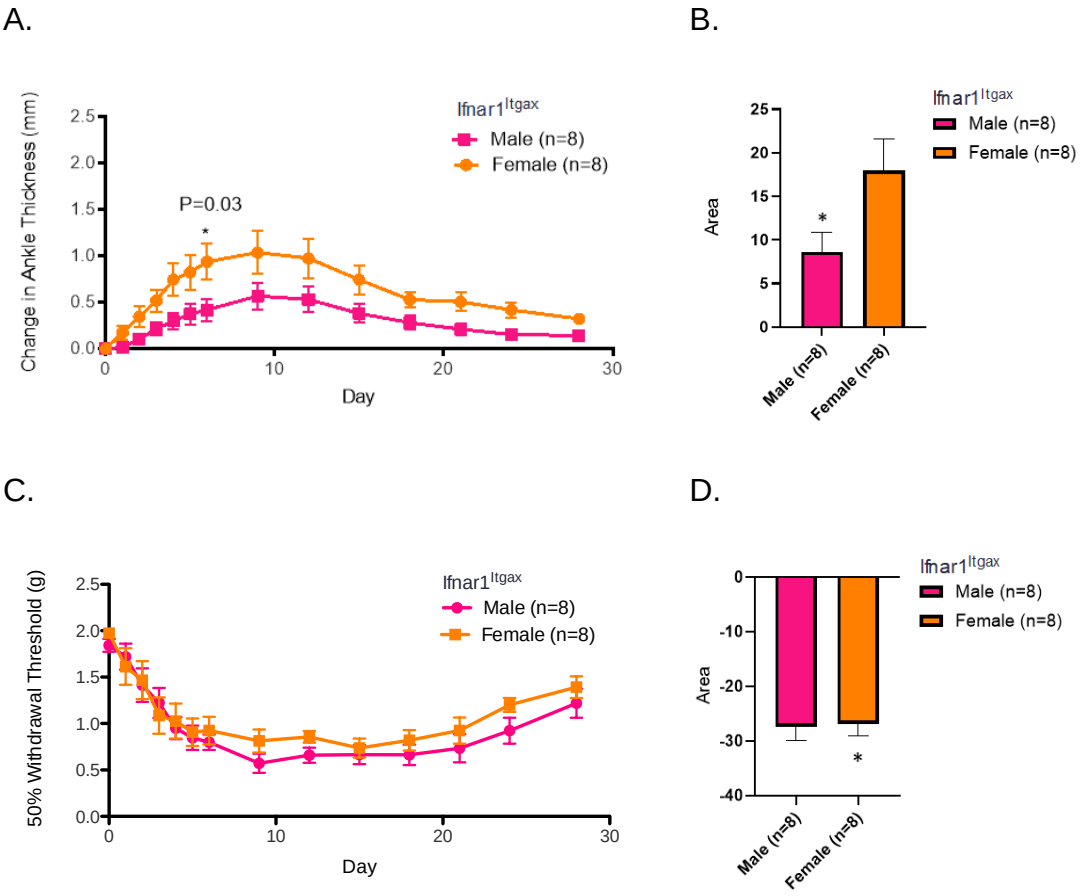


Figure 7: K/BxN serum induced ankle swelling and allodynia in *Ifnar1*$^{\Delta Itgax}$ to monitor pain and swelling in Itgax conditional Ifnar1 gene deletion. K/BxN serum was administered to male and female mice. Ankle swelling (A and B) and mechanical withdrawal thresholds (C and D) were assessed on the indicated days. Means of the area under the curves $\pm$ SEM were calculated (B and D). Females have increased swelling compared to males [Two-way ANOVA; F(1, 196) = 48.38, P<0.0001]. Males and females developed similar initial tactile allodynia with slight resolution by day 28 in both sexes [F(1, 196) = 4.481, P=0.0355].

Interferon regulatory factors (IRF) are transcription factors that induce type I interferon production and then are themselves induced by the produced type I interferons. As these transcription factors are simaltaneously upstream and downstream of type I interferons, theoretically, knocking them out should significantly reduce levels of types I interferons systemwide. To evaluate the signaling pathway involving these transcription factors mice deficient in both Irf3 and Irf7 were examined for the clinical signs of arthritis. Male and female Irf3.Irf7 deficient mice returned similar patterns and averages of both ankle swelling and tactile allodynia (Figure 8A-8D). Males and females had an onset of ankle swelling around day 3 with maximum swelling on day 9 and 12. Like the other models tested, complete resolution of swelling down to baseline was not achieved (Figure 8A). The overall trend of pain-like behavior was similar to the *Ifnar1*$^{\Delta Itgax}$ mice with a minimum mechanical withdrawal threshold measured at the highest incidence of ankle swelling (day 9) and an observable resolution in both males and females towards the end of the 28 day experiment (Figure 7A, 7C, 8A and 8C).

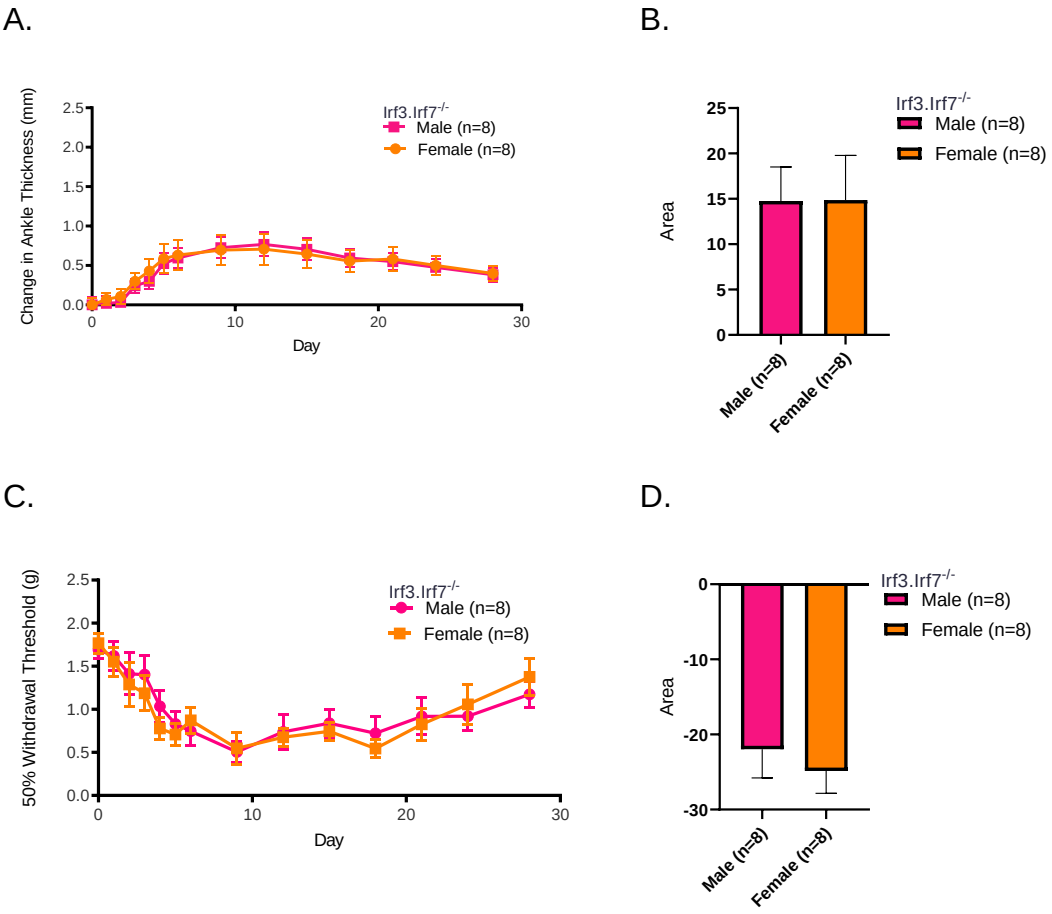


Figure 8: K/BxN serum induced ankle swelling and allodynia in *Irf3/Irf7*$^{-/-}$ mice. K/BxN serum was administered to male and female mice. Ankle swelling (A and B) and mechanical withdrawal thresholds (C and D) were assessed on the indicated days. Means of the area under the curves ± SEM were calculated (B and D). Irf3/Irf7 deficient males and females had similar swelling [Two-way ANOVA; F(1, 196) = 0.1291, P=0.7197]. Males and females both developed tactile allodynia, which resolved itself towards the end of the experiment [F(1, 196) = 0.4544, P=0.5011].

To evaluate immune modulating effects even further upstream of the interferon pathway, K/BxN-induced arthritis was employed on Tmem173 deficient mice. Tmem173 gene encodes for the STING receptor, an endoplasmic receptor which induces the production of type I interferons upstream of interferon regulatory factors (Li et al., 2017). Unlike any other model tested, the severity of swelling in the males appeared to be greater than the females, but overall, the Tmem173 deficient mice had very low ankle swelling with a peak days 9 and 12 (Figure 9A and 9B). Despite the reduced swelling, Tmem173 deficient mice had similar levels of tactile allodynia as the WT mice with a similar initial trend; however, neither sex appeared to have resolution of the pain-like behavior towards the end of the experiment (Figure 4C and 9C). Tmem173 deficient female mice also had less allodynia overall compared to the Tmem173 deficient males (Figure 9D) with a significant difference on day 21 and 25 and (Figure 9C).

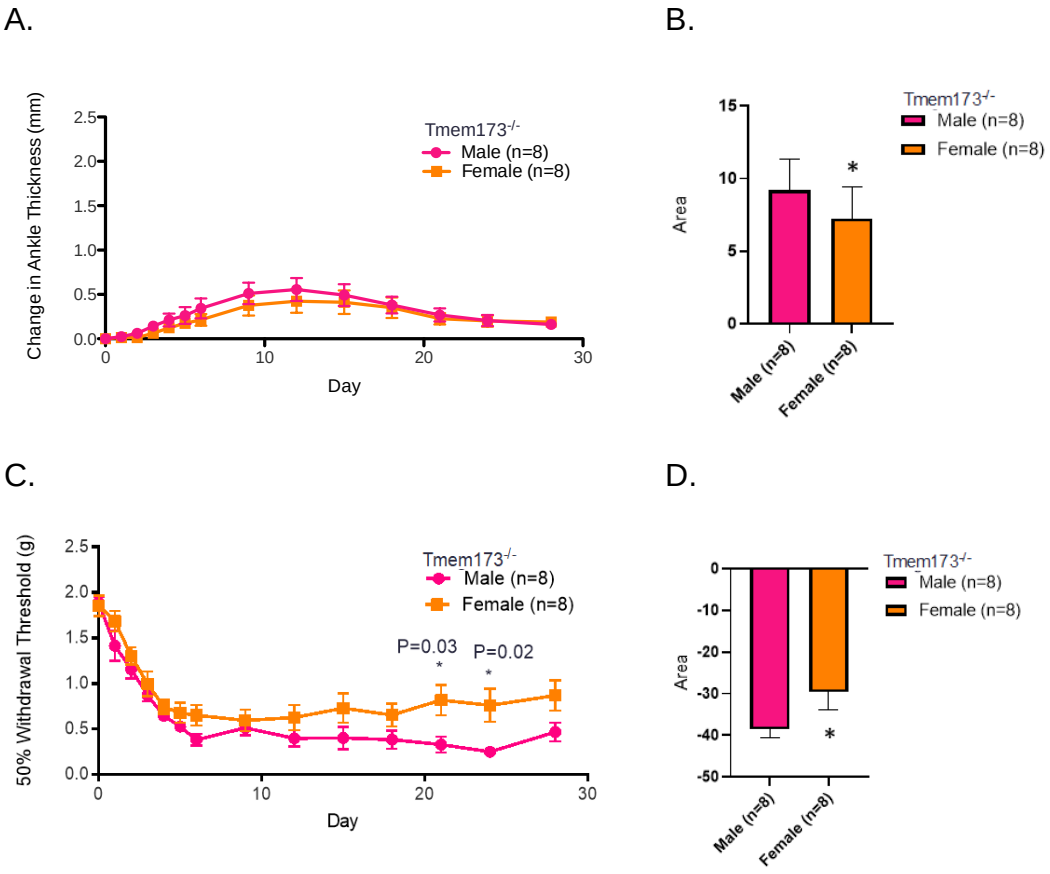


Figure 9: K/BxN serum induced ankle swelling and allodynia in *Tmem173*$^{-/-}$ mice. Ankle swelling (A and B) and mechanical withdrawal thresholds (C and D) were assessed on the indicated days. Means of the area under the curves ± SEM were calculated (B and D). Male *Tmem173*$^{-/-}$ mice had slightly more swelling [Two-way ANOVA; F(1, 196) = 3.981, P=0.0474]. Both sexes developed similar tactile allodynia initially; however, females overall had less [F(1, 196) = 30.60, P<0.0001].

To achieve a visual comparison of each strain a heatmap summary using means of area under the curve for ankle swelling and allodynia was constructed. The darker shades are representative of increased ankle swelling or pain-like behavior and the map is ordered by severity of ankle swelling (greatest to smallest). In general, females appeared to have a higher incidence of swelling compared to their male counterparts, except *Tmem173*$^{-/-}$ mice which the opposite was true. Mice deficient in Ifnar1 universally and in lysozyme expressing cells on average appeared to have more severe swelling than WT mice and other immune deficient strains; however, a higher threshold for mechanical withdrawal. The Tmem173 deficient mice appeared to have the lowest comparable amount of ankle swelling, but their pain-like behavior was similar to the WT mice. Strains deficient in Irf3.Irf7 and Itgax controlled Ifnar1 deletion overall had slightly reduced swelling and allodynia compared to the WT mice (Figure 10).

The visual comparison of these strains suggests that IFNAR1 receptors, specifically in lysozyme expressing cells, direct pain-like behavior in male and female K/BxN arthritic murine mice, but not the occurrence of ankle swelling. The reduction of IRF3 and IRF7 directed production of interferons or the signaling capacity of type I interferons in Itgax expressing cells may have a small inhibitory effect on swelling and allodynia compared to the WT; however, a deletion in a receptor upstream of type I interferons, STING, decreases the swelling while having no impact on allodynia. These observations suggest that STING may have proinflammatory properties that are not directly linked to the occurrence of allodynia.

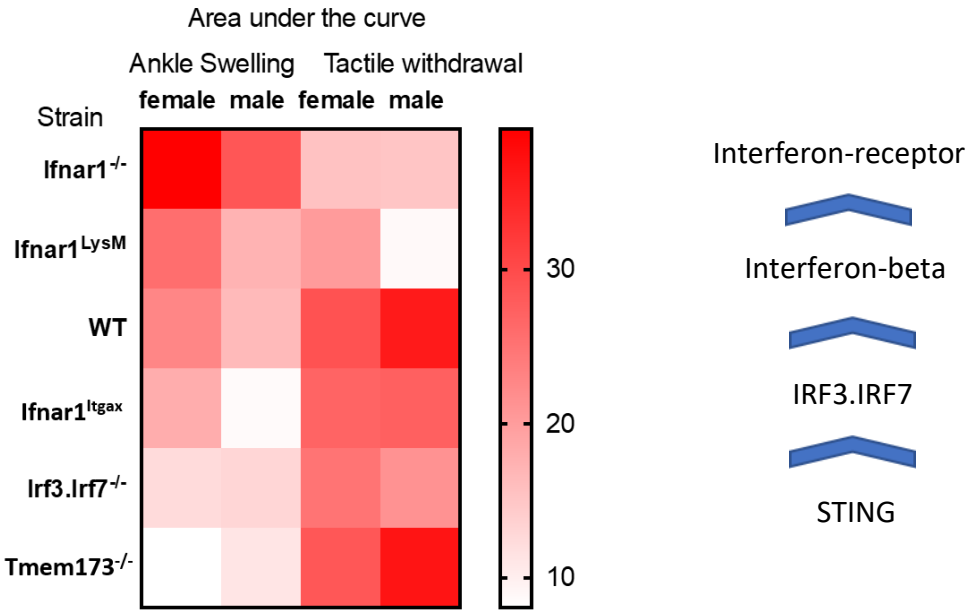


Figure 10: Heatmap summary of ankle swelling and mechanical allodynia for strains with K/BxN arthritis. Groups of male and female mice were given K/BxN serum transferred arthritis. The swelling in the *Ifnar1*$^{-/-}$ mice was the most severe for both males and females; however, there was little allodynia. The mice with the LysM cre driven deletion in Ifnar1 best recapitulated this phenotype. Mice that were STING deficient had reduced swelling but developed allodynia disproportionately to the swelling.

3.2 Specificity of R-INH#4-6 Compound Activity

Compounds previously identified as potential immunomodulators by high throughput screening were repurchased commercially for further investigation. Studies performed by this lab investigated the effectiveness and specificity of these repurchased compounds narrowing it down to 3 from the 2-Nitrofuran Arylamide chemotype (Table 3). The compounds were renamed R-INH#4, R-INH#5 and R-INH#6 (repurchased inhibitor, R-INH) and their inhibitory effects tested further on different innate pathways by quantifying specific cytokines with and without compound treatment on WT murine BMDCs (bone marrow-derived dendritic cells). The studies found, among other things, that compounds R-INH#4-6 suppressed the expression of mTNF-α, mIFN-β and mIP-10 from moderate to significant levels when co-treated with a STING agonist (Fujita, 2019).

To further investigate the specificity of compounds R-INH#4-6, BMDC's were harvested from WT murine males and stimulated with agonists for TLR4 (LPS), TLR7 (1V270) or STING (DMXAA). A subset of cells was incubated with only media as a control. The cells were then treated with DMSO (control), 5μM of R-INH#4, R-INH#5 or R-INH#6 and incubated overnight. After an overnight incubation, the supernatant from these cells was isolated and analyzed through an ELISA for the relative concentrations of mIP-10, mIFN-β and mTNF-α in DMSO or compound treated cells.

Cells stimulated with only media returned little to no expression of cytokines mIP-10, mIFN-β and mTNF-α when co-treated with DMSO or the 3 compounds (Figure 11-13). Production of mIP-10 was mainly observed in cells

simulated with LPS and DMXAA, agonists for TLR4 and STING, respectively. IP-10 is induced by IFN-gamma (Gotsch et al., 2007), which is a mediator of LPS stimulation (Varma, 2002), and is present at high levels in the synovial fluid of RA (Hanaoka et al., 2003). 1V270, a TLR7 ligand (Sato-Kaneko et al., 2017), stimulated cells returned minimal levels of mIP-10 in DMSO and compound treated cells that were comparable to the media control (Figure 11). Treatment with compounds R-INH#4-6 suppressed mIP-10 production in both LPS and DMXAA stimulated cells when compared to cells treated with DMSO only (Figure 11). For LPS stimulated cells, all 3 compounds returned similar levels of suppression, but in cells stimulated with DMXAA, the compound R-INH#5 had the highest suppression of mIP-10 (Figure 11).

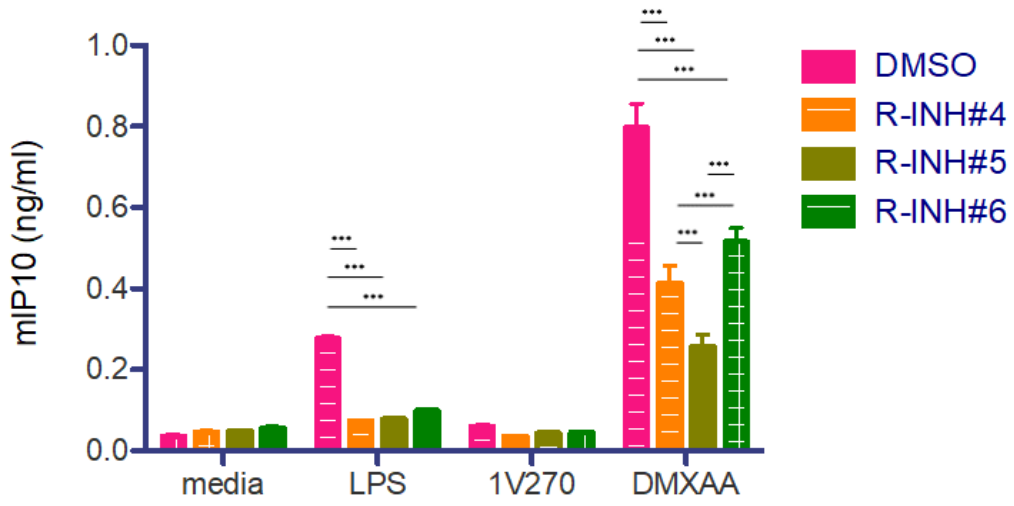


Figure 11: Production of mIP-10 in LPS and DMXAA stimulated WT BMDC inhibited after R-INH#4-6 compound treatment. Media stimulated cells had no mIP-10 production. LPS and DMXAA stimulated cells returned mIP-10 inhibition after treatement of R-INH#4-6 when compared to cells treated with DMSO only. R-INH#5 had highest supression in DMXAA stimulated cells. Low detection of mIP-10 from the 1V270 stimulated cells [Two-way ANOVA calculated; *p < 0.05, **p < 0.01, ***p < 0.001, n=3].

The production of mIFN-β was only observed in cells stimulated by the STING agonist, DMXAA. Treatment with compounds R-INH#4-6 significantly suppressed mIFN-β production in DMXAA stimulated cells with the greatest reduction observed in the R-INH#5 treated cells (Figure 12). These observations were in concordance with the production of mIP-10 in DMXAA stimulated cells treated with each compound (Figure 11).

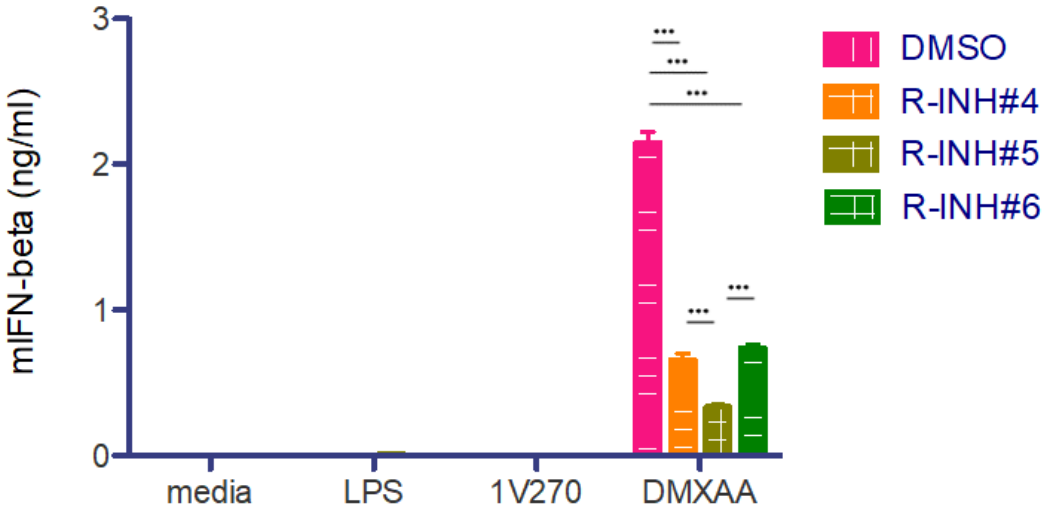


Figure 12: Production of mIFN-beta by DMXAA stimulated WT BMDC inhibited after R-INH#4-6 compound treatment. Media stimulated cells had no mIFN-β production. DMXAA stimulated cells returned mIFN-β inhibition after treatement of R-INH#4-6 when compared to cells treated with DMSO only. R-INH#5 had the most significant supression of mIFN-β. mIFN-β was undetectable in the LPS or 1V270 stimulated cells [Two-way ANOVA calculated; *p < 0.05, **p < 0.01, ***p < 0.001, n=3].

The presence of mTNF-α was observed in all DMSO treated cells stimulated with LPS, 1V270 and DMXAA. mTNF-α was strongly produced in DMSO treated cells stimulated by 1V270 and LPS with lower relative levels in cells stimulated by DMXAA (Figure 13). Treatment with compounds R-INH#4-6 reduced the production of mTNF-α in all experimental cells with the greatest significance in LPS and 1V270 stimulated cells. In 1V270 stimulated cells, R-INH#4 had the greatest inhibition of mTNF-α production compared to R-INH#5 and #6 (Figure 13).

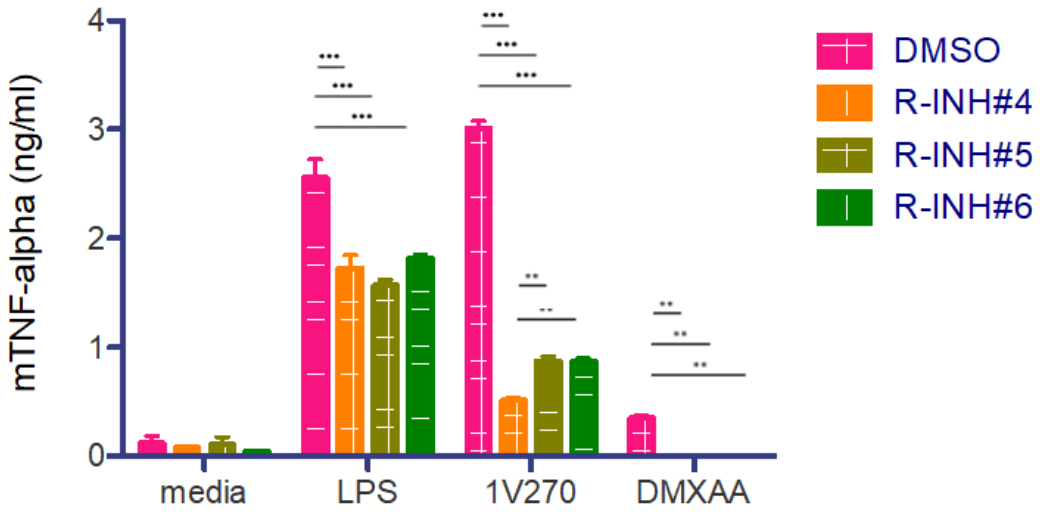


Figure 13: Production of mTNF-alpha in LPS, 1V270 and DMXAA stimulated WT BMDC inhibited after R-INH#4-6 compound treatment. Media stimulated cells had minimum mTNF-α production. LPS, 1V270 and DMXAA stimulated cells returned mTNF-α inhibition after treatment of R-INH#4-6 when compared to cells treated with DMSO only. R-INH#4 had the most significant supression of mTNF-α in 1V270 stimulated cells [Two-way ANOVA calculated; *p < 0.05, **p < 0.01, ***p < 0.001, n=3].

3.3 Investigation of 2-Nitrofuran Arylamides as Potential STING Antagonists

Prior studies have indicated that 2-Nitrofuran derivatives may function by inhibiting the STING adaptor protein (Haag et al. 2018). To further investigate R-INH#4, R-INH#5 and R-INH#6 as potential STING antagonists, BMDC's were harvested from *Tmem173*$^{-/-}$ murine males and stimulated with agonists for TLR4 (LPS), TLR7 (1V270) and STING (DMXAA) with a subset incubated with only media as a control. Similar to the WT cells, the *Tmem173*$^{-/-}$ cells were co-treated with DMSO (control) or 5µM of R-INH#4, R-INH#5 or R-INH#6 and incubated overnight. After an overnight incubation, the supernatant from these cells was isolated and analyzed through an ELISA for the relative concentrations of mIP-10, mIFN-β and mTNF-α. The relative cytokine concentrations were compared within the *Tmem173*$^{-/-}$ cells and to the WT cells from the previous mentioned experiment.

The cytokine production for cells stimulated with the media was negligent, except for a minimal level of mIP-10 in cells treated with R-INH#6 (Figure 14). Murine IP-10 production was also observed in 1V270 and DMXAA stimulated cells when treated with R-INH#5-6 compared to the DMSO treated cells (Figure 14). All LPS stimulated cells returned mIP-10 production with a suppression upon treatment with compounds R-INH#4-6. R-INH#4 and #5 inhibition were significantly greater than R-INH#6 (Figure 14). Significant inhibition was also seen in WT cells stimulated with the TLR4 agonist, LPS (Figure 11). DMXAA is a STING agonist so reduced cytokine concentration in DMXAA stimulated cells compared to the WT was expected (Figure 14).

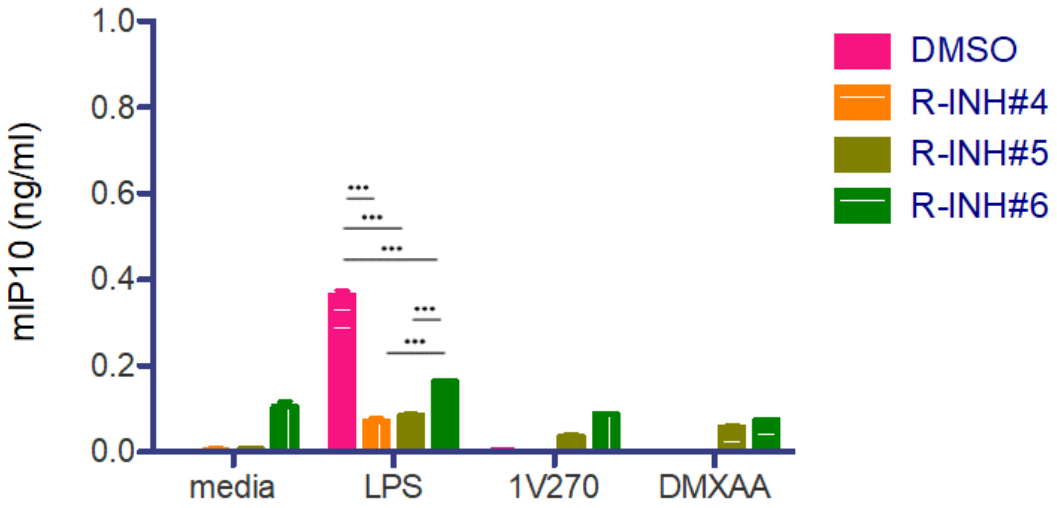


Figure 14: Production of mIP-10 by LPS stimulated *Tmem173*$^{-/-}$ BMDC inhibited after R-INH#4-6 compound treatment. Media stimulated cells had mIP-10 production in R-INH#6 treated cells. 1V270 and DMXAA stimulated cells returned mIP-10 productin with R-INH#5 and #6 treatment compared to DMSO only treatment. LPS stimulated cells returned mIP-10 inhibition after treatment with R-INH#4-6 compared to cells treated with DMSO only [Two-way ANOVA calculated; *p < 0.05, **p < 0.01, ***p < 0.001, n=3].

Bone marrow derived cells acquired from *Tmem173*$^{-/-}$ males returned no observable production of mIFN-β upon media or agonist stimulation (Figure 15). In the WT cells, the production of mIFN-β was only observed in cells stimulated with DMXAA (Figure 12), so the lack of production in the LPS and 1V270 stimulated cells in the *Tmem173*$^{-/-}$ was already expected by comparison. The absence of m-IFN-β in DMXAA stimulated cells is thought to be due to the STING deficiency.

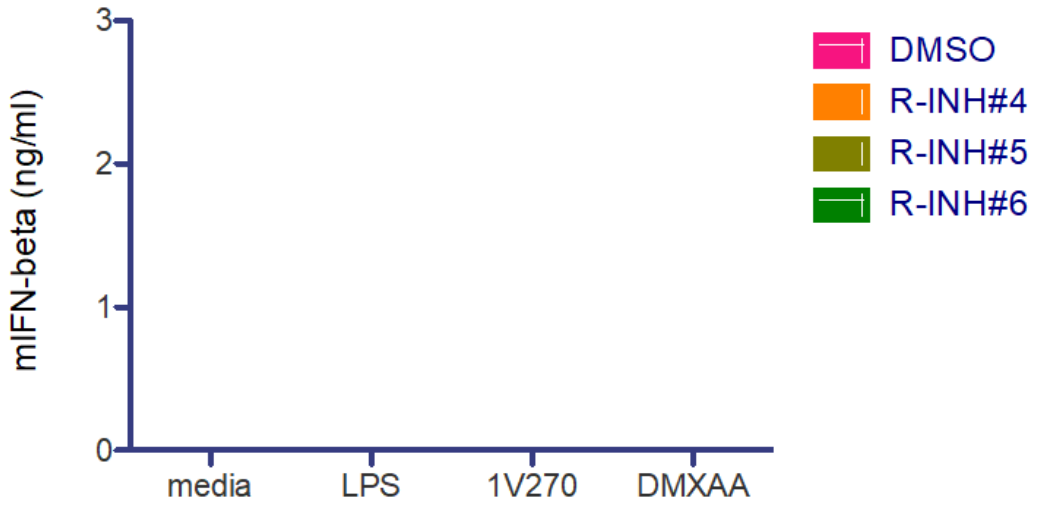


Figure 15: No production of mIFN-beta in LPS, 1V270 or DMXAA stimulated *Tmem173*$^{-/-}$ BMDC. Media, LPS, 1V270 and DMXAA stimulated cells returned no mIFN-β production regardless of treatment.

The inhibition of mTNF-α in 1V270 *Tmem173*$^{-/-}$ stimulated cells was originally observed to be reminiscent of their WT counterpart; however, significant inhibition was only achieved when treated with R-INH#4 (Figure 16). R-INH#4 returning the greatest inhibition was also seen in WT cells stimulated with 1V270 (Figure 13). There was no significant difference between DMSO and compound treated cells stimulated with LPS or DMXAA, which was contrary to what was seen in the WT cells (Figure 13 and 16). Visually, there appeared to be little to no production of mTNF-α in all cells (WT and *Tmem173*$^{-/-}$) stimulated with DMXAA (Figure 13 and 16).

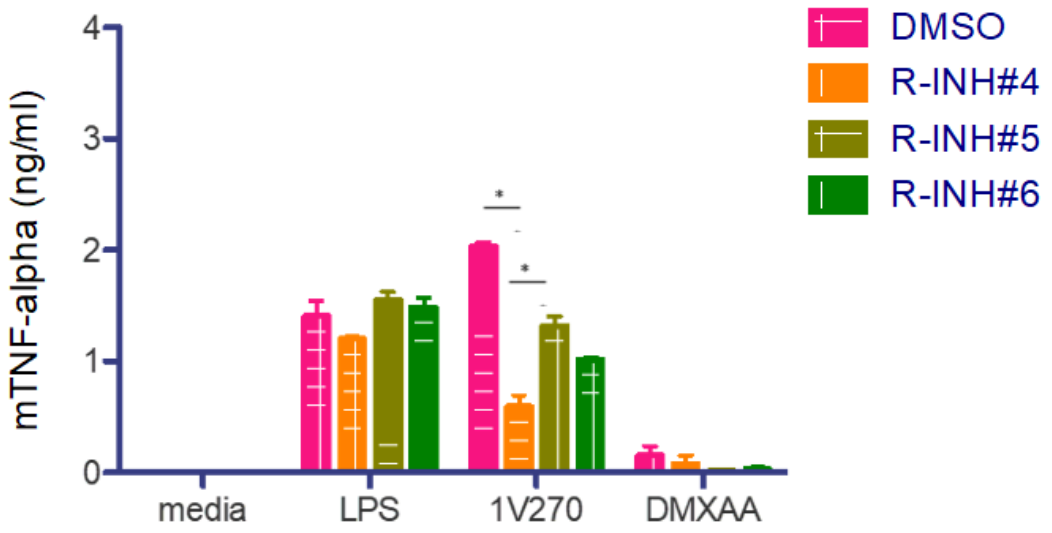


Figure 16: Production of mTNF-alpha in 1V270 stimulated *Tmem173*$^{-/-}$ BMDC inhibited after R-INH#4 compound treatment. Media stimulated cells had no mTNF-α production. 1V270 stimulated cells returned mTNF-α inhibition after treatement of R-INH#4 compared to cells treated with DMSO only. No inhibition of mTNF-α was observed in LPS stimualted cells treated with R-INH#4-6 [Two-way ANOVA calculated; *p < 0.05, **p < 0.01, ***p < 0.001, n=3].

3.4 Treatment of K/BxN Induced Arthritis in C57BL/6 Mice with R-INH#5

The selection of R-INH#5 for live animal testing was reflective of past studies suggesting a decrease in TNF-α and an increase in IFN-β expression leads to the resolution of K/BxN-induced arthritic neuropathic pain (Woller et al., 2019). Past studies testing the specificity of R-INH#5 indicated that it not only significantly reduced the production of TNF-α, but also increased the production of IFN-β in LPS stimulated cells (Fujita, Y. 2019). In the ELISA's recently performed, R-INH#5 comparatively reduced mIP-10, mTNF-α and mIFN-β in all cells and had the highest inhibition of mIFN-β and mIP-10 production via the STING mediated pathways (Figure 11-13). These findings were in slight contradiction to the previous studies but did not disqualify R-INH#5 as a candidate for further study.

Other studies performed out of this lab have also shown a potential reduction in pain-like behavior in WT C57BL/6 male mice when treated with 750 mmol of R-INH#5 twice a day at the onset of K/BxN-induced arthritis; however, late application of the compound was inconclusive. The inconclusiveness of previous experiments may be in part to the small sample size used. To further investigate the impact of R-INH#5 on allodynia and swelling, replications of the previous experiment were performed using WT C57BL/6 male mice. K/BxN serum was administered through IP injections on day 0 and 2 to induce a passive rheumatoid arthritis phenotype in all mice. The experiments each lasted 28 days with pain and swelling measured days 0-6, 9-15, 18, 21, 25 and 28. The mice were placed into 2 groups to test the

effectiveness of the compound at different stages during the progression of K/BxN-induced arthritis.

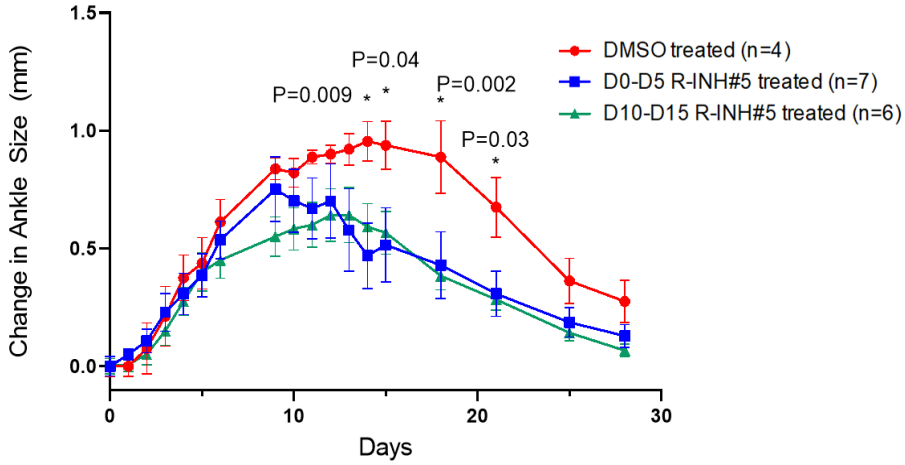
The first treatment group was comprised of 3-4 WT C57BL/6 male mice (7 total). Twice daily IP injections of 750 nmols of R-INH#5 prepared in a DMSO stock were administered from days 0 to 5 (D0-D5). This group represented the effectiveness of the compound if treatment was administered at the onset of arthritis. Von Frey testing indicated that the mice treated with R-INH#5 from D0-D5 had little to no allodynia on days 4-6; however, after day 6, their mechanical withdrawal threshold (level of allodynia) regressed until leveling off around day 14, becoming consistent with the DMSO controls (Figure 18A). Overall ankle swelling, as measured by a caliper, was significantly less in compound treated mice compared to DMSO treated mice (Figure 17B). Compound R-INH#5 appeared to significantly reduce swelling in the D0-D5 treated mice from days 14-21 when compared to the DMSO treated controls (Figure 17A).

The second treatment group was comprised of 3 WT C57BL/6 male mice (6 total). Twice daily IP injections of 750 nmols of R-INH#5 prepared in a DMSO stock were administered from days 10 to 15 (D10-D15). This group represented the effectiveness of the compound if treatment was administered at later stages, or at the peak, of arthritis. From days 0 to 10, prior to compound treatment, there was a steep incline reflecting a rapid increase in allodynia after serum administration. On day 10, when treatment was initiated, there was no significant attenuation of allodynia compared to the DMSO treated mice, which lasted throughout the course of treatment (Figure 18A). As with the D0-D5

group, there was a significant decrease in ankle swelling in the D10-D15 treated mice from days 14-21 when compared to the DMSO mice (Figure 17A). On average, both D0-D5 and D10-D15 treated mice had lower overall ankle swelling (Figure 17B).

The results returned suggest that compound R-INH#5 mediates swelling in early and late phase treatment of K/BxN-induced arthritic C57BL/6 males; however, early treatment is necessary to alleviate the associated allodynia.

A.



B.

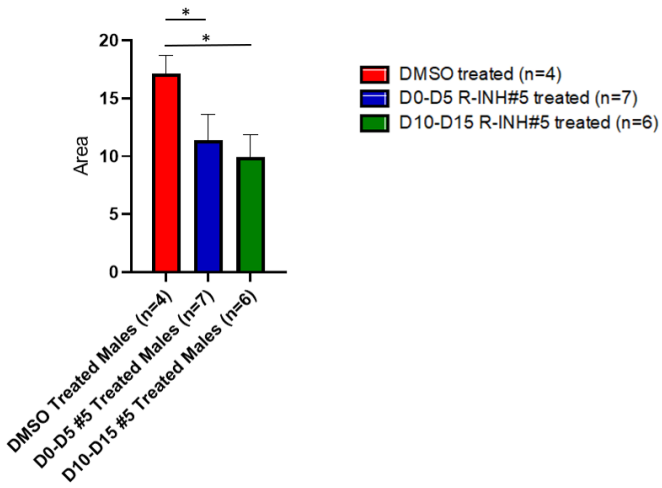
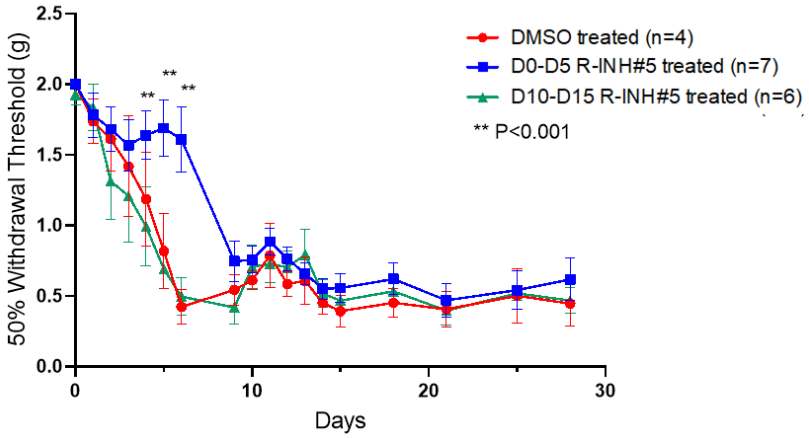


Figure 17: R-INH#5 treatment reduces development of swelling in K/BxN induced arthritis. WT males were injected on days 0 and 2 with K/BxN sera and were treated twice daily from D0-D5 or D10-D15 with R-INH#5. Two experiments performed and the data pooled. Individual measurements (A) and averaged change over the 28 day period (B) depicted. Ankle swelling was significantly reduced in both the D0-D5 and D10-D15 treated mice compared to the DMSO treated WT mice from days 14 - 21. Data are represented as mean ± SEM [Two-way ANOVA; $F(2, 244) = 18.42$, $P<0.0001$].

A.



B.

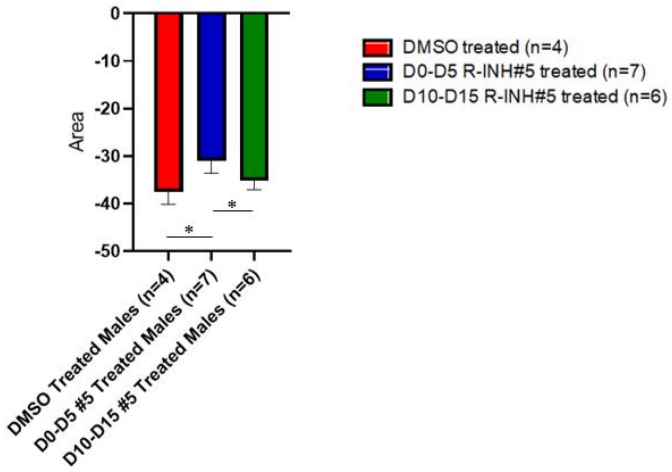


Figure 18: Early treatment of R-INH#5 reduces development of allodynia in K/BxN induced arthritis. WT males were injected on days 0 and 2 with K/BxN sera and were treated twice daily from D0-D5 or D10-D15 with R-INH#5. Two experiments performed and the data pooled. Individual measurements (A) and averaged change over the 28 day period (B) depicted Tactile allodynia in mice treated with R-INH#5 on D0-D5 was less severe than the controls treated with DMSO. Data are represented as mean ± SEM [Two-way ANOVA; $F(2, 244) = 14.52$, $P<0.001$].

3.5 Treatment of K/BxN Induced Arthritis in *Tmem173*$^{-/-}$ Mice with R-INH#5

Cytokine evaluation in *Tmem173*$^{-/-}$ murine cells suggested that compounds R-INH#4-6 may have some inhibitory effects in STING deficient cells, despite claims they are STING antagonists (Haag et al. 2018). In WT male mice, compound R-INH#5 did alleviate allodynia when treated early, but those experiments did not specify the compound's mechanism of action. To further investigate R-INH#5 as a STNG antagonist *in vivo* experiments were performed using *Tmem173*$^{-/-}$ male mice to determine if the compound still maintained it's clinical effects. As with the WT C57BL/6 male mice, K/BxN serum was administered through IP injections on days 0 and 2 to induce a passive rheumatoid arthritis phenotype in all mice. The experiment lasted 28 days with allodynia and swelling measured days 0-6, 9, 12, 15 18, 21, 25 and 28. As compound treatment in WT C57BL/6 male mice was mainly successful if given at the onset of arthritis, male mice deficient in STING only underwent compound treatment on days 0-5. The mice were placed into 4 groups to determine the effectiveness of the compound in *Tmem173*$^{-/-}$ males.

This experiment had 3 separate controls. The first control was comprised of 1 WT C57BL/6 male mouse. Twice daily IP injections of DMSO stock were administered from days 0-5. The DMSO treated WT mouse returned ankle swelling and allodynia consistent with what has been previously observed; maximum ankle swelling between 1 and 1.5 mm and allodynia that persisted despite the resolution of swelling (Figure 4A, 4C, 17A, 18A, 19A and 20A). The second control, also comprised of 1 WT C57BL/6 male mouse, received twice daily IP injections of 750 nmols of R-INH#5 prepared in a DMSO

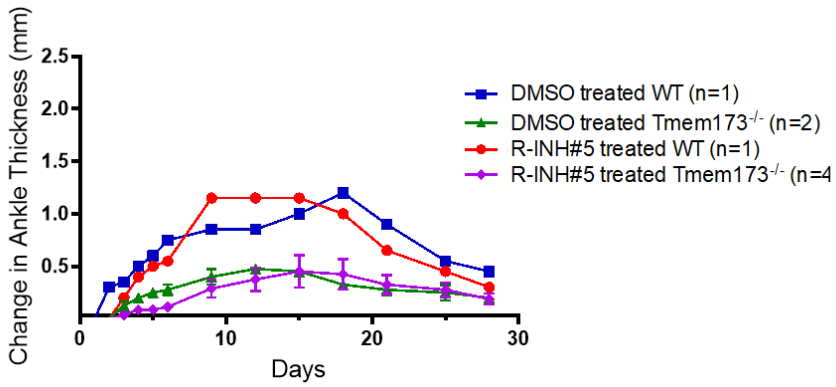
stock from D0-D5. Surprisingly, the R-INH#5 treated WT had swelling and pain-like behavior inconsistent to what was previously observed. Not only was the ankle swelling similar to the DMSO treated WT male(s) (Figure 17A and 19A), but there was also no abatement in allodynia during treatment (Figure 20A). Treatment with R-INH#5 in the WT mouse had no observable impact on allodynia and the pain-like behavior was similar to the DMSO treated WT mice seen in the current experiment and prior experiments (Figure 18A and 20A).

The final control was comprised of 2 *Tmem173*$^{-/-}$ male mice that received twice daily IP injections of DMSO from days 0-5. Ankle swelling in the DMSO treated *Tmem173*$^{-/-}$ was observed to be lower than both WT controls (Figure 19A and 19B); however, there was no significant difference in allodynia (Figure 20A and 20B). The level of allodynia and ankle swelling was in concordance with previous studies on non-DMSO treated arthritic *Tmem173*$^{-/-}$ mice (Figure 9A and 9C).

The treatment group consisted of 4 male *Tmem173*$^{-/-}$ mice who received twice daily IP injections of 750 nmols of R-INH# 5 prepared in a DMSO stock. If the inhibition of STING was involved in R-INH#5 mechanism of action, it was expected that the allodynia and swelling of the compound treated *Tmem173*$^{-/-}$ mice would be consistent with the DMSO treated *Tmem173*$^{-/-}$ mice. In fact, this was what was seen. The compound treated *Tmem173*$^{-/-}$ mice mimicked the DMSO treated *Tmem173*$^{-/-}$ in ankle swelling and allodynia (Figure 19A and 20A). These results were inconclusive however, as there was no appreciable difference between the DMSO or compound treated WT control mice in allodynia or swelling (Figure 19A and 20A). The similarities in allodynia seen in

all groups indicate that the compound was ineffective as a whole in this experiment.

A.



B.

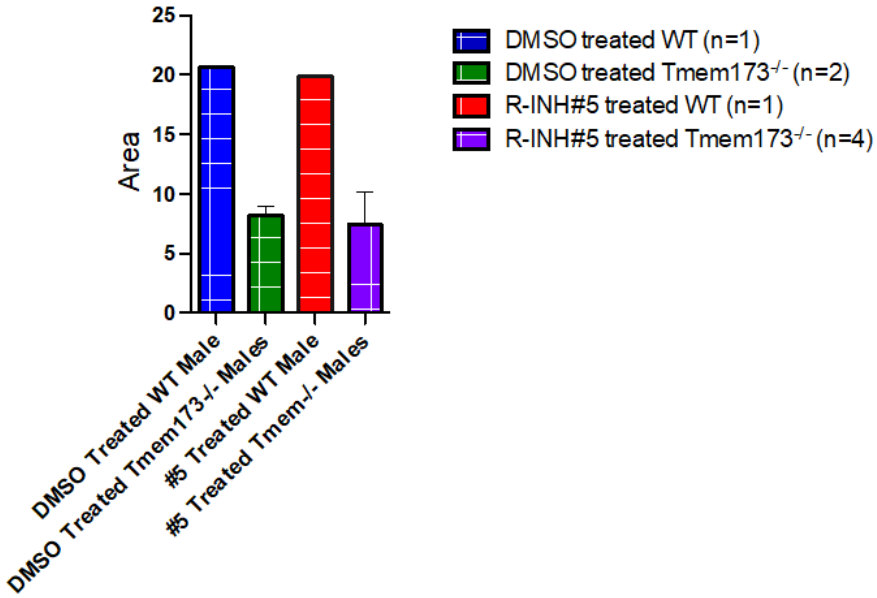
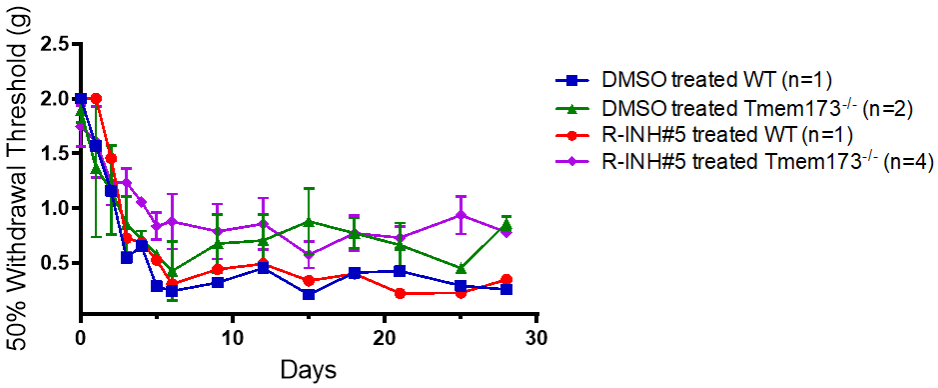


Figure 19: R-INH#5 treatment in *Tmem173*$^{-/-}$ male mice did not impact development of swelling in K/BxN induced arthritis. *Tmem173*$^{-/-}$ males were injected on days 0 and 2 with K/BxN sera and were treated twice daily from D0-D5 with R-INH#5. Individual measurements (A) and averaged change over the 28 day period (B) depicted. No significant reduction in ankle swelling was observed in the *Tmem173*$^{-/-}$ or WT males compared to the DMSO treated controls. Data are represented as mean ± SEM [Two-way ANOVA; F(3,4)= 9.952, P=0.0251].

A.



B.

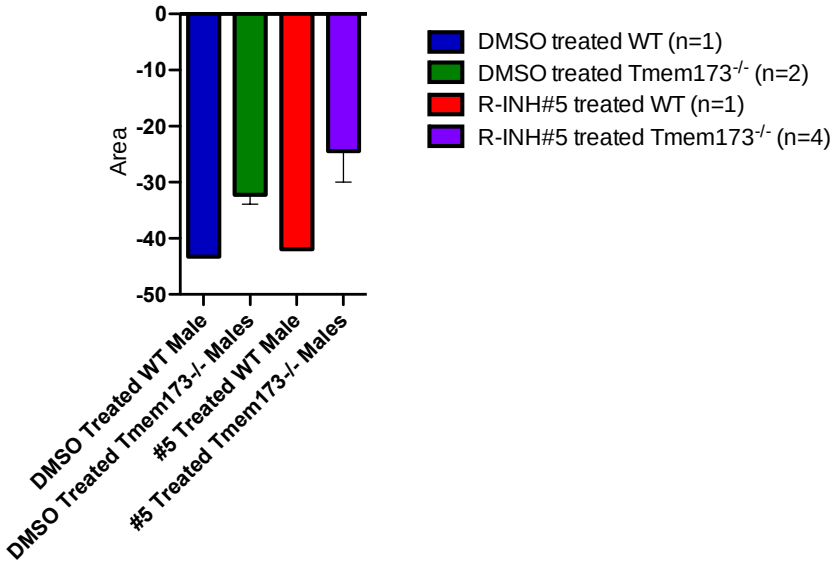


Figure 20: R-INH#5 treatment of *Tmem173*$^{-/-}$ male mice had no significant impact on allodynia in K/BxN induced arthritis. *Tmem173*$^{-/-}$ males were injected on days 0 and 2 with K/BxN sera and were treated twice daily from D0-D5 with R-INH#5. Individual measurements (A) and averaged change over the 28 day period (B) depicted. Tactile allodynia in *Tmem173*$^{-/-}$ mice treated with R-INH#5 was similar to the *Tmem173*$^{-/-}$ DMSO control and WT controls. Data are represented as mean ± SEM [Two-way ANOVA; F(3,4) = 1.025, P=0.4704].

4. Discussion

In these experiments the role of the innate immune system in RA was investigated by utilizing a K/BxN arthritis mouse model. The main area of interest centered on type I interferons' role in inflammation and the initiation and transition from acute to chronic pain. The K/BxN-induced arthritis model used best exemplified these characteristics allowing inferences on arthritis induced inflammation and pain in multiple immune deficient environments. Signaling pathways are complex and complete with redundancies and regulations and there can also be spatial and temporal specificity (Kholodenko, 2006), making the identification of particular mechanisms extremely difficult. The initial experiments indicated that knocking out a subunit component of an interferon receptor (IFNAR1) had the ability to reduce allodynia in males and females. In this model, interferons are still being produced, but their effects were modulated by removing a primary receptor. Interferon stimulated genes are numerous and have multiple functions, which might be why attenuating their expression lessened allodynia, but also increased swelling. Cell types potentially associated with the *Ifnar1*$^{-/-}$ phenotype were macrophages and microglia. In regard to immune cells, LysM directed lysozyme M is mainly expressed in macrophages; however, some expression is seen in a small percentage of microglia after injury (Greenhalgh et al., 2018). Microglia has been implicated in acute and chronic pain associated with arthritic disease. The persistence of pain despite the resolution of synovial swelling seen in RA patients points to sensitizing central pain pathways (Nieto et al., 2016). After nerve injury, the activation of microglia in the dorsal horn of the spinal cord has

been linked to synaptic alterations and potentially hypersensitivity (Inoue and Tsuda, 2018). Peripheral macrophages are also involved in pain responses through secretion of pro-inflammatory cytokines and nociceptor interactions (Barry and Haberberger, 2019); however, when dendritic cells and macrophages were targeted through an Itgax directed Ifnar1 deletion, the level of attenuation was not as significant as with the LysM directed deletion. This suggests inhibiting an IFNAR1 directed response in dendritic cells and macrophages may impact pain by attenuating a generalized immune response, but microglia have a greater involvement in centralized pain through the action of IFNAR1, which was also magnified by the reduced pain-like response despite the increased severity of swelling.

Investigating the impact of type I interferons further upstream by knocking out key transcription factors, IRF3 and IRF7, resulted in reduced inflammation, but initial pain reminiscent of the WT mice. Interferon regulatory factor 3 is constitutively expressed in RA patients and the high levels of phosphorylated IRF3 in their synovial tissue has been associated with high levels of ISG expression (Jefferies, 2019). In chronic constriction injury, studies have shown IRF3 mediates the transition to neuropathic pain in rats. High IRF3 expression in DRG's of rats led to high levels of NF-κB and their resultant pro-inflammatory cytokines (i.e. TNF-α and IL-1β). Silencing IRF3 attenuated allodynia in these rats possibly through inhibiting NF-κB expression. High levels of NF-κB in DRG's has also been seen in inflammatory pain models (Li et al., 2017). Interferon regulatory factor 7 has also been implicated in RA; however, it may have an inhibitory role. K/BxN-induced arthritic mice deficient in IRF7

had increased severity of arthritis compared to the WT, potentially due to macrophage directed IFN-β production and other anti-inflammatory effects on fibroblast-like synoviocytes (Sweeney et al., 2012). Knocking out IRF3 potentially explains the resolution of allodynia towards the end of the experiment, while knocking out IRF7 may explain why the mice still had initial pain reminiscent of the WT.

The investigation of 2-Nitrofuran Arylamide's as potential immunosuppressants in K/BxN-induced arthritis through the antagonization of STING was an extension of prior studies performed out of the Corr lab. High throughput screening and *in vivo* testing in murine models highlighted 3 compounds, renamed R-INH#4-6, as a potential immunosuppressants in an arthritic model (Fujita, 2019). Cytokine expression is an indicator of an immune response and its inhibition provides insight to the immunosuppressant abilities of different treatments. To confirm the immunosuppressant abilities of compounds R-INH#4-5, their inhibitory effects were tested on the production of mIP-10, m-IFN-β and mTNF-α in WT bone marrow-derived dendritic cells using an ELISA. These cytokines were chosen due to past success (Fujita, 2019) and because they are all implicated in RA. The dendritic cells were stimulated for agonists against TLR4, TLR7 and STING and it was found that R-INH#4-6 significantly reduced cytokine production in all stimulated cells whenever DMSO treated cell cytokine production was observed (compared to the media control). These results confirmed that compounds R-INH#4-6 can act as immunosuppressants in dendritic cells.

Differences between the inhibitory abilities of each compound were significant in 3 cases. Compound R-INH#5 had significantly greater inhibitory abilities in STING induced production of mIP-10 and mIFN-β. Compound R-INH#4 inhibited TLR7 directed production of mTNF-α to a greater extent than R-INH#5-6, which was unexpected, but also seen in prior experiments (Fujita, 2019). The ability of these compounds to inhibit these molecular pathways could be essential in treatment of arthritis, especially pathways induced by TLR4 and STING. Toll-like receptor 4 has been implicated in the transition from acute to chronic mechanical hypersensitivity in a K/BxN mouse model (Christianson et al., 2011) possibly though it's induction of both proinflammatory cytokines and type I interferons (Huang and Pope, 2009). The STING pathway is also an important pathway for the production of type I interferons and has been implicated in autoimmune diseases through leaked mitochondrial or nuclear DNA self-reactivity (Barber, 2015). Dysregulation of type I interferon production and signaling, a term coined interferonpathologies, is linked to several diseases including rheumatic diseases (Volpi et al., 2016).

Testing the efficacy of these compounds on Tmem173 deficient bone marrow-derived dendritic cells indicated that these compounds may work by antagonizing the STING pathway. Unlike the WT dendritic cells, significant inhibition was only achieved by all compounds in LPS directed mIP-10 production. Compound R-INH#4 had a significant impact on mTNF-α, but to a lesser extent. Based on these results we can speculate that these compounds primarily function by inhibiting the STING pathway; however, there may be secondary modes of action.

Compound R-INH#5's ability to significantly inhibit STING associated cytokine production made it a reasonable candidate for *in vivo* studies. Treatment of WT C57BL/6 K/BxN arthritic males with this compound attenuated allodynia close to baseline levels if given at the onset of arthritis; however, attenuation was transient and not achieved if treatment was administered after the arthritis had progressed. Due to this pattern it can be speculated that the basis of this attenuation may partially rely on TLR4. Blocking TLR4 in K/BxN serum transfer arthritic mice attenuates allodynia if administered at the onset, but it cannot reverse it once the arthritis has progressed (Christianson et al., 2011). This pattern mimics what was seen in the R-INH#5 treated mice. This speculation, however, is in contradiction with prior experiments indicating R-INH#5 as a STING adaptor protein antagonist (Haag et al. 2018), and it is possible other mechanisms are at play.

Compound treated mice overall had reduced ankle swelling regardless of when it was administered. The reduction in swelling in the late-phase treated mice without attenuation of allodynia, indicates the reduced swelling was not causative of the reduced allodynia. Dissociation between allodynia and swelling was already observed in a subset of the Ifnar1 deficient mice tested. Antagonizing STING could potentially reduce inflammation in the arthritic mice. Studies have shown that the cGAS-STING signaling mediates systemic and localized inflammation. Macrophage induced post-MI cardiac inflammation in ischemic myocardial infarction can effectively be reversed if this pathway is ablated (Wan et al., 2020). Toll-like receptor 4 also has inflammatory effects,

however, and inhibiting its mode of action could also lead to reduced inflammation.

The hypothesized significance of STING in attenuating K/BxN-induced allodynia was undermined in the *Tmem173*$^{-/-}$ experiments. Male and female mice deficient in the gene that encodes for STING returned allodynia that was consistent with their WT counterparts. If blocking STING reduces allodynia, which the compound was hypothesized to do, it was expected that knocking it out completely would have the same effect. The ankle swelling, however, in the *Tmem173*$^{-/-}$ mice was visibly reduced like the R-INH#5 treated WT C57BL/6 male mice. In humans, gain-of-function mutations in STING have been linked to diseases such as SAVI and FLS and even potentially to systemic lupus erythematosus (SLE) (Wan et al., 2020). However, STING deficient mice have been linked to hyperresponsiveness to endosomal TLR ligands and an increase in proinflammatory cytokines (Barber, 2015). Perhaps, momentarily inhibiting STING can alleviate allodynia by avoiding over-reactively, but ablating STING completely results in a pre-established over-reactively through hyperresponsiveness and, if treated long term, the compound may return a similar phenotype. Studies in long-term treatment may shed light on this assumption.

Treatment of *Tmem173*$^{-/-}$ with compound R-INH#5 unfortunately was inconclusive. The DMSO and R-INH#5 treated *Tmem173*$^{-/-}$ mice did return similar allodynia and ankle swelling; however, in this experiment R-INH#5 also did not attenuate allodynia in the WT control. As R-INH#5 was previously tested in 6 separate WT C57BL/6 males, all which returned attenuated allodynia if

treated D0-D5, only 1 WT C57BL/6 R-INH#5 treated mouse was used in this experiment as a comparison. Both the DMSO and R-INH#5 treated WT mice had similar allodynia and ankle swelling, contrary to what was seen previously. It is possible that the efficacy of the compound was compromised or diluted through accumulated condensation as a consequence of multiple rounds of thawing and opening. At this point repeating the experiment with a fresh stock of compound is warranted.

Dysregulation of type I interferons and their associated pathways may play a role in many autoinflammatory and autoimmune diseases; however, the exact role can be controversial. Experiments performed highlighted the complexities of these pathways and how attenuation at certain stages and in certain cells can have a range of effects. Further exploration of the temporal and spatial role of type I interferons in the progression of RA may result in more efficient treatments by increasing the specificity of the therapeutics used.

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