

Investigating galectin-3 in vasculopathy associated with
inflammatory arthritis



By

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This thesis is submitted to Cardiff University in fulfilment of the
requirement for the Degree of Doctor of Philosophy in the
School of Medicine.

November 2021

“Strength does not come from what you can do.

It comes from overcoming the things you once thought you couldn’t.”

- Rikki Rogers

Acknowledgments

The past three and a half years have been a rollercoaster of intense but rewarding experiences and I am so appreciative to many people who have helped me along this journey. I am extremely grateful to Professor Anwen Williams and Dr. Gareth Jones for guiding me through this process, for pushing me when I needed to be pushed and for letting me take control and fly on my own when I was ready. Your blend of expertise as scientists and as supervisors are second-to-none and your patience and understanding did not go unnoticed. Thank you.

Thank you to Galecto Biotech for funding this project, for being incredibly understanding when things didn't quite go to plan and for allowing us to determine the direction of the research. A special thank you to Dr. Alison MacKinnon at Galecto who was a pleasure to both work with and get to know personally, and for making me feel so welcome during my stay in Edinburgh in February 2020. Thank you also to Dr. Ross Mills for his never-ending patience during my time in Edinburgh and afterwards, sending numerous reagents and samples to Cardiff, much appreciated.

Thank you to Professor Simon Jones for welcoming me into your lab and lab meetings and making me feel like I was one of your crew even though I still don't really understand what a circos plot is and every heatmap still looks the same to me. To the Simon Jones lab group - there have been many faces come and go over the past 3.5 years, but I have thoroughly enjoyed getting to know all of you and I am honoured to call many of you my friends. To Dr. Anna Cardus - possibly the most patient woman I've ever met - thank you for your constant support, both professionally and personally and always offering and willing to help with anything and everything, even when you really didn't have the time. To Dr. Rob Jenkins, my fellow Celtic coffee pal, thank you for being so grounded and the voice of reason of the office, and for all the help with my i.a. injections! To Dr. Alicia Derrac Soria - who would've thought that performing countless oral gavage on hectic little DBA mice would make us such close friends! You were there through all the ups and downs of this thesis. I am so grateful for your support, listening to my rants, drying my tears and words of encouragement and the odd glass of wine in between!! To Dr. Dave Hill, thank you for all of the help in the early stages of my PhD, for always having sound advice at hand and bringing the laughs to the office. To Dr. Javier Uceda, thank you for being a ray of sunshine in the early months of my PhD and for being my first friend in Cardiff. I miss you everyday and still find it so hard to believe you are gone. RIP. To Dr James Burston, thank you for always taking time to discuss data with me and Dr. Ben Cossins, thank you for always being up for a cheeky little gossip session! To the wider Simon Jones lab group past and present, thank you for your company and friendship. To the staff dotted around the Tenovus building - Magda, Liz, Duncan and John - most days you were the only faces I saw during the isolating writing up period, thanks for the laughs and keeping me smiling!

This PhD has been a journey of 2 halves - pre and post Covid19 pandemic. Navigating a PhD through a pandemic has come with its own unique set of challenges that I couldn't have overcome if it wasn't for the immense support that I received from friends and family.

When I moved to Cardiff 3.5 years ago, little did I realise how lucky I was about to become with the people I was going to meet. The friends I have made in Wales truly are my family abroad and I am so grateful for the support they have given me, particularly in the last 18 months when going to Ireland has not been an option. I have so many fond memories of nights out, coffee dates, mountain hikes, beach days, concerts and everything in between. To my fellow PhD friends, thank you for understanding, and listening to the rants; to my Richards Street (& wider circle) group of girls, thank you for the many laughs; to Dr. Katie Smith, thank you for seeing me at lunch one day and offering to be my mentor – I'm sure you regretted that soon after! What started as a mentor/mentee relationship ended as being close friends. Thank you for always keeping things in perspective for me and reminding me that everything will work out. To the street pub crew – Mike, Rosie, Will, Elena, Mark, Farook, Adam and Dips thank you for being the most random but fabulous bunch of people and the best support network. Muireann – thank you for being my home away from home, for always lending an ear and never making me feel like a burden. To Harry - you deserve a PhD in patience. Living with you has been a huge part of my PhD and thank you for letting me adopt Shadow as my therapy dog some days and as my stress ball on other days! Thank you for listening and pretending to be interested in whatever random stories I had to tell from my day. I couldn't imagine living through a global pandemic and lockdowns with anyone else. It's been one hell of a time and I am truly going to miss not having you as a housemate.

Finally, to my army of support in Kildare. Thank you to my friends and family for your constant and unwavering support. I always felt a renewed source of strength and faith every time I left Richardstown that fuelled me to keep going. To Lydia – thank you for the endless voice notes, they have got me through many tough days. To my extended family - aunts, uncles and cousins - your faith and encouragement went above and beyond, and I am so grateful. Thank you. Stephen and Fiona – thank you for the care packages and for always looking out for me. To Michelle – I am indebted to you for all of your support, for all the random gifts I got to my front door, for you organising things for me that I didn't even know needed to be organised, to just being ready to answer a text at the drop of a hat at any time of the day. You've allowed me to completely focus on my PhD so for that, thank you. Leighton – thank you for being a light at the end of the tunnel! And finally, to Mam and Dad. Thank you for your unconditional love and support, for being my on-call counsellors, car mechanics, career advisors and most of all, my friends who listened to my rambles every Sunday night. I simply could not have done this without you both.

While there were some days where I felt isolated in this process, these acknowledgements just shows that I was never really alone and that I am extremely fortunate to be surrounded by such fantastic people. I promise I won't put ye through another PhD – I'm done now!!

Aisling Morrin

November 2021

Summary

Cardiovascular comorbidities are the primary cause of death in patients with rheumatoid arthritis. Galectin-3, a pro-inflammatory binding protein, regulates a broad range of biological processes such as cell adhesion, chemoattraction and cell activation and is involved in pathological processes, including inflammation, organ fibrosis, cancer and autoimmunity. Galectin-3 is expressed in immune cells, including T cells, yet the mechanisms by which galectin-3 links systemic T cell dysregulation in arthritis associated vasculopathy are largely unknown.

The studies presented in this Thesis test the ability of a small-molecule inhibitor of galectin-3, GB1107, to prevent vascular pathology in inflammatory arthritis. Initial studies used the collagen induced arthritis (CIA) mouse model to investigate galectin-3 inhibition on vascular inflammation. Immunohistochemistry was used to track leukocyte accumulation in the thoracic perivascular adipose tissue (PVAT) and myography to measure constriction responses in the thoracic aorta as a marker of vascular dysfunction. Consistent with cardiovascular associations in rheumatoid arthritis, immunohistochemistry of thoracic aortas showed a significant increase in galectin-3-positive cells and CD3 T cell staining in the vascular tissue of mice with CIA. Treatment with GB1107 caused a reduction in T cells within thoracic PVAT. However, treatment with GB1107 did not improve vascular constriction defects at this chronic stage of CIA. To determine if galectin-3 may be mediating its effects via immune cells, galectin-3 expression was characterised in CD4 T cell differentiation assays. A unique and significant increase in galectin-3 expression and secretion was observed in Th17-polarising conditions. Notably, Th17 cell differentiation was inhibited by GB1107 in a concentration dependent manner. Additionally, the use of GB1107 and galectin-3^{-/-} cells (*Lgals3*^{-/-}) identified a role of galectin-3 in Th17 proliferation, establishing galectin-3 as a potential therapeutic target in diseases where Th17 cells are involved and are pathogenic. Later studies used the antigen induced arthritis (AIA) model of inflammatory arthritis which features a Th17 driven synovitis. Galectin-3 expression was upregulated in CD4 T cells during AIA and treatment with GB1107 led to a reduction in CD4 cells in the inflamed synovium. Furthermore, treatment with GB1107 led to the full restoration of vascular constriction responses, suggesting that inhibition of galectin-3 could be beneficial in the application of arthritis associated vasculopathy.

Galectin-3 promotes Th17 cell differentiation and proliferation and regulates vascular T cell accumulation in CIA. Inhibiting galectin-3 using GB1107 fully restores vascular constriction responses in AIA and has potential to limit Th17-mediated pathology in chronic inflammatory diseases.

Presentations

Oral presentations

- “Investigating the role of galectin-3 in T cells associated with vascular inflammation”. **December 2020**. *Infection and Immunity Annual Meeting, Cardiff*.
- “Investigating the role of galectin-3 in vascular inflammation associated with rheumatoid arthritis”. **November 2020**. *PhD Student Seminars, Division of Infection and Immunity, School of Medicine, Cardiff University*.
- “Role of galectin-3 in rheumatoid arthritis and its associated co-morbidity, cardiovascular disease”. **November 2019**. *PhD Student Seminars, Division of Infection and Immunity, School of Medicine, Cardiff University*.
- “Role of galectin-3 in rheumatoid arthritis and its associated co-morbidity, cardiovascular disease”. **February 2019**. *PhD Student Seminars, Division of Infection and Immunity, School of Medicine, Cardiff University*.

Poster Presentations

- “Galectin-3 and its role in rheumatoid arthritis and its associated co-morbidity, cardiovascular disease”. Morrin AS, Jones GW, Williams AS. **January 2019**. 33rd Annual Schools of Medicine and Dentistry Postgraduate Research Day, Cardiff.

Abbreviations

A

ACPA	Anti-citrullinated protein antibodies
AIA	Antigen induced arthritis
APC	Antigen presenting cell

B

Bcl-2	B-cell lymphoma-2
BSA	Bovine serum albumin

C

CD	Cluster of differentiation
CFA	Complete Freund's adjuvant
CFR	Coronary flow reserve
CFSE	Carboxyfluorescein succinimidyl ester
CIA	Collagen induced arthritis
CRD	Carbohydrate recognition domain
CRP	C reactive protein
CVD	Cardiovascular disease

D

dH ₂ O	Deionised water
DNA	Deoxyribonucleic acid

E

EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase

F

FBS	Foetal bovine serum
Foxp3	Forkhead box P3
FSC-A	Forward scatter-area
FSC-H	Forward scatter-height

G

G3BP	Galectin-3 binding protein
------	----------------------------

	GM-CSF	Granulocyte-macrophage colony stimulating factor
H		
	HLA	Human leukocyte antigen
	HPMC	Hydroxypropyl methylcellulose
	HRP	Horseradish peroxidase
I		
	IFN	Interferon
	Ig	Immunoglobulin
	IL	Interleukin
	iNOS	Inducible nitric oxide synthase
L		
	LAG	Lymphocyte activation gene-3
M		
	mBSA	Methylated bovine serum albumin
	MCP-1	Modified citrus pectin-1
	MHC	Major histocompatibility complex
	MMP	Matrix metalloproteinases
	mN	Millinewtons
N		
	NO	Nitric oxide
	NSAID	Non-steroidal anti-inflammatory
O		
	OA	Osteoarthritis
P		
	PADI	Peptidylarginine deiminase
	PBS	Phosphate buffered saline
	PEG300	Polyethylene glycol 300
	PMA	Phorbol 12-myristate 13-acetate
	PTPN22	Protein tyrosine phosphatase protein non receptor type 22
	PVAT	Perivascular adipose tissue

Q

qPCR Quantitative polymerase chain reaction

R

RA Rheumatoid arthritis

RANKL Receptor activator of nuclear factor kappa- β ligand

RNA Ribonucleic acid

ROR γ t Retinoic acid-related receptor orphan gamma-T

ROS Reactive oxygen species

S

SSC-A Side scatter-area

SSC-H Side Scatter-height

T

TBS Tris buffered saline

Tc Cytotoxic T cell

Tfh T-follicular helper

TGF β Transforming growth factor β

Th T-helper cell

TMB 3,3',5,5'-tetramethylbenzidine

TNF Tumour necrosis factor

Treg Regulatory T cell

TREM-2 Triggering receptor expressed on myeloid cells 2

W

WT Wild-type

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Chapter 1

General Introduction

1.1 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is an autoimmune disease resulting in chronic inflammation. Inflammation primarily occurs in synovial joints and ultimately leads to joint destruction - but can have systemic effects on vital tissues and organs. RA affects between 0.5% to 1% of the worldwide population (Silman and Pearson 2002). It is characterised by the production of autoantibodies against IgG, also known as rheumatoid factor, and anti-citrullinated protein antibodies (ACPAs). RA is a highly heterogeneous disease influenced by genetic and environmental factors that are associated with susceptibility and progression of disease. For example, heterogeneity is observed at the genetic and epigenetic level, in local joint inflammation, and in systemic immune and metabolic signatures. Women are at 2-3 times higher risk of developing RA than men. RA has a genetic component and patients with the HLA-DRB1*01 and HLA-DRB1*04 alleles are at significant risk of developing RA (Gregersen et al. 1987). Variants in PTPN22 and IL-6ST genes are associated with both seropositive and seronegative patients (Viatte et al. 2012), whereas variants in CD28 are only found in seropositive RA patients and variants in PRL genes are only found in seronegative patients. Many variants in genes associated with RA are found at enhancer regions of genes, indicating that they may play a role in regulating genes at distant locations. Epigenetic modifications have also been identified with the risk of developing RA – DNA methylation of the gene EXOSC1 which is involved in RNA degradation, showed differences in a RA patient and their identical twin (Gomez-Cabrero et al. 2016). While genetics are a risk factor for RA, environmental impact can also have a role in developing RA. Smoking doubles the risk of developing RA, and smokers who have alleles on the HLA-DRB1 gene have 20-fold increased risk (Källberg et al. 2011). Other environmental risk factors are periodontal disease, low levels of gut microbiota and previous history of Epstein-Barr virus (EBV).

There are four stages in the pathogenesis of RA – susceptibility to RA, preclinical RA, early RA and established RA. While factors associated with disease susceptibility can be genetic and/or environmental and have been outlined previously, pre-clinical RA is considered as the first indications of damage to synovial joints. During this phase, proteins such as histones and vimentin become citrullinated by PADI enzymes, altering their chemistry, and leading to the activation of T cells and production of antibodies by B cells, resulting in synovial inflammation (Yamada et al. 2005; Holers 2013). Patients in this stage are asymptomatic. Progression to early RA is characterised by inflammation in the synovium and infiltration of immune cells, including CD4⁺ T-helper cells and macrophages, to the

joint. Early RA and established RA is separated by further synovitis and irreparable damage to the joint architecture.

The synovium is the most affected tissue in RA. In healthy conditions the synovium produces lubricant and provides nutrients to cartilage to allow for a low friction environment. The synovium has a variety of cells in the form of macrophage-like synoviocytes, fibroblasts, adipocytes, and blood vessels. During RA, the innermost layer - also known as the intimal lining - of the synovium containing macrophage like synoviocytes, fibroblasts and resident immune cells, proliferates and ultimately results in the production of cytokines (e.g. IL-6, TNF α and IL-1) (Smolen et al. 2018). Another feature of RA is the infiltration of immune cells to the synovium lining, which can result in the formation of ectopic lymphoid structures of B cells that further proliferate and produce antibodies. The joint becomes damaged by the proliferation of these cells and the subsequent production of pro-inflammatory cytokines and matrix metalloproteinases (e.g., MMP-9). Bone destruction occurs when these mediators invade and degrade the cartilage, leaving the articular surface exposed. When exposed, bone resorbing cells called osteoclasts are matured and activated by RANKL. Osteoclasts degrade bone matrix by protease and hydrochloric acid production. Cytokines have been recognised as playing a key role in synovitis and are produced by synovial cells which further enhance inflammation. Cytokines produced activate other T cells and macrophages, which in turn produce their own cytokines forming a feedback mechanism by which cytokine production is dysregulated and uncontrolled.

While the joint is the main feature of inflammation during RA, inflammation also occurs at other sites in the body including the eyes, lungs, and heart. The most common co-morbidity of RA is cardiovascular disease. Approximately 40% of RA patients die from cardiovascular diseases, of which include hypertension, atherosclerosis and myocardial infarction (Dhawan and Quyyumi 2008). Key features of CVD-associated RA are due to immune dysregulation and inflammation. Inflammation leads to vasculitis and may lead to the formation of atherosclerotic plaques in the blood vessels. Similar to joint pathology, cytokines play a role in fuelling vascular inflammation and TNF α , IL-6 and IL-17 have been associated with vascular inflammation.

1.2 Cardiovascular System

The cardiovascular system is comprised of the heart and blood vessels. The function of blood vessels is to carry nutrient-rich blood away from the heart to tissue and to return nutrient depleted blood back to the lungs for gas exchange. Blood vessels are complex structures with many layers. The tunica intima is the innermost layer of arteries and is comprised of single layer of endothelial cells, called the endothelium, which provide a semi permeable barrier, inhibiting the passage of harmful chemicals while allowing for the passage of required molecules such as oxygen and nutrients between blood and tissues. The tunica media is a dense population of vascular smooth muscle cells that support the vessels mechanical function by constriction and relaxation of the vessel. The tunica externa, also known as the adventitia, is an external layer and is comprised of elastic lamina that is rich in collagenous extracellular matrix (ECM) molecules.

1.2.1 Vascular Function and Dysfunction

The network of blood vessels, also known as the vascular system, is used as a transport system for nutrients, red blood cells, immune cells and cytokines around the body to maintain homeostatic mechanisms such as regulation of blood pressure. The endothelium plays a vital role in maintaining these mechanisms by release of vasoactive substances such as nitric oxide, bradykinin and histamine. The endothelium has been identified in having roles in platelet and leukocyte interaction, by allowing the passage of white blood cells to sites of tissue damage for repair (Nourshargh and Alon 2014). To allow for this to occur, endothelial cells become activated and can rapidly produce and express cell surface receptors such as P-selectin that allow for the adhesion of leukocytes to the endothelium and for the subsequent migration of leukocytes through the junctions in the endothelium to surrounding tissues (Ley et al. 2007). While rapid induction of the endothelium is generally in response to inflammatory stimuli such as histamine and platelet activation factor, additionally endothelium activation can occur more slowly in response to cytokines such as IL-1 β and TNF α .

The endothelium also plays a role in the regulation of vascular tone and releases vasodilatory (e.g., prostacyclin and nitric oxide) and vasoconstriction substances (e.g., serotonin, endothelin, angiotensin II) (Rajendran et al. 2013). As blood passes through the vessel, frictional force is exerted on the endothelium which is termed as shear stress. The level of shear stress is dependent on the flow rate and viscosity of blood and plays a role in regulation of vessel diameter and therefore blood flow. Molecules on the surface of the

endothelium such as platelet endothelial cell adhesion molecule-1 (PECAM-1) and vascular endothelial growth factor II (VEGFR2) sense the rate of blood flow and release molecules to induce vasodilatory or vasoconstriction effects. For example, the endothelium senses increased frictional force and shear stress and so can lead to the production of NO in the artery wall, resulting in vasodilation (Nyberg et al. 2015).

Vascular dysfunction, also known as endothelial dysfunction, is characterised by a switch in the silent phenotype of the endothelium to a host defence response (Deanfield et al. 2007). This “switch” involves the decreased availability of vasodilators (e.g., NO) and increased contractile mediators. This leads to an imbalance in contraction and relaxation - resulting in shear stress, the production of superoxides, and damage to vessel walls (Hadi et al. 2005). Vascular dysfunction is a feature of many cardiovascular diseases such as atherosclerosis, hypertension and coronary artery disease. The production of superoxides and free radicals can drastically change the environment of the endothelium and beyond. In healthy conditions, the endothelium does not allow for the passage of macromolecules (e.g., LDL) into the media layer, externa layer or tissue. However, the heightened levels of superoxides results in a “leaky” endothelium, that becomes more permeable to toxins, proteins, and immune cells. The passage of immune cells across the endothelium can lead to the formation of atherosclerotic plaques – resulting in arterial stiffness, narrowing of the blood vessel lumen, hypertension and ultimately myocardial infarction following plaque rupture. Vascular dysfunction is a feature of RA and can occur both in large arteries and the microvasculature.

1.2.2 Vascular Dysfunction in RA

Vascular dysfunction in RA is evidenced by heightened levels of von Willebrand factor (vWf), soluble E-selectin and intracellular cell adhesion molecule-1 (sICAM-1) (Blann et al. 1995). The risk factors for the development of vascular dysfunction in RA is the same as those for the development of RA itself. The combination of genetic factors (presence of certain HLA-DRB1 alleles), immune dysregulation (by the presence of rheumatoid factor and citrullinated proteins), inflammation (production of proinflammatory cytokines IL-1 β , TNF α , IL-6); presence of co-existing comorbidities such as hypertension or diabetes; and age or sex may lead to the induction of vascular dysfunction. Vascular dysfunction leads to stiffening of large arteries resulting in increased pulse pressure to the microvasculature, damaging microvasculature capillary beds and initiating damage to organs by means of dysregulated passage of metabolites, leukocytes and oxygen transport in and out of tissue.

For example, damage to the microvasculature in organs can result in hypoperfusion of oxygen to tissues, increasing the risk of ischemic damage (Bordy et al. 2018). Vascular dysfunction occurs in early RA – in a study by Foster *et al*, patients were classified as early RA when having symptoms for minimum of 1 month, and a median duration of symptoms was 2 months (Foster et al. 2012). Patients with RA had increased number of circulating endothelial cells (indicating endothelial shedding which is suggestive of disease and increased risk of leukocyte adhesion and thrombosis (Georgiadou and Cunnington 2019)) when compared to healthy controls. Methods of measuring endothelial dysfunction in patients include coronary flow reserve (CFR), finger plethysmography or laser Doppler flowmetry in the skin. Vascular dysfunction has been noted in RA, as measured by CFR (Ciftci et al. 2008), but CFR is considered an invasive technique and therefore is not routinely used in clinical practice. An overview of types of cardiovascular diseases associated to each type of inflammatory arthritis is outlined in **Table 1.1**.

1.3 Animal Models of RA

As measurements of vascular dysfunction in the clinic are invasive and therefore not typically adopted, mechanisms underlying how vascular dysfunction develops and leads to CVD co-morbidities in RA is poorly understood. Animal models have been used to study human diseases for a long time. There are many different animal models of inflammatory arthritis including collagen-antibody induced arthritis, zymosan induced arthritis and antigen induced arthritis. The most common model used for studying the systemic effects of inflammatory arthritis is the collagen induced arthritis mouse model (CIA). CIA exhibits many similarities to human RA – both are polyarticular, have similar circulating markers (e.g., ACPAs and anti-type II collagen antibodies) and similar joint pathology, as characterised by immune cell infiltration and pannus formation. While CIA was first shown in rats, DBA/1 mice have become the most common choice for the model as they possess the MHC Class II^q haplotype, making them susceptible towards the development of CIA. The H-2^q haplotype gives rise to a stronger autoantibody response to type II collagen when compared to non-H2^q carriers (Holmdahl et al. 1986). Macroscopic arthritis typically presents Day 21 – 25 after immunisation of chicken collagen in complete Freund's adjuvant (CFA) and peaks at Day 30 - 35 (Brand et al. 2007; Jones et al. 2018). CIA has been performed in C57Bl/6 mice, however CIA in these mice was milder, as determined by measurement of paw diameter (Inglis et al. 2007) and so the use of DBA/1 mice is recommended. CIA is associated with B and T cell responses by the production of anti-collagen type II antibodies and collagen-specific T cells. CIA was chosen as the animal

model of inflammatory arthritis in **Chapter 3** of this Thesis as it exerts a systemic inflammatory effect and can be used to study co-morbidities (Asquith et al. 2009). Vascular inflammation is a feature of CIA. In a study by Denys *et al*, mice with CIA had heightened levels of VCAM-1, and when run in conjunction with a high fat diet, inflammation in vessels was observed (Denys et al. 2016). Furthermore, a study by Tozzato *et al* show the presence of vascular dysfunction in DBA/1 mice with CIA by enhanced iNOS expression in the aorta, heart, kidneys and blood, suggesting a decrease in nitric oxide bioavailability (Palma Zochio Tozzato et al. 2016). In this Thesis, vascular dysfunction is measured in CIA by determination of constriction response of aortic rings to vasoconstrictor serotonin (5HT). Mice with CIA have shown to have significantly decreased constriction responses to 5HT when compared to healthy age-matched control mice (Reynolds et al. 2012; Sime et al. 2017). The defective responses are discussed in detail in **Chapter 3** of this Thesis.

As discussed above, other animal models of inflammatory arthritis can be used to study various aspects of disease. Zymosan-induced arthritis involves the administration of polysaccharide zymosan intra-articularly to the knee joint. Zymosan binds to TLR2 in macrophages, induces the production of pro-inflammatory cytokines and activates the complement pathway. These mechanisms result in an inflammatory arthritis with mononuclear cell infiltration in the affected knee joint. However, this model is restricted to one articular joint and peak of disease occurs at Day 3 and subsides at Day 7, giving a small window of opportunity to study pathology (Keystone et al. 1977). The antigen induced arthritis (AIA) model also uses an intra-articular injection of methylated BSA in CFA, but these mice are also primed with an antigen in the weeks prior to arthritis onset. AIA is used to study adaptive-mediated articular disease. AIA is typically used on C57Bl/6 mice and given that C57Bl/6 mice can be genetically modified, knockout models can be used to study the role of varying cytokines and receptors in the pathogenesis of adaptive mediated joint pathology (Jones et al. 2018). Vascular dysfunction in AIA has not been reported in the literature and is investigated in **Chapter 5** of this Thesis. An overview of animal models of inflammatory arthritis is shown in **Table 1.2**.

Inflammatory disease	Associated CVD	Treatment	Reference
Osteoarthritis	Ischaemic heart disease	Joint replacement surgery; non-steroidal anti-inflammatories (NSAIDs), but long-term use of NSAIDs increase the risk of CV events.	(Rahman et al. 2013; Wang et al. 2016).
Osteoarthritis	Congestive heart failure	Joint replacement surgery; non-steroidal anti-inflammatories (NSAIDs), but long-term use of NSAIDs increase the risk of CV events.	(Rahman et al. 2013).
Ankylosing spondylitis	Myocardial infarction	Joint replacement surgery; non-steroidal anti-inflammatories (NSAIDs), but long-term use of NSAIDs increase the risk of CV events.	(Bengtsson et al. 2017).
Psoriatic arthritis	Myocardial infarction	Etanercept reduces CVD risk via reducing overall inflammation.	(Lauper et al. 2018).
Rheumatoid arthritis	Left ventricular heart disease	Etanercept reduces CVD risk via reducing overall inflammation.	(Midtbø et al. 2014).
Rheumatoid arthritis	Atherosclerosis	Etanercept reduces CVD risk via reducing overall inflammation.	(Evans et al. 2011).

Table 1.1: Types of cardiovascular disease associated to each form of inflammatory arthritis and effect of arthritis treatment on each CVD.

Name of model	Species/Strain	Description	Reference
Collagen induced arthritis	DBA/1 mice	Immunisation with type II collagen leads to anti-collagen antibody response and systemic inflammation.	(Brand et al. 2007).
Adjuvant induced arthritis	Lewis/Sprague Dawley Rat	Rats immunised with complete Freund's adjuvant results in destruction of cartilage and bone in distal joints.	(Whiteley and Dalrymple 2001).
Antigen induced arthritis	C57Bl/6 mice	Intraperitoneal injection of pertussis toxin and subcutaneous administration of mBSA, followed by intra-articular injection of the same antigen leading to arthritis in one joint characterised by synovitis, leukocyte infiltration and bone erosion.	(Jones et al. 2018).
Streptococcal cell wall induced arthritis	Lewis Rat	Initial immunisation peptidoglycan-polysaccharide polymer results in macrophage and B cell response whereas subsequent immunisations result in T cell responses.	(Bevaart et al. 2010).
Zymosan induced arthritis	C57Bl/6 mice	Intra-articular immunisation of polysaccharide zymosan induces activation of macrophages via TLR2 resulting in mononuclear cell infiltration and synovial hypertrophy.	(Keystone et al. 1977).
Collagen antibody induced arthritis	DBA/1 mice, BALB/c mice	Administration of serum from an immunised mouse to a naïve mouse results in the development of arthritogenic antibody cocktails against the antibodies delivered. This leads to macrophage responses, but not B or T cell responses, making this model useful for studying separate roles of innate and adaptive immunity in arthritis.	(Asquith et al. 2009).
K/BxN	K/BxN mice	Transgenic mice expressing T cell receptor KRN and MHC class II A ^{g7} haplotype develop severe arthritis and serum transfer from K/BxN mice to naïve mice induces arthritis.	(Monach et al. 2008).
<i>Il1ra</i>^{-/-} transgenic mice	BALB/c	Spontaneous development of arthritis that has synovial and periarticular inflammation as a feature.	(Choudhary et al. 2018).

Table 1.2: An overview of animal models of human RA

1.4 Galectin-3

Galectin-3 is a 30kDa β -galactoside binding protein that is widely expressed in various cell types including macrophages, T cells, fibroblasts, epithelial cells, gastric mucosa and osteoclasts (Dumic et al. 2006). The expression and localisation of galectin-3 is highly context dependent. Galectin-3 has a carbohydrate recognition domain (CRD) with a C and N terminal, both of which play a role in aiding galectin-3 to bind to targets. The C and N terminals aid galectin-3 binding and activation in both intracellular and extracellular environments. Galectin-3 is predominantly synthesized on ribosomes in the cytoplasm, but may be found in the nuclear compartment, on the cell surface or in the extracellular environment. Cytosolic galectin-3 has roles in cell proliferation, differentiation, anti-apoptosis and connective tissue growth and development (Fowles et al. 1995; Boileau et al. 2008). Galectin-3 is highly expressed in the mitochondria and has shown to prevent cytochrome c release and prevent oxidative stress via synexin (Yu et al. 2002). Nuclear galectin-3 is mainly localised in the interchromatin space and can function in acting as a precursor mRNA splicing factor and as a regulation of Wnt/ β -catenin signalling in embryogenesis and tissue regeneration (Wang et al. 2013). All galectin-3 binding occurs via its N terminal or CRD. While the mechanism underlying galectin-3 activation intracellularly hasn't yet been fully elucidated, it has been suggested that tyrosine phosphorylation (Menon et al. 2011) aids the shuttling of the protein from the nucleus to cytoplasm whereas translocation from the cytoplasm to the mitochondria is mediated by synexin.

Galectin-3's export from the cell is via a non-classical secretory mechanism and does not require the ER/golgi complex (Hughes 1999). Following secretion, extracellular galectin-3 binds to glycan ligands on the cell surface or in the extracellular matrix (e.g., growth factor receptors, cadherins and integrins). While intracellular galectin-3 has anti-apoptotic effects, extracellular galectin-3 can induce apoptosis of T cells, highlighting that galectin-3 roles are highly context dependent on localisation and type of cell it is involved with (Rabinovich et al. 2007). Enhanced extracellular galectin-3 expression has been linked with fibrosis, tumour development and atherosclerosis (Yu et al. 2013). Extracellular galectin-3 elicits its actions by undergoing oligomerisation. This binding results in a conformational change in galectin-3, and galectin-3 undergoes oligomerisation, therefore increasing the number of carbohydrate recognition domains on the protein. This oligomerisation acts as a support for signalling receptors on the cell surface (Henderson and Sethi 2009). While galectin-3 has many ligands, as outlined in **Table 1.3**, there is still unclear mechanisms as to how galectin-3 elicits its effects.

1.4.1 Expression and function of galectin-3 in cells and tissues

Galectin-3 is expressed in mouse lung, spleen, colon, uterus and ovary but its expression is low in mouse heart, pancreas and cerebrum (Kim et al. 2007). While galectin-3 is expressed in specific tissues, it is also expressed in monocytes, macrophages, dendritic cells, neutrophils and activated T and B cells and is considered as a regulator of host immune response (Breuilh et al. 2007). Galectin-3 acts as a chemoattractant in monocytes and macrophages in a concentration dependent manner (Sano et al. 2000); as an adhesion molecule for neutrophil extravasation (Sato et al. 2002); and it has functions in the regulation of growth and apoptosis of T cells (Yang et al. 1996). Galectin-3 is constitutively expressed in regulatory and memory T cells, but is only expressed in activated CD4⁺ and CD8⁺ T cells (Hsu et al. 2009). Galectin-3 has similar sequence to Bcl-2, a suppressor of apoptosis and when located in the cytoplasm can significantly decrease cell apoptosis. Therefore, cancers that are cytoplasmic galectin-3 positive are generally more aggressive than nuclear galectin-3 positive cancers (Califice et al. 2004). However, galectin-3 has been linked with survival of B cells and is essential in supporting the effects of IL-4 on B cell differentiation and lineage (Acosta-Rodríguez et al. 2004).

Receptor	Cell type the receptor is present on	Function associated with ligand binding	Reference
CD32	PBMCs	Negative regulator of IL-5 transcription resulting in decreased/inhibition IL-5 gene transcription.	(Cortegano et al. 2000).
CD29, CD7	T cell lines (Jurkat, CEM, MOLT-4)	Apoptosis - induction of cytochrome c release resulting in caspase 3 activation.	(Fukumori et al. 2003).
CD11b	Macrophage	Cell adhesion.	(Dong and Colin Hughes 1997).
Bcl-2	T cell line (Jurkat)	Apoptosis.	(Yang et al. 1996).
CD95	T cell line (CEM, Jurkat)	Apoptosis – galectin-3 interacts with CD95 on the cell surface to determine if CD95 initiates type I or type II apoptotic signalling.	(Fukumori et al. 2004).
Synexin	BT459 cell line	Regulation of apoptosis - synexin mediates translocation of galectin-3 to perinuclear mitochondrial membrane to protect from apoptosis.	(Yu et al. 2002).
β-catenin	Galectin-3 in nucleus	Galectin-3 co-localizes with β -catenin in the nucleus and induces transcriptional activity of Tcf4 to result in overall regulation of Wnt/ β -catenin signalling.	(Shimura et al. 2004).
CD45, CD71	T cell lines (Jurkat, MOLT4, CEM)	Apoptosis.	(Stillman et al. 2006).
CD98	BMDMs wild type mice	Alternative macrophage activation.	(MacKinnon et al. 2008).
TREM2	Microglia	Microglia activation from galectin-3 ^{-/-} 5xFAD mice reduces Alzheimer's disease pathology.	(Boza-Serrano et al. 2019).
LAG-3	Murine CD8 T cells	Binding of galectin-3 to LAG3 on CD8 T cells results in suppression of CD8 T cell effector function and poorer outcome in tumour environments.	(Kouo et al. 2015).

Table 1.3: Binding partners for galectin-3.

1.4.2 Role of galectin-3 in inflammatory disease

As galectin-3 is so abundantly expressed, it has been shown to play a role in influencing various biological processes. Galectin-3 has been heavily implicated in inflammation, organ fibrosis, cancer and auto-immune disease. Galectin-3 was shown to activate pro-inflammatory gene signatures via the Nf- κ B complex (Weinmann et al. 2016), it is expressed in macrophages and foam cells and its expression levels are heightened in atherosclerotic plaques. Galectin-3 also promotes tumour cell proliferation and therefore may influence oncogenesis and metastasis. However, the role of galectin-3 in cancer has been shown to be specific for the type of cancer and can have positive or negative effects (Sciacchitano et al. 2018). Nuclear galectin-3 expression is decreased while cytoplasmic galectin-3 is increased in colon cancers; and levels of cytoplasmic galectin-3 differed in patients with squamous cell lung carcinomas and lung adenocarcinomas (Thijssen et al. 2015). The function of galectin-3 in these contexts remains to be elucidated. However, galectin-3 is known as a regulator of tissue fibrogenesis and is now a prognostic biomarker of all-cause mortality in kidney fibrosis (de Boer et al. 2012). Galectin-3 is expressed in human cartilage and chondrocytes of patients with osteoarthritis and exposure of chondrocytes to galectin-3 led to further expression of disease markers such as IL-1 β , TNF α , MMP-1 and 3.

1.4.3 Galectin-3 in RA and CVD

Galectin-3 is increased in human synovial membranes and synovial fibroblasts in arthritic joints and is correlated to CRP and IL-6 serum levels in RA patients (Filer et al. 2009). Galectin-3 is also increased in the serum of patients with RA (Issa et al. 2015). Furthermore, elevated circulating galectin-3 is found in the serum of mice with CIA (de Oliveira et al. 2015), which strengthens the use of CIA as an appropriate model of systemic inflammatory arthritis to investigate galectin-3 biology. Individual susceptibility to RA may be in part due to the presence of the LGALS3 +292C galectin-3 gene allele, as this polymorphism is more common in RA patients than healthy controls (Hu et al. 2011). Fibroblast-like synoviocytes (FLS) are present in RA and produce pro-inflammatory cytokines and chemokines to promote pathology in the joint and are considered as key drivers in joint destruction. Galectin-3 is highly expressed in the FLS taken from RA patients. The FLS adhere to cartilage and induce the expression of galectin-3. Furthermore, galectin-3 can induce FLS to produce and secrete pro-inflammatory cytokines such as IL-6 and GM-CSF (Neidhart et al. 2005). Galectin-3 is also heightened in many forms of cardiovascular disease (e.g. atrial fibrillation,

hypertension) and it has been suggested that galectin-3 may serve as a prognostic biomarker of mortality (Dong et al. 2018). Galectin-3 is known to contribute to cardiac fibrosis by increasing the avidity of cell adhesion molecules and inhibiting apoptosis (Ochieng et al. 2002). It is unknown if the increased risk of CVD observed in RA is due to a direct effect of galectin-3. Regardless, inhibition of galectin-3 is an attractive therapeutic target in RA associated cardiovascular disease as it plays roles in both joint pathogenesis and cardiovascular disease.

1.4.4 Galectin-3 in clinical trials

Targeting galectin-3 for therapeutic benefit has been investigated in clinical trials. Modified citrus pectin-1 (MCP-1) is a pectin commonly found in the peel of fruits that has been considered as a potential anti-oxidant and anti-inflammatory. MCP-1 was thought to bind and inhibit the functions of extracellular galectin-3. This has been shown in mouse models of atherosclerosis whereby treatment of MCP-1 inhibited galectin-3 and reduced atherosclerotic lesion size by inhibition of leukocyte adhesion to endothelial cells (Lu et al. 2017). Treatment of MCP-1 also reduced galectin-3 expression and disease severity in a mouse model of acute kidney injury (Kolatsi-Joannou et al. 2011). However, recent studies have found that MCP-1 does not bind the CRD of galectin-3 and has failed to show galectin-3 inhibition (Stegmayr et al. 2016). Furthermore, while there is no current clinical trials investigating the modulation of galectin-3 expression by MCP-1 in rheumatoid arthritis, a trial investigating galectin-3 inhibition in osteoarthritis by treatment with MCP-1 was conducted from 2016 - 2018. In the trial, OA patients received either 4 grams MCP-1 or placebo twice daily for 12 weeks. Circulating galectin-3 levels were quantified at baseline and at 12 weeks by ELISA, and disease activity was measured by WOMAC-knee, SF-36 and RAPID3 surveys at baseline and at 12 weeks. The Phase III trial of inhibition of galectin-3 by MCP-1 in osteoarthritis showed no change in galectin-3 levels or OA disease severity in MCP-1 versus placebo treated groups (Andrews et al. 2020). This further highlights the need for specific galectin-3 inhibitors for treatment of arthritis and associated comorbidities.

Other clinical trials involving galectin-3 inhibition to treat endometriosis, hypertension and idiopathic pulmonary fibrosis (IPF) are currently ongoing. Clinical trials evaluating the use of galectin-3 as a prognostic biomarker in thyroid cancer is currently progressing. However, there are no trials underway for the treatment of rheumatoid arthritis or rheumatoid arthritis associated CVD. As described in **Section 1.3**, animal models of inflammatory

arthritis are invaluable in understanding the pathology and investigating potential new therapies for the treatment of these disease.

1.4.5 Galectin-3 inhibition in animal models

The role of galectin-3 has been investigated in experimental models of arthritis. A study by Hafsia, 2014 showed that galectin-3 had a protective role in murine osteoarthritis (OA). OA was induced by partial medial meniscectomy in wild-type and galectin-3 mutant mice in 1 knee joint. Galectin-3 deficient mice had more severe OA than wild-type mice (Hafsia 2014). The role of galectin-3 in joint inflammation has also been studied. Administration of galectin-3 intra-articularly to the knee joints of 129c/c mice induced joint swelling, altered the structure and cellularity of cartilage and subchondral bone underwent remodelling when compared to controls (Janelle-Montcalm et al. 2007). The role of galectin-3 has also been studied in an equine model of osteoarthritis. Horses were induced with osteoarthritis via carpal fragmentation and synovial fluid was collected. Disease controls of naturally occurring carpal osteoarthritis were used (Reesink et al. 2018). The concentration of galectin-3 in synovial fluid was present in healthy and OA synovial fluid but is significantly increased in the experimental OA group (Reesink et al. 2018). To my knowledge there are no papers exploring the effect of specific inhibition of galectin-3 in OA, however the data discussed above highlights pharmacological modulation of galectin-3 is an attractive therapeutic target of OA.

There has been no clinical trials investigating galectin-3 inhibition in rheumatoid arthritis. However, there are studies investigating the role of galectin-3 in animal models of RA. In a study by Shou *et al* the blood from rats with collagen induced arthritis was analysed for reliable biomarkers of disease progression. The study showed that galectin-3 is secreted in the plasma and synoviocytes of CIA rats, indicating not only that galectin-3 is a valid biomarker in inflammatory arthritis but also may promote the development of inflammation in arthritis (Shou et al. 2006). Furthermore, the loss of galectin-3 by genetic knockdown in antigen induced arthritis showed alleviated joint pathology. This was exhibited by significantly decreased synovial inflammation and immune cell infiltration when compared to wild-type controls. Additionally, re-administration of recombinant galectin-3 had restored inflammation as shown by increased synovitis similar to that of wild-type mice with AIA. Furthermore, there was decreased circulating TNF α and IL-6 in galectin-3 knockout (*Lgals3*^{-/-}) mice compared to controls. Following the re-administration of galectin-3 to *Lgals3*^{-/-} mice, TNF α and IL-6 levels were restored to levels comparable to

wild-type mice. There was no difference in IFN γ production in lymph node supernatants between *Lgals3*^{-/-} and wild-type (WT) mice, but there was a trend towards decreased IL-17 production in *Lgals3*^{-/-} mice, indicating that Th1 mediated responses are not involved in galectin-3 signalling, but Th17 responses are (Forsman et al. 2011). The relationship between galectin-3 and Th17 cell responses is investigated in **Chapter 4** of this Thesis. The studies discussed here show a clear rationale for investigating inhibition of galectin-3 in inflammatory arthritis. While there has been studies investigating the role of galectin-3 in inflammatory arthritis, via the use of genetically modified mice, to my knowledge there is no studies focussing on pharmacological inhibition of galectin-3 in inflammatory arthritis.

Pharmacological inhibition of galectin-3 in models of CVD have shown alleviated myocardial inflammation and cardiac remodelling, reduced collagen production and improved cardiac function (Zhong et al. 2019). Animal models have aided in evaluating the effects of galectin-3 inhibition in CVDs. In a rat model of hypertension, galectin-3 was expressed 5 times higher in the heart of experimental rats compared to controls and administration of galectin-3 to pericardial sacs led to increased collagen I/III ratio resulting in cardiac remodelling (Sharma Umesh et al. 2004). Induction of cardiac remodelling via angiotensin II infusion in *Lgals3*^{-/-} mice and wild-type mice showed no cardiac fibrosis in the *Lgals3*^{-/-} mice. Pharmacological inhibition of galectin-3 in wild type mice by treatment with N-acetyl-lactosamine (N-Lac) showed reduced left ventricular dysfunction and fibrosis when compared to untreated wild-type mice. The untreated wild type mice exhibited left ventricular hypertrophy, fibrosis and remodelling. This indicated that galectin-3 plays a direct role, but is not fully responsible for the mechanisms underlying cardiac remodelling (Yu et al. 2013). The effect of galectin-3 on vascular dysfunction was investigated by Ou *et al.* The relationship between galectin-3 and the LOX-1 receptor on endothelial cells was studied. LOX-1 is a receptor for oxidized LDL (oxLDL) and contributes to endothelial dysfunction. When HUVEC cells were treated with galectin-3 and oxLDL led Ou *et al.* found that the expression of the LOX-1 receptor was enhanced, ROS production was increased and the p38 subunit of the Nf- κ B complex was subsequently induced. Galectin-3 may therefore promote vascular dysfunction via LOX-1/ROS/Nf- κ B signalling mechanism (Ou et al. 2019). Given these studies, galectin-3 is considered an attractive therapeutic target for the treatment of RA associated vascular dysfunction leading to CVDs.

1.5 The immune system

The immune system is a complex set of structures that function in protecting the body from invading pathogens and microbes. Broadly, the immune system is comprised of cells (leukocytes), cytokines, chemokines, antibodies, and organs (thymus, spleen, lymphatic system and the bone marrow). These components work together to produce and store leukocytes, mark foreign pathogens to the body for destruction, and the inactivation and destruction of those pathogens. Blood cells are comprised of granulocytes (i.e., neutrophils), monocytes and lymphocytes such as T and B cells. The response of the immune system can be divided in two: the initial innate response and the later adaptive response.

1.5.1 The innate immune system

The innate immune system is the first line of defence in the body and the main aim of these responses to inhibit the spread of foreign pathogens in the body. The innate immune system is comprised of physical barriers (tight epithelial junctions in the skin and mucous membranes to inhibit entry to the body), phagocytic white blood cells such as monocytes, macrophages and neutrophils, inflammatory-related proteins such as complement proteins and c-reactive protein, cytokines that are released to aid in clearance of pathogens and receptors (e.g., toll-like receptors, TLRs) that sense invaders and signal to mount an immune response. Cellular defences include the migration of neutrophils to a site of infection or inflammation and initiate the phagocytosis cascade by lysis. Macrophages phagocytose pathogens and cellular debris, stimulate the production and secretion of cytokines and aid in tissue repair by production of collagen and matrix metalloproteinases (MMPs). Macrophages possess the ability to polarise to subsets, depending on the inflammatory context of the situation. M1 macrophages typically exhibit pro-inflammatory effects and function in enhancing the ability of the cell to kill microbial pathogens while M2 macrophages exhibit anti-inflammatory effects and function in tissue repair. However it is now recognised that both subsets can interchange and change their phenotype when the microenvironment changes (Aristizábal B 2013).

Galectin-3 inhibitor	Specificity to galectin-3	Other targets	Disease indication	Route of administration	Stage/Outcome in clinical trial	Reference
MCP-1	No	Galectin-1, galectin-9	Hypertension	Oral	No efficacy observed.	Clinical trial reference number NCT01960946.
MCP-1	No	Galectin-1 , galectin-9	Osteoarthritis	Oral	Phase III showed no efficacy.	(Andrews et al. 2020); Clinical trial reference number NCT02800629.
GM-CT-01	No	Galectin-1	Metastatic melanoma	Intravenous infusion	Terminated during Phase II.	(Klyosov et al. 2012); Clinical trial reference number NCT01723813.
GCS-100	No	Galectin-1	Chronic kidney disease	Intravenous infusion	Terminated at phase II	(Drechsler et al. 2015); Clinical trials reference number NCT01843790.
GR-MD-02	No	Galectin-1	Metastatic melanoma	Intravenous infusion	Phase I ongoing.	Clinical trials reference number NCT02117362.
GR-MD-02	No	Galectin-1	Portal hypertension	Intravenous infusion	No efficacy observed at Phase II.	(Chalasanani et al. 2020); Clinical trial reference number NCT02462967.
GBO139	Yes	Poor affinity for galectin-1	Idiopathic pulmonary fibrosis	Inhaled	Yes – Phase IIb	Clinical trial reference number NCT03832946.
GB1211	Yes	Poor affinity for galectin-1	NASH	Oral	Yes – Phase I passed	Clinical trial reference number NCT03809052.
GB2064	Yes	Poor affinity for galectin-1	Myelofibrosis	Oral	Yes – Phase IIa	Clinical trial reference number NCT04679870.

Table 1.4: Galectin-3 inhibitors in clinical trials

1.5.2 The adaptive immune system

The adaptive immune response takes longer to establish than the innate responses but is more specific and provides immunological memory to give long term protection against pathogens. It is activated to eliminate pathogens that evade the innate immune responses. The mechanisms of adaptive responses are mediated by 2 elements – cell mediated immunity, conducted by T cells against intracellular pathogens, and humoral immunity which is elicited by B cells against extracellular pathogens and toxins (Snyder 2017). Macrophages possess pattern recognition receptors (PRRs) which contribute towards the phagocytosis of pathogens, but macrophages also function as antigen presenting cells (APCs) for T cell recognition and stimulus for activation. During antigen presentation, molecules called major histocompatibility complexes (MHC) present fragments of the antigen for recognition by T cells. MHC molecules can be divided into two classes. MHC Class I presents antigens that originate from a nucleated cell's cytoplasm and are shuttled to the cell surface on MHC Class I molecules for destruction by CD8⁺ cytotoxic T cells. MHC Class II are expressed in macrophages and dendritic cells lead to CD4⁺ T-helper cell activation and the subsequent downstream activation of effector subtypes. Both B and T cells possess the unique ability to develop receptors to recognise and eliminate specific antigens that are harmful to the body, without creating antibodies against harmless antigens or against the body itself. The B cell receptor (BCR) is formed by 2 heavy chain and 2 light chain molecules. The T cell receptor (TCR) is formed by a heterodimer of alpha and beta polypeptide. TCRs differ from BCRs by their ability to recognise amino acids on MHC class molecules.

1.5.3 T cell activation

T cells are cells of the adaptive immune system and are grouped based on the receptor they express on their surface - CD8 cytotoxic T cells, Foxp3⁺ regulatory T cells and CD4 T helper cells. CD8 cytotoxic T cells (Tc cells), as the name suggests, kills cells that are infected with pathogens. Regulatory T cells (Tregs) fall under the umbrella of CD4 T-helper cells but have a distinct function in protecting the immune system from over activation and stopping immune responses when no longer required to avoid unnecessary damage to cells and tissues. They mediate their effects through various mechanisms including the release of inhibitory cytokines such as IL-10 and TGFβ; cytolysis and inhibition of dendritic cell maturation and function (Vignali et al. 2008). CD4 T-helper (Th) cells recognise the peptide on MHC molecules on APCs and subsequently become activated. When activated, they

produce cytokines to signal to the rest of the immune system to remove the pathogen. CD4 Th cells can be further divided into groups dependent on the cytokines produced and effector functions of those cells. They can be classed as Th1, Th2, Th9, Th17 and T-follicular (Tfh). Each type of cell, the major cytokines they produce, and their function can be found in **Table 1.5**.

These cells play a vital role in adaptive immunity by not only activating B cells and cytotoxic T cells, but also by releasing their own cytokines to regulate the immune response. Th cells require a 2-hit signal to activate. The first signal is from a foreign antigen on antigen presenting cells and the second signal is provided by co-stimulatory molecules which become recognized by CD28 on the T cell surface - resulting in enhanced TCR signal and decreased threshold for Th cell activation. The third key signal for sustained T cell activation is from cytokines produced by APCs. T cells require all three signals to activate and will undergo apoptosis or senescence if those signals are not received (Alberts B 2002; Wherry and Masopust 2016).

1.5.4 The role of T cells in autoimmune diseases

While all immune cells are present to protect the body from infection and harmful toxins, autoimmunity may arise which causes dysregulation of immune cell activation. T cells are especially involved in the pathogenesis of several autoimmune diseases - such as diabetes, RA and atherosclerosis - and they can become "self-reactive". When T cells become self-reactive their production of pro-inflammatory cytokines contribute to inflammation which signal towards the activation of auto-reactive B cells. Auto-reactive B cells then produce auto-antibodies (antibodies directed against the own immune system) which can further contribute to tissue inflammation. T-helper cells can also expand and proliferate into various effector subtypes which vary in their contribution to inflammation depending on the cytokines they produce (Jäger and Kuchroo 2010).

In RA, T cells activate macrophages and fibroblasts at sites of inflammation, which result in macrophages becoming tissue destructive cells by differentiation to osteoclasts and the release of pro-inflammatory cytokines and chemokines. Th1 cells produce IFN γ and TNF α which signal to macrophages and can act as APCs to present MHC Class II molecules to other T cells. Th2 cells secrete IL-4 and IL-5 which act as anti-inflammatory cytokines. However, Th2 cells also play a role in the immune system by contributing to B cell activation during parasitic infection and promote the switching of IgG antibodies to IgE antibodies (Schulze-Koops and Kalden 2001). Th17 cells secrete IL-17 which can act as

further stimulation to produce other pro-inflammatory cytokines and MMPs. Th17 cells have been noted as key players in the pathogenesis of RA and serum IL-17 levels positively correlate with CRP levels, erythrocyte sedimentation rate (ESR) and disease activity by DAS28 in RA patients. IL-17 can also increase the production of vascular endothelial growth factor (VEGF), IL-6, MMP1 and MMP3 in RA fibroblasts. Th9 cells also positively correlate with DAS28 score and ESR of RA patients. Furthermore, Th9 cells enhance the survival of neutrophils and contributed to Th17 cell differentiation by induction of ROR γ t transcription factor (Yap et al. 2018). IL-17 further contributes to inflammation by recruiting neutrophils to sites of inflammation resulting in further perpetuation of the innate immune response (Tesmer et al. 2008).

Abnormal T-helper cell polarisation has been identified in vascular diseases. Vascular inflammation often co-exists with autoimmune and chronic inflammatory diseases, and a subset of CD4 T cells have been identified in contributing to autoimmune disease, called effector memory T (TEM) cells. TEM cells contribute to tissue injury by release of pro-inflammatory cytokines, which in turn results in the recruitment of macrophages, neutrophils, T and B cells to the site of inflammation. T cells can also contribute to vascular inflammation by promoting the development of hypertension. This has been shown in mice subjected to daily stress had increased blood pressure, but also increased activation of circulating T cells and increased infiltration of T cells to the vasculature (Marvar et al. 2012). T cells are involved in atherosclerosis and approximately 10% of all leukocytes expressed in atherosclerotic plaques are CD4 T cells (Jonasson et al. 1986). Atherosclerosis is reported as a Th1 mediated disease, as evidenced by the recruitment of vascular smooth muscle cells by IFN γ to sites of inflammation, and the activation of macrophages and dendritic cells by IFN γ . Furthermore, in mice, *Ifn γ ^{-/-}*, *Ifn γ r^{-/-}* or transcription factor *Tbx21^{-/-}* mice shown to be protected from the development of atherosclerosis (Wolf and Ley 2019). This is recapitulated in human atherosclerotic plaques whereby plaques isolated from patients have a strong IFN γ presence and lack of IL-4 secreting cells (Frostegård et al. 1999). In a study by Hart *et al* mouse CD4 T cells were found to be present and activated in the vascular smooth muscle cells and were required to induce vasculitic lesions (Baiu et al. 2010). The role of various Th cell subsets and effector functions vary between disease and stage of disease. ANCA associated vasculitis (AAV) is a condition whereby blood vessels become inflamed caused by the generation of anti-neutrophilic cytoplasmic autoantibodies.

Type of Th cell	Function	Master transcription factor	Cytokines produced	Reference
Th1	Promote immune responses against intracellular bacteria and enhance microbicidal activity of macrophages.	T-bet	IFN γ , IL-2	(Goswami and Kaplan 2017).
Th2	Protect against parasitic helminth infections and promotes allergic responses. Secretion of cytokines stimulates the production of IgE antibodies by B cells and recruitment of eosinophils.	GATA3	IL-4, IL-5, IL-13	(Maynard and Weaver 2015).
Th9	Pleiotropic functions including allergic inflammation, by recruitment of eosinophils, anti-tumour immunity by recruitment of CD8 T cells and autoimmune inflammation.	STAT6, PU.1, IRF4, GATA3	IL-9, IL-10	(Kaplan 2013).
Th17	Mediate responses to fungal and bacterial infections by recruitment of neutrophils and macrophages to infected tissues.	ROR γ t	IL-17A, IL-17F, IL-22	(Ouyang et al. 2008).
T-follicular helper (Tfh)	Regulate B cell proliferation.	Bcl6	IL-21	(Nurieva and Chung 2010).
Treg	Suppress the immune response by production of anti-inflammatory cytokines.	FoxP3	IL-10	(Kondělková et al.).

Table 1.5 Subtypes of T-helper cells and their effector functions.

ANCA autoantibodies attack neutrophils and leads to inflammation of small blood vessels. However, neutrophils are not exclusively implicated in AAV. Patients with AAV have increased circulating IFN γ or IL-4 depending on disease state. This has been shown in patients with active localised disease (i.e. in vasculature in upper respiratory tract) have heightened levels of IFN γ , indicating a Th1 mediated response where patients active generalised disease (i.e. systemic multi-organ involvement) have heightened IL-4, indicating a Th2 response (Müller et al. 2000). Furthermore, Th17 cells have also been implicated in the pathogenesis of AAV. Neutrophils produce Th17 effector cytokines IL-17A and IL-23 in response to ANCA autoantibodies which thereby promote Th17 activation and localised autoimmunity in the blood vessel by means of further increased release of Th17 cytokines IL-17, IL-23 and IL-6 (Hoshino et al. 2008). **Chapter 4** of this Thesis will investigate the galectin-3 dependent mechanisms in T cells.

1.6 Summary

The mechanisms underlying the heightened rate of cardiovascular events and morbidities in rheumatoid arthritis is poorly understood. However, the inflammatory response is the principal driver of triggering cardiovascular events. Inflammation in rheumatoid arthritis is not confined to the joint, it is a systemic disease with systemic effects. Targeting these early inflammatory insults is advantageous as it halts the sequence of events that follow and lead to autoimmune responses. Understanding the cells involved in the systemic inflammation, and the mediators released from these cells can allow for early intervention in treating arthritis associated cardiovascular comorbidities. While cardiovascular events are clearly linked with rheumatoid arthritis, the reasons underlying why this occurs is poorly understood. This project investigates the potential mechanisms underlying this by targeting the protein galectin-3 and unravelling the galectin-3 dependent mechanisms involved in vascular inflammation.

1.7 Hypothesis and Aims

The overarching hypothesis of this Thesis is that galectin-3 promotes the inflammatory response to result in vascular inflammation. In order to test this hypothesis, the following aims were set:

1. Investigating galectin-3 inhibition in the collagen induced arthritis mouse model.
 - **Chapter 3** of this Thesis uses a model of inflammatory arthritis that features vascular inflammation, the collagen induced arthritis model, to investigate the role of galectin-3 in vascular inflammation.
2. Evaluating the role of galectin-3 in T cells.
 - The aim of **Chapter 4** of this Thesis was to investigate the galectin-3 dependent mechanisms in CD4 T cells by developing an *in vitro* bioassay to measure CD4 effector responses in response to galectin-3 inhibition.
3. Evaluating galectin-3 inhibition in antigen induced arthritis.
 - **Chapter 5** investigates the effect of galectin-3 inhibition using a T cell driven model of inflammatory arthritis to determine the effect of galectin-3 dependent mechanisms of vascular and joint inflammation in a controlled and well-defined model.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Consumables

All chemicals were purchased from Sigma-Aldrich, unless stated otherwise. All plasticware was obtained from Greiner Bio-One Ltd. Deionised water (dH₂O) from a Millipore Milli-Q system was used to prepare buffers. Roswell Park Memorial Institute 1640 Medium (RPMI) from ThermoFisher was supplemented with 10% (v/v) foetal bovine serum (Corning), 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco), 1 mM sodium pyruvate and 50 µM β-mercaptoethanol. Iscove's Modified Dulbecco's Medium (IMDM; ThermoFisher) was used for Th17 cell cultures and was supplemented with 10% foetal bovine serum (Corning), 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco), and 55 µM (v/v) β-mercaptoethanol.

Galectin-3 recombinant protein (R&D Systems) was dissolved in 0.1% (w/v) Bovine Serum Albumin (BSA). GB1107 (ID: x209; c95D) was supplied directly from Galecto AB, Copenhagen in powder form.

2.1.2 Buffers

Krebs buffer and high potassium Krebs Buffer for myography were made up in 1 litre dH₂O on the day of the experiment (**Table 2.1**). Flow activated cell sorting (FACs) buffer was comprised of 0.5% BSA, 5 mM EDTA and 7.5 mM sodium azide. Magnetic activated cell sorting (MACs) buffer was comprised of 0.5% BSA and 2 mM EDTA. Citrate buffer for immunohistochemistry was made by dissolving 2.94 g tri-sodium citrate in 1 L dH₂O and pH adjusted to 6.0. Tris/EDTA buffer for immunohistochemistry was made by dissolving 1.21 g Tris, 0.37 g EDTA and 0.5 mL Tween20 in 1 L dH₂O, pH 9.0.

Chemical Name	Chemical Formula	Krebs Buffer (g/L)	High Potassium Krebs Buffer (g/L)
Sodium Chloride	NaCl	6.38	2.3
Potassium Chloride	KCl	0.2	4.5
Monopotassium Phosphate	KH ₂ PO ₄	0.16	0.16
Magnesium Sulphate	MgSO ₄ .7H ₂ O	0.3	0.3
Sodium Bicarbonate	NaHCO ₃	2.1	2.1
Glucose	C ₆ H ₁₂ O ₆	1.98	1.98
Calcium Chloride	CaCl ₂ .H ₂ O	0.22	0.22

Table 2.1: Components of Krebs buffer

2.1.3 Primers for quantitative PCR

All TaqMan qPCR primers and probe sets were sourced from Applied Biosystems, ThermoFisher UK.

2.1.4 Antibodies for immunohistochemistry

All antibodies and dilutions used are summarised in **Table 2.2**.

2.1.5 Antibodies for flow cytometry

Antibodies for flow cytometry were purchased from the sources listed in **Table 2.3**.

Gene Name	Assay ID	Species
<i>Lgals3</i>	Mm00802901_m1	Mouse
<i>Il17a</i>	Mm00439618_m1	Mouse
<i>Ifny</i>	Mm01168134_m1	Mouse
<i>Il4</i>	Mm00445259_m1	Mouse
<i>Act6</i>	Mm01205647_m1	Mouse

Table 2.2: List of primers used for qPCR

Target	Antigen Retrieval	Primary Antibody	Dilution and concentration	Isotype Control and stock concentration	Isotype control dilution and final concentration	Secondary antibody	Dilution	Serum Block	Commercial Source
CD3	Citrate buffer	Rabbit anti-human CD3	1:100 (6 µg/mL)	Rabbit IgG (15 mg/mL)	1:2500 (6 µg/mL)	Biotinylated swine anti-rabbit	1:300	Swine (10%)	Dako (A045229-2)
Galectin-3	Tris/EDTA buffer	Rabbit anti-mouse galectin-3	1:250 (2 µg/mL)	Rabbit IgG (1 mg/mL)	1:500 (2 µg/mL)	Biotinylated goat anti-rabbit	1:750	Goat (10%)	Abcam (ab76245)

Table 2.3: List of antibodies for immunohistochemistry

Species	Target	Clone	Stock Concentration	Dilution	Source
Mouse	CD4	RM4-5	0.2 mg/mL	1:200	eBioscience
	CD3	1452c11	0.2 mg/mL	1:200	Biologend
	IFN γ	XMG12	0.5 mg/mL	1:200	eBioscience
	IL-4	11B11	0.2 mg/mL	1:200	eBioscience
	IL-17A	TC11-18H10.1	0.5 mg/mL	1:200	Biologend
	Galectin-3	M3/38	0.2 mg/mL	1:200	Bio-techne
	Foxp3	FJK-16S	0.5 mg/mL	1:200	eBioscience
	Ki67	11F6	0.5 mg/mL	1:200	Biologend

Table 2.4: List of antibodies used for flow cytometry.

Methods

2.2 Myography

Myography was used to assess the vascular constriction responses in aortic rings of mice. A schematic representation of the myograph is outlined in **Figure 2.1**.

Mice were culled by inhalation of a rising concentration of carbon dioxide and death was confirmed by palpitation. The thoracic aorta was perfused with 2 mL Krebs buffer and was carefully dissected from the thoracic cavity before placing in Krebs buffer. Using a dissection microscope, the aorta was placed in a Petri dish containing Krebs buffer and the perivascular adipose tissue (PVAT) was carefully removed using a high precision tweezers (size 6) and a dissecting scissors. Two rings, each 2 mm in length, were cut from each aorta and mounted onto two wire prongs in each bath of the myograph, as shown in **Figure 2.1**. Rings were continuously bathed in oxygenated Krebs buffer at 37°C once mounted onto the myograph. Baseline tension was set at zero millinewtons (mN) for 20 minutes. Following equilibration, tension was manually increased by 0.5 mN every minute until a baseline of 5 mN was set. Rings were maintained at 5 mN for 20 minutes. Smooth muscle cells were then re-activated with 6×10^{-2} M high-potassium (K^+) Krebs solution for 20 minutes. High K^+ Krebs solution was removed by washing in Krebs solution 3 - 4 times until tissues returned to baseline. Following the addition and subsequent removal of 6×10^{-2} M high-potassium K^+ Krebs solution, tissues were bathed in Krebs solution for 20 minutes to allow to re-equilibrate to 5 mN. Tissues were then treated with increasing concentrations of serotonin (5HT) in half log increments (**Table 2.4**). Maximum contraction was noted following each addition of 5HT. Data was analysed using MyoDaq software and GraphPad Prism 9.

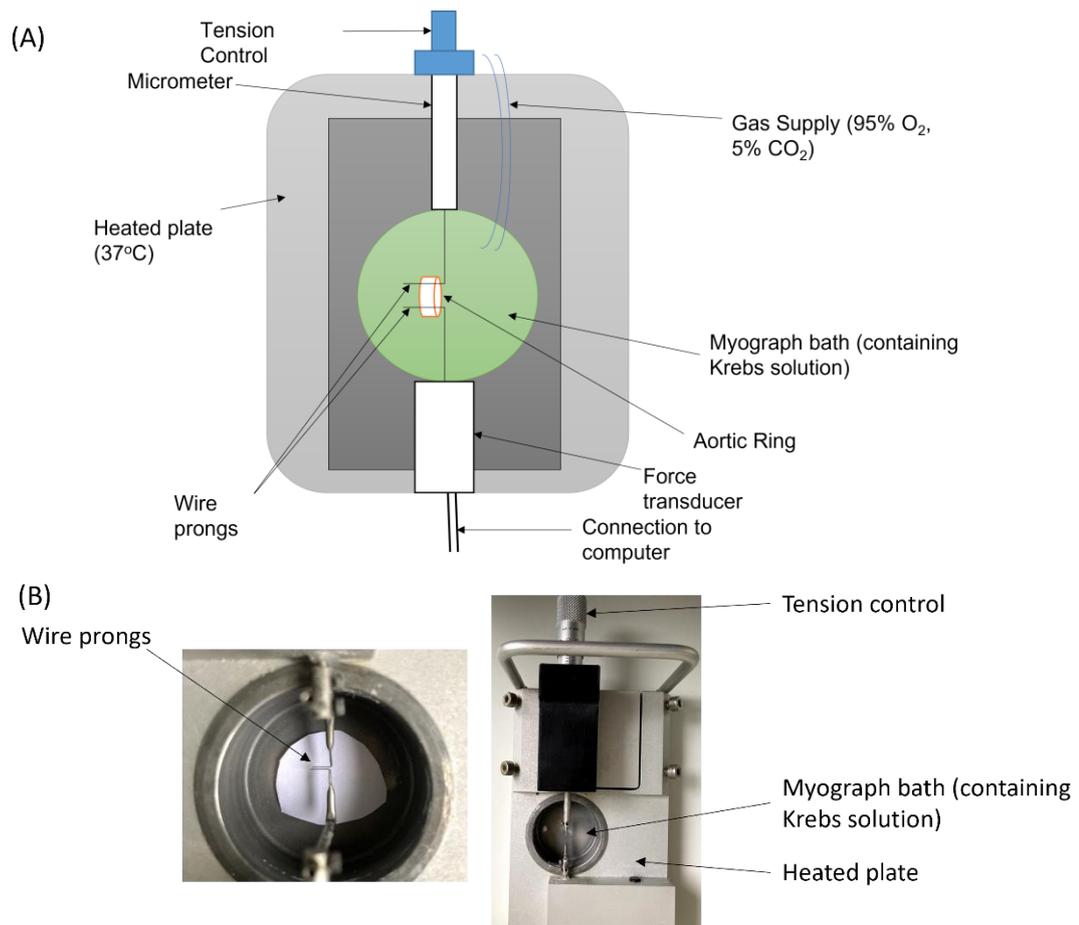


Figure 2.1: Layout of the myograph

(A, B) A representative diagram and a photograph of a wire myograph bath. The wire myograph consists of two wires, on which the aortic ring is situated between. The bath is connected to a force transducer connected to a computer which gives a readout of constriction responses and to a tension control which allows the baseline tension of the aortic ring to be set. Aortic rings were submerged in physiologically relevant Krebs buffer and the bath was supplied with 95% O₂ 5% CO₂ to ensure tissue viability throughout measurement.

Volume to add to bath	Starting Concentration	Final Concentration in bath (mM)
50 μL	1×10^{-7}	1×10^{-9}
100 μL	1×10^{-7}	3×10^{-9}
35 μL	1×10^{-6}	1×10^{-8}
100 μL	1×10^{-6}	3×10^{-8}
35 μL	1×10^{-5}	1×10^{-7}
100 μL	1×10^{-5}	3×10^{-7}
35 μL	1×10^{-4}	1×10^{-6}
100 μL	1×10^{-4}	3×10^{-6}
35 μL	1×10^{-3}	1×10^{-5}
100 μL	1×10^{-3}	3×10^{-5}

Table 2.5: Serial dilutions of 5-HT added to myograph bath

5-HT induces constriction of aortic rings and is added in increments to the organ bath to result in a maximum final concentration of 3×10^{-5} mM.

2.3 Animal Models of Inflammatory Arthritis

2.3.1 Mice

Male DBA/1 mice (Envigo, UK and Charles River, UK) were used for the collagen induced arthritis (CIA) model as they exhibit a high disease incidence of 80-100% (Brand et al. 2007). Wild-type C57Bl/6 mice were used for the antigen induced arthritis model. Mice were purchased at 8 - 9 weeks old and were housed under specific pathogen free (SPF) conditions at Cardiff University for 1 - 2 weeks prior to undergoing procedure. All animals were housed at a stable temperature, on a 12-hour light/dark cycle and with food and water *ad libitum*. For CIA and AIA experiments, mice were kept to 4 per group per experiment to keep within the constraints of the myograph upon harvesting of aortas. Experiments were repeated where necessary if the data was statistically underpowered. A separate control aged-matched group (n=4) was run alongside the experimental groups and was harvested for tissues within 24 hours of the vehicle/GB1107 treated mice.

2.3.2 Induction of Murine Collagen Induced Arthritis

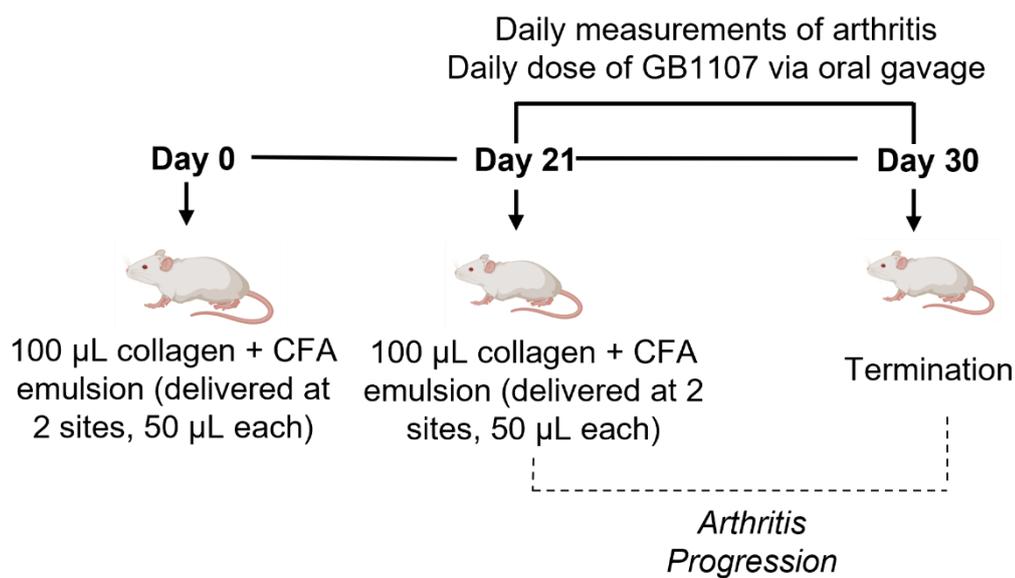
A suspension of Type II collagen from chicken sternum (5 mg/mL) was prepared by adding a 10 mM solution of acetic acid (prepared in deionized water) to lyophilized collagen (Sigma-Aldrich). The collagen was then left to dissolve overnight at 4°C. To prepare complete Freund's adjuvant (CFA), heat-killed *Mycobacterium tuberculosis* (100 mg, strain H37Ra, 231141, BD Biosciences) was ground to a fine powder using a glass mortar and pestle. Using a glass syringe fitted with a 19-gauge needle, 20 mL of incomplete Freund's Adjuvant (F5506, Sigma-Aldrich) was mixed thoroughly with the ground *Mycobacterium tuberculosis*. The CFA suspension (5 mg/mL) was aliquoted and stored at -20°C until needed. On the day of immunizations, an emulsion of collagen in CFA was prepared. A single aliquot of CFA was defrosted and *M. tuberculosis* (*M. tb*) thoroughly resuspended by vortexing. CFA (2.5 mL) was added to an equal volume of the Type II chicken collagen solution in acetic acid. An emulsion (1 mg Type II chicken collagen per 2.5 mg *M.Tb* per mL) was formed by passing the liquid through a glass syringe, fitted with a 19-gauge needle, 20 - 30 times. The emulsion was tested for stability by adding a drop into a petri dish of water. A successful emulsion did not dissociate upon contact with the water and was considered stable when it remained intact.

All experimental procedures were approved by Local Research Ethics Committee and were carried out under the personal licence IC3B6F923 and were performed under the authority of Home Office project licence PF34A3DC8 (and previously PB3E4EE13 to April 2019).

On the day mice were to be immunised (Day 0), male DBA/1 mice were injected with 1 mg/mL Type II chicken collagen emulsion (100 μ L). The injection was administered via the intradermal route in 2 sites on the left side of the base of the tail to minimise adjuvant-induced ulceration. Mice received another identical intradermal injection of 1 mg/mL collagen in CFA on the right side of the base of the tail on Day 21. The procedure timeline is outlined in **Figure 2.2**. Male mice were used as oestrogen is considered as atheroprotective (Arnal and Bayard 2001) and may interfere with the vascular readouts of the experiments. Mice were monitored for the clinical progression of arthritis and examined for wellbeing throughout the 30-day procedure.

2.3.3 Induction of Antigen Induced Arthritis

Antigen induced arthritis (AIA) was induced in 8-week-old male C57Bl/6 mice. On Day -21, mice were immunised with 100 μ L 1 mg/mL methylated bovine serum albumin (mBSA) emulsified in equal volume CFA via subcutaneous injection to the right flank. A CFA emulsion was prepared by passing the liquid through a syringe fitted with a 19-gauge needle 20 - 30 times and tested for stability by adding a drop into a petri dish of water. Mice also received 100 μ L 160 ng heat inactivated *Bordetella pertussis* toxin (p. toxin) via intraperitoneal injection. The administration of mBSA in CFA and p. toxin served to prime an immune response in the mice. One week later, on Day -14, mice received a booster injection of mBSA in CFA via subcutaneous injection on the left flank to further prime the immune response. On Day 0, twenty-one days after the initial injection, inflammatory arthritis was induced by administration of 10 μ L 1 mg/mL mBSA to the knee joint. Mice were monitored daily for wellbeing and for the development of arthritis - as determined by knee swelling – by the measurement of knee diameters using a micrometer - before termination on Day 3 and Day 10 post intra-articular injection. A timeline of the protocol is outlined in **Figure 2.3**.



Paw Score	Pathological Feature
1	Normal (no swelling)
2	Mild/moderate erythema and swelling
3	Severe swelling covering whole paw
4	All joints affected by arthritis
5	Deformed paw/anklyosis

Figure 2.2 Overview of the collagen induced arthritis model

Male DBA/1 mice were administered with Type II collagen in CFA via intradermal injection on Day 0 and Day 21 on the left and right flank respectively. Mice were weighed, monitored and had regular health checks throughout the experiment. Mice were dosed with GB1107 from Day 21 until termination. Arthritis progression was monitored daily from Day 21 until termination by scoring swelling in individual paws, as per the criteria outlined above.

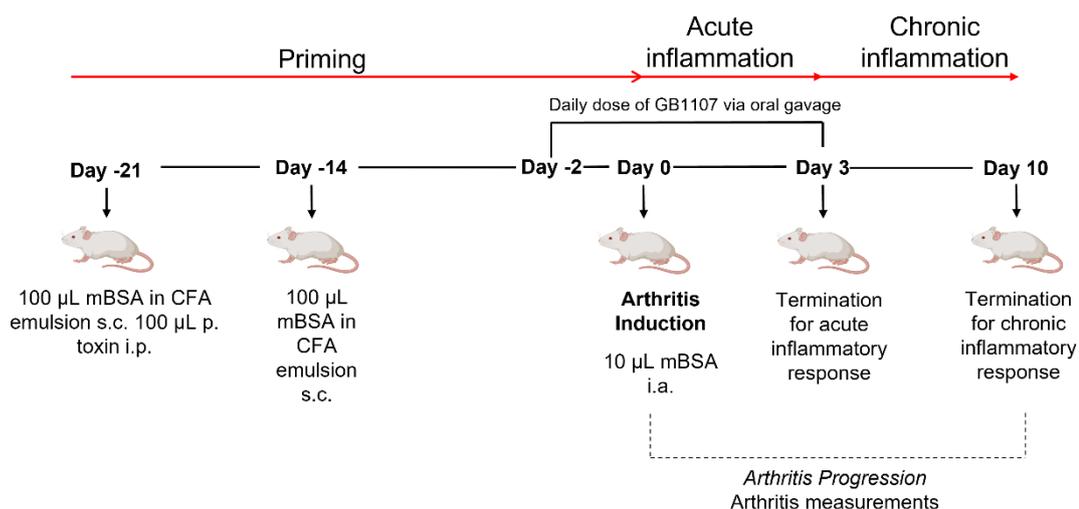


Figure 2.3: Overview of the antigen induced arthritis protocol

Administration of mBSA in CFA at Day -21 and Day -14 and pertussis toxin at Day -21 primes the immune response and establishes an antigen specific T cell and antibody specific response to mBSA in the absence of synovitis. Inflammatory arthritis is initiated on Day 0 by intra-articular (i.a.) injection of mBSA to the knee joint which is followed by an acute inflammatory response. The chronic-like inflammation phase features a T cell effector and B cell infiltration. Joint swelling is measured on Day 0, 1, 2, 3 and 10 post i.a. injection and GB1107 is administered via oral gavage from Day -2 to Day 3. There are two termination timepoints in the AIA protocol – Day 3 and Day 10.

2.3.4 Administration of therapeutic modalities during CIA and AIA

The CIA and AIA mouse models were used to test novel therapies. Small group sizes were used (n=4 per group) to enable the optimal use of the myograph upon termination. Experiments were run minimally in duplicate and resource equations were used to estimate the number of mice required to show significant differences in clinical scores and constriction responses between groups.

Vehicle Formulations

GB1107, a small molecule inhibitor of galectin-3, was administered by daily oral gavage. Initial experiments used 10% propylene glycol in 0.5% HPMC as vehicle for GB1107. Briefly, 50 mg HPMC was dissolved in 10 mL hot water (75°C) to make a 0.5% solution. 10 mg GB1107 was added to 1 mL propylene glycol to create a 10 mg/mL solution and this was then diluted by adding 9 mL 0.5% HPMC to create a 1 mg/mL final working solution. Later experiments used 10% solutol in PEG300 as vehicle. Solutol is solid at room temperature and so was melted at 37°C until it reached a liquid state and a 100 mL aliquot was taken. The solutol was autoclaved at 121°C to ensure sterility. 1 mL solutol was added to 9 mL PEG300 to create a 10% solutol:90% PEG300 solution. This vehicle was incubated at 37°C and thoroughly mixed until a homogenous solution formed. 20 mg GB1107 was added to this solution and mixed thoroughly to create a 2 mg/mL stock solution of GB1107 and the solution put back into the water bath until a fully homogenous solution was formed. The drug suspension was then aliquoted to volumes required per day of dosing and each aliquot was discarded at the end of each day. Mice were dosed with GB1107 via oral gavage from Day 21 in the CIA model or from Day -2 in the AIA model until termination.

2.3.5 CIA and AIA Assessments

A macroscopic assessment of arthritis was made by measuring individual paw diameters (hind paws only), paw scores (for each paw) and clinical scores (combined score from all four paws) were measured daily from Day 21 until termination in the CIA model. Scoring was determined by pathological features observed in the paws, as outlined in **Figure 2.2**. Swelling was assessed in the AIA model by measuring knee diameters on Day 0 (prior to the induction of arthritis), Day 1, 2, 3, 7 and 10 .

2.3.6 Tissue Harvest

Animals were culled by inhalation of a rising concentration of carbon dioxide, and death was confirmed by palpitation. The thoracic aorta was harvested by dissection. The synovium was harvested from the AIA model by creating an incision at the patella ligament and peeling back to expose the synovium. The synovium was collected using a fine tweezers and placed into RNAlater for qPCR analysis, or cRPMI for flow cytometry analysis. Tissues harvested for histology were fixed in 10% neutral buffered formalin saline for 48 hours before transferring to 70% ethanol for long-term storage. The thoracic aorta was used for myography, as outlined in **Section 2.2**. Blood was collected by cardiac puncture and was transferred to a vacutainer coated with the calcium chelator EDTA (BD Biosciences) to prevent coagulation. Whole blood was kept on ice before separation of erythrocytes, leukocytes and platelets from plasma by centrifugation at 2000 x g for 10 minutes at 4°C. The pellet was discarded and supernatant stored as plasma at -20°C.

Digestion of Synovium for flow cytometry

Synovium was isolated from inflamed joints as outlined in **Section 2.3.6** and placed in 10 mL cRPMI containing 1 mg/mL Type IV Collagenase (Worthington, Biochemicals). The tissue underwent enzymatic digestion in collagenase at 37°C for 2 hours, vortexing every 10 minutes. Cells were passed through a 40 µM cell strainer and washed with cRPMI before centrifuging at 400 x g for 5 minutes. Cells were then seeded in a 96 well plate and stained for flow cytometry (**Section 2.11**).

Isolation of Inguinal Lymph Nodes

Inguinal lymph nodes were identified and dissected from the mouse before mashing and passing through a 40 µM cell strainer to form a single cell suspension. Cells were centrifuged and resuspended in cRPMI before seeding onto a 96 well plates for flow cytometry analysis (**Section 2.11**).

2.4 Ex Vivo Myography

Ex vivo myography experiments were carried out in healthy male DBA/1 mice to determine individual contributions of galectin-3 to vascular constriction responses. DBA/1 mice were terminated by Schedule one method and thoracic aortas were harvested and mounted on the myograph. Baseline tensions were set according to **Section 2.2**. Aortic rings were incubated with either 1 ng/mL galectin-3 in 0.1% (w/v) carrier protein bovine serum albumin (BSA) or 0.1% (w/v) BSA only for 2 hours prior to measuring constriction responses to 5HT.

2.5 Enzyme Linked Immunosorbent Assay (ELISA)

2.5.1 Sandwich ELISA

Sandwich enzyme-linked immunosorbent assay (ELISA) was used to detect the concentration of cytokines in plasma from mice with inflammatory arthritis, and also to detect the concentration of cytokines releases from cells in *in vitro* cell cultures. All kits were sourced from R&D Systems and performed according to the manufacturer's instructions. Briefly, high-binding plates were coated overnight with capture antibody before non-coated areas of wells were blocked with 1% BSA to avoid non-specific binding. This was followed by addition of sample and known concentrations of the analyte of interest (protein standards). For detection, a biotinylated detection antibody was followed by the use of horseradish peroxidase (HRP)-conjugated streptavidin. The chromogenic substrate 3,3',5,5'-Tetramethylbenzidine (TMB) was added to each well to quantify IgG binding to antigen and the plate kept in the dark at room temperature until a colour change was observed. Stop solution (25 μ L; Seracare, UK) was added to terminate the enzyme substrate reaction and the absorbance was measured at 450 nm using a FLUOstar Omega Microplate Reader. A standard curve was prepared using the OD450 nm readings from the protein standards. Interpolation of the standard curve gave the concentration of protein of interest in each sample.

2.5.2 Direct ELISA

Antigen specific responses to mBSA were determined by measuring mBSA-specific IgG titres in plasma from mice with AIA. High binding plates were coated with 5 μ g/mL mBSA in PBS overnight at room temperature before washing in PBS Tween20 (0.05% v/v) and blocking in 5% (w/v) milk extract in PBS Tween20 for 1 hour at room temperature. Plates

were washed and plasma samples were diluted 1/100 – 1/100000 in 5% (w/v) milk extract in PBS and added to the plates at room temperature for 2 hours. mBSA specific antibodies were detected by addition of a horseradish peroxidase conjugated to goat anti-mouse IgG (0.5 mg/mL, 2 hours, Dako). The chromogenic substrate 3,3',5,5'-Tetramethylbenzidine (TMB) was added to each well to quantify IgG binding to antigen and the plate kept in the dark at room temperature until a colour change was observed before adding stop solution and measuring absorbance (450 nm) using FLUOstar Omega plate reader (BMG Labtech).

2.6 Multiplex Assays

LEGENDplex assay kits (Biolegend, UK) were used to probe for multiple cytokines simultaneously from the same sample. The procedure was performed following the manufacturer's instructions, where plasma samples were incubated with mixed beads, detection antibodies and assay buffer to create a sandwich-based assay. A standard curve was constructed using "matrix B" and mixed beads and standards of known concentrations from the kit. The mixed beads contain beads of varying size and fluorescence that are conjugated to a specific antibody. All samples were subsequently incubated with PE fluorochrome which bound to each detection antibody. Samples were then run on a flow cytometer on a low flow rate and 4000 events were recorded for each sample. Analytes in each sample were distinguished based on their size (according to forward and side scatter) and fluorescent intensity. Data was exported in the form of FCS files and analysed using the LEGENDplex software provided.

2.7 Histology

2.7.1 Tissue processing

Thoracic aortas harvested for immunohistochemical analysis were fixed in 10% non-buffer formalin saline (NFBS; Merck) for 48 hours to preserve tissue structure prior to being transferred to 70% ethanol for long term storage. Tissues were processed in the Histology Suite, Bristol University. Tissues were processed through increasing concentrations of methanol to dehydrate the tissue and allow for the infiltration of wax to the tissue and xylene to remove any residual methanol. Tissues were then permeated with paraffin wax. Details of the cycles are outlined in **Table 2.5**.

Reagent	Length of cycle (minutes)
70% methanol (v/v)	90
90% methanol (v/v)	90
100% methanol (v/v)	60
Xylene	90
Xylene	120
Xylene	120
Wax	120
Wax	90
Wax	90
Wax	60

Table 2.6: Tissue processor cycles for soft tissue samples

2.7.2 Sectioning

Processed samples were embedded in paraffin wax to make blocks at Bristol University Histology Suite. Aortas were embedded perpendicular to the histology cassettes to allow for transverse section of the aorta to be cut. Tissues were sectioned at 7 μ M thick using a microtome and placed onto a SuperFrost Microscope slide (ThermoFisher). Slides were incubated at 60°C overnight to remove any excess wax and stored in boxes until staining.

2.8 Immunohistochemistry

Formalin-fixed paraffin-embedded (FFPE) tissue sections were deparaffinised by immersion in three changes of xylene (5 minutes each) and rehydrated in 100%, 90% and 70% ethanol for 5 minutes before washing in dH₂O for 5 minutes. Antigen retrieval was performed by placing slides in 10 mM citrate buffer; pH 6.0 at 95°C for 40 minutes to break crosslinking of proteins and therefore unmask antigens. Citrate buffer acts as calcium chelators, therefore breaking protein bonds and revealing antigens for antibody binding. The higher the temperature, the more effective the unveiling of antigens is.

Slides were incubated with 3% hydrogen peroxide for 10 minutes at room temperature to block endogenous peroxidase activity. Serum diluted in TBS/T was added to each slide and incubated at room temperature for one hour to prevent non-specific antibody binding. Each slide contained 2 sections and so one section was stained for the antigen of choice and the other was stained with an isotype control. Slides were incubated with primary antibody in TBS/T at 4°C overnight to allow antibody-antigen binding. After removal of the primary antibody by washing in TBS/T, biotinylated secondary antibodies were used to immunolabel target antigens. Visualisation of antibody labelling was achieved by using Vectastain ABC kit and DAB (3'3-diaminobenzidine) staining kit (Vector Laboratories). DAB added to the sections and forms a precipitate at sites of antibody binding, which results in the development of brown staining. The development of specific DAB staining to the sites of antibody binding was monitored under a light microscope (approx. 2-3 minutes) and then stopped by immersion in water. After antigen detection, tissue sections were counterstained with Harris haematoxylin for 10-15 seconds before washing in water to remove excess stain. Sections were then dehydrated by immersion in 70%, 90% and 100% ethanol followed by three changes of xylene (all 5 minutes each). Slides were finally mounted in DPX.

2.8.1 Quantification of IHC Staining

Sections were visualised using a Leica DM 2000 microscope and Leica Application Suite v4.9 software. Positive staining was quantified by ImageJ software, which quantifies the area of staining based on the number of DAB positive pixels. The protocol used was based on enhancing contrast to allow the software to detect and quantify the number of DAB-positive pixels.

2.9 *In vitro* T cell cultures

2.9.1 Isolation of murine CD4⁺ T cells

96 well U-bottomed plates were coated in 1 µg/mL anti-CD3 antibody (clone 145 2c11, R&D Systems) for 24 hours at 4°C prior to seeding cells. Spleens were harvested from wild type (WT) C57Bl/6 male mice aged 9 - 14 weeks. Single cell suspensions were prepared by homogenising spleens in a small Petri dish in media. Tissue was transferred to a 40 µM cell strainer and washed through with media. Cells were centrifuged at 400 x g for 5 minutes at 4°C. Red blood cells were lysed by treating with 5 mL red blood cell lysis buffer (BioLegend, UK) according to manufacturer's instructions. Splenocytes were isolated by centrifugation and the pellet resuspended in 400 µL MACS (0.5% BSA and 2 mM EDTA) buffer. The isolation of CD4 cells was performed using T cell isolation kit (Miltenyi Biotec, UK) according to manufacturer's instructions. Briefly, any non CD4 cells were labelled with a biotin antibody cocktail conjugated to magnetic microbeads. The cell suspension was passed through a magnetic column (Miltenyi Biotec) which allowed for the attachment of biotin labelled cells (i.e., any cell not expressing the CD4 antigen on their surface) to the magnet and CD4 cells were passed through the column to a fresh collecting tube. The use of untouched selection was essential as it prevents the binding of antibodies to the cell, which can result in premature cell activation and proliferation. The CD4 cell suspension was passed through a fresh magnetic column to ensure a CD4 pure population. Cells were centrifuged and the pellet resuspended in 1 mL cRPMI.

2.9.2 Cell Count

10 µL cell suspension was added to 90 µL cRPMI and 10 µL of this dilution was added to a glass haemocytometer, with a cover slip placed over the cell suspension at an angle to minimise air bubbles. The cells were counted in four of the 3x4 squares under a microscope and an average cell count was calculated. The average cell count (cells/mL) was multiplied by the dilution factor and by 10⁴, to account for the surface area of the haemocytometer.

2.9.3 Differentiation of CD4 T cells

Plates coated with anti-CD3 in PBS were aspirated to remove PBS before the addition of purified CD4 T cells. Cells were cultured at 1×10^5 cells per well in complete RPMI or complete IMDM followed by media containing a cytokine combination to model the conditions required for the differentiation of CD4 T cells to Th1, Th2, Treg and Th17 cells respectively (as outlined in **Table 2.6**). Th0 cells were included as controls and are defined as cells that were TCR activated but were not differentiated to an effector subset. For T cell activation, in addition to plate-bound anti-CD3 - which activates the TCR complex - cultures also contained soluble anti-CD28 (5 $\mu\text{g}/\text{ml}$; clone 37.51, eBioscience) to provide secondary activation and promote T cell proliferation. Cells were incubated at 37°C 5% CO_2 until harvest. An overview of T cell cultures is outlined in **Figure 2.4**.

2.9.4 Addition of GB1107 to cultures

For *in vitro* studies, GB1107 was dissolved in DMSO to form a 10 mM concentration. GB1107 was then further diluted in IMDM media to the desired concentration.

T Cell Subset	Media	Cytokine(s)	Concentrations
Th1	cRPMI	IL-12	20 ng/mL
Th2	cRPMI	IL-4	40 ng/mL
Treg	cRPMI	TGF β	5 ng/mL
Th17	cIMDM	TGF β	1 ng/mL
		IL-6	20 ng/mL
		IL-23	20 ng/mL
		IL-2 Antibody	10 $\mu\text{g}/\text{mL}$

Table 2.7: Required cytokines and concentrations for T cell cultures.

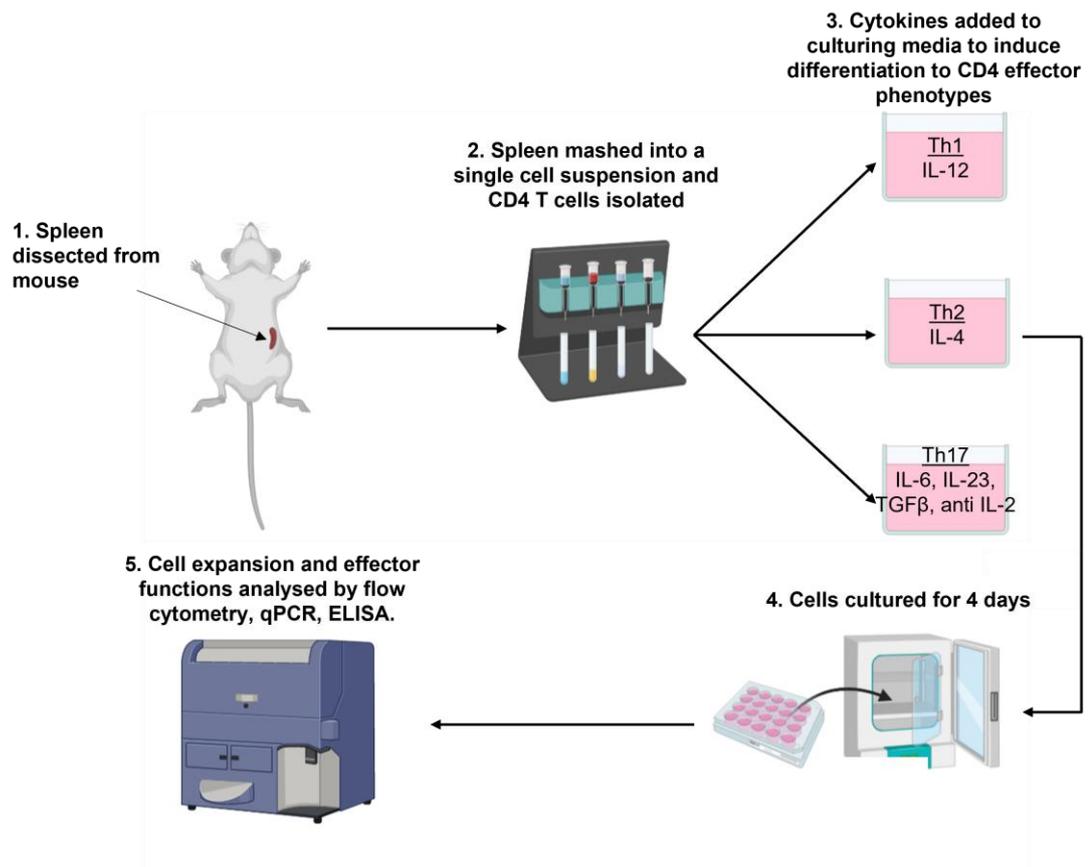


Figure 2.4: Overview of T cell cultures

Spleen was harvested from male C57Bl/6 mice and CD4 T cells isolated by MACs separation. Cells are then cultured in medium containing cytokine combination to promote differentiation to a Th1, Th2 or Th17 phenotype for 4 days at 37°C before analysing expansion and effector functions by flow cytometry. Images obtained from BioRender.com.

2.10 Quantitative Polymerase Chain Reaction (qPCR)

2.10.1 RNA Isolation from cells

RNeasy Mini kit (Qiagen) was used to isolate RNA from lysed cells. CD4 cells were harvested in 250 μ L lysis buffer containing 1% (v/v) β -mercaptoethanol after 48 hours in culture. RNA was isolated and purified using the RNeasy Mini kit (Qiagen). Total RNA was extracted following manufacturer's instructions (Qiagen) and RNA was eluted in 20 μ L RNase free water. RNA quantity and purity was quantified by adding 1 μ L RNA to the spectrophotometer, NanoDrop 2000 (ThermoFisher), measured by reading absorbance at 260 nm and 280 nm and a ratio of the two was defined. A 260/280 ratio of approx. 2.0 is considered pure for RNA.

2.10.2 RNA Isolation from tissues

Synovium, thoracic aortas and thoracic PVAT was harvested from AIA experiments and stored in RNAlater at -80°C . Tissues were defrosted on ice and weighed. Following weighing tissues were placed in TRIzol to maintain RNA integrity and disrupted using a handheld homogeniser. Chloroform was added to the homogenized tissue in TRIzol and centrifuged 16000 x g for 15 minutes resulting in a phase separation of RNA. The top aqueous layer was removed and washed in 100% ethanol. From this, the RNA-ethanol mix was added to columns from miRNeasy micro isolation kit (Qiagen) and further steps followed as per the manufacturers instructions. RNA was quantified using Nanodrop 2000 (ThermoFisher).

2.10.2 Complementary DNA (cDNA) Synthesis

Complementary DNA was reverse transcribed from isolated RNA, using a random oligonucleotide primer and dNTP mix (Precision nanoScript 2 Reverse Transcription kit; PrimerDesign and a C1000 Touch thermocycler (BioRad)). Briefly, total RNA was incubated with 1 μ L reverse transcriptase primer, RNase/DNase free water and sufficient RNA sample, so that all samples had 500 ng RNA, at 65°C for 5 minutes. Samples were then incubated with 10 μ L master mix containing nanoScript 4X buffer, 10 mM dNTP mix, RNase/DNase free water and nanoScript 2 enzyme at 25°C for 5 minutes, followed by 42°C for 20 minutes, followed by 75°C for 10 minutes. cDNA was stored at -20°C until use for qPCR.

2.10.3 Quantitative Polymerase Chain Reaction (qPCR)

Gene targets for identification of T cell subsets and galectin-3 was amplified using Taqman Gene Expression Assays (Applied Biosystems, ThermoFisher). Taqman assays are composed of two primers and two fluorescent probes, of which is cleaved upon interaction with the

primer on the target sequence of cDNA, releasing fluorescence. The amount of fluorescent signal intensity is directly proportional to the amount of RNA. To achieve this, a 9 μL master mix was made comprised of 5 μL Taqman fast advanced master mix, 0.5 μL Taqman primer for the gene of interest and 3.5 μL RNase/DNase free water. The 9 μL master mix was added to 1 μL cDNA sample. Gene expression was quantified by measurement of fluorescence by QuantStudio 12K Flex Real-Time PCR (Applied Biosystems) and using the comparative delta Ct method. All data was normalised against housekeeping gene *Actb*. The delta Ct method calculates the fold change in gene expression, relative to an internal control “housekeeping” gene.

2.11 Flow Cytometry

Flow cytometry is commonly used to characterise and quantify heterogenous cell populations by labelling cellular proteins or receptors with a specific antibody that is conjugated to a single fluorochrome. The cell population is passed through a laser which hits the fluorochromes and excites them, therefore increasing their emission of light. This is then picked up by detectors that can determine the size of the cell – as measured by forward scatter - and the intracellular complexity of the cell – as measured by side scatter - of the deflected light beam. The level of excitation of the fluorochrome translates to a specific wavelength, which then allows the user to identify the phenotype of the cell (Adan et al. 2017).

2.11.1 Intracellular Staining Protocol

Cytofix/Cytoperm solution (BD) was used to permeabilise cell membranes for intracellular staining. Cells were stimulated with 50 ng phorbol myristate acetate (PMA) and 5 μg Ionomycin (to activate intracellular pathways therefore increasing production of cytokines) in the presence of 30 μM Monensin (which blocks golgi transport, therefore inhibiting the transfer of cytokines and proteins to the outside of the cell) for 4 hours at 37°C 5% CO₂. Wells were stained with 25 μL Live/Dead Zombie Aqua (diluted 1:500 in PBS) and incubated at 4°C for 5 mins. Cells were centrifuged at 500 x g for 3 mins at 4°C and resuspended in 50 μL FACs buffer (0.5% BSA, 2 mM EDTA and 7.5 mM sodium azide) containing 4 $\mu\text{g}/\text{mL}$ Fc block (BD, UK) and incubated in the fridge for 20 minutes. Wells were incubated with 50 μL master mix containing antibodies for target surface antigens for 30 minutes at 4°C. Cells were washed twice with 200 μL FACs buffer and centrifuged at 500 x g for 3 minutes. Cell membranes were permeabilised with 100 μL Cytofix/Cytoperm solution and incubated at 4°C for 30 mins. Cells were washed twice with 200 μL PermWash, diluted 1 in 10 in water,

and centrifuged at 500 x g for 3 minutes. All antibodies for intracellular staining were diluted in 1X Permwash and 50 μ L of the antibody mix was added to each well. Cells were washed with Permwash (BD) thereafter and were fixed with 1X Cellfix solution (1 in 10 in water; BD) to denature proteins to avoid degradation of the sample prior to running through the cytometer.

2.11.2 Cytometer Set-Up

Compensation is essential when analysing flow cytometry data to exclude spectral overlap between fluorochromes. This was calculated by the addition of a single fluorochrome antibody to a protein that is constitutively expressed on the cells (e.g., CD4 on Th cells) in separate wells. Gates were applied to the positively stained population and compensation was calculated by the cytometer. A single unstained sample was run to determine the side scatter (SSC) and forward scatter (FSC) of the cells. Once the cells were identified, the SSC and FSC were applied to all samples. The samples were recorded at 50,000 events and at a medium flow rate. All data was analysed by FlowJo Software.

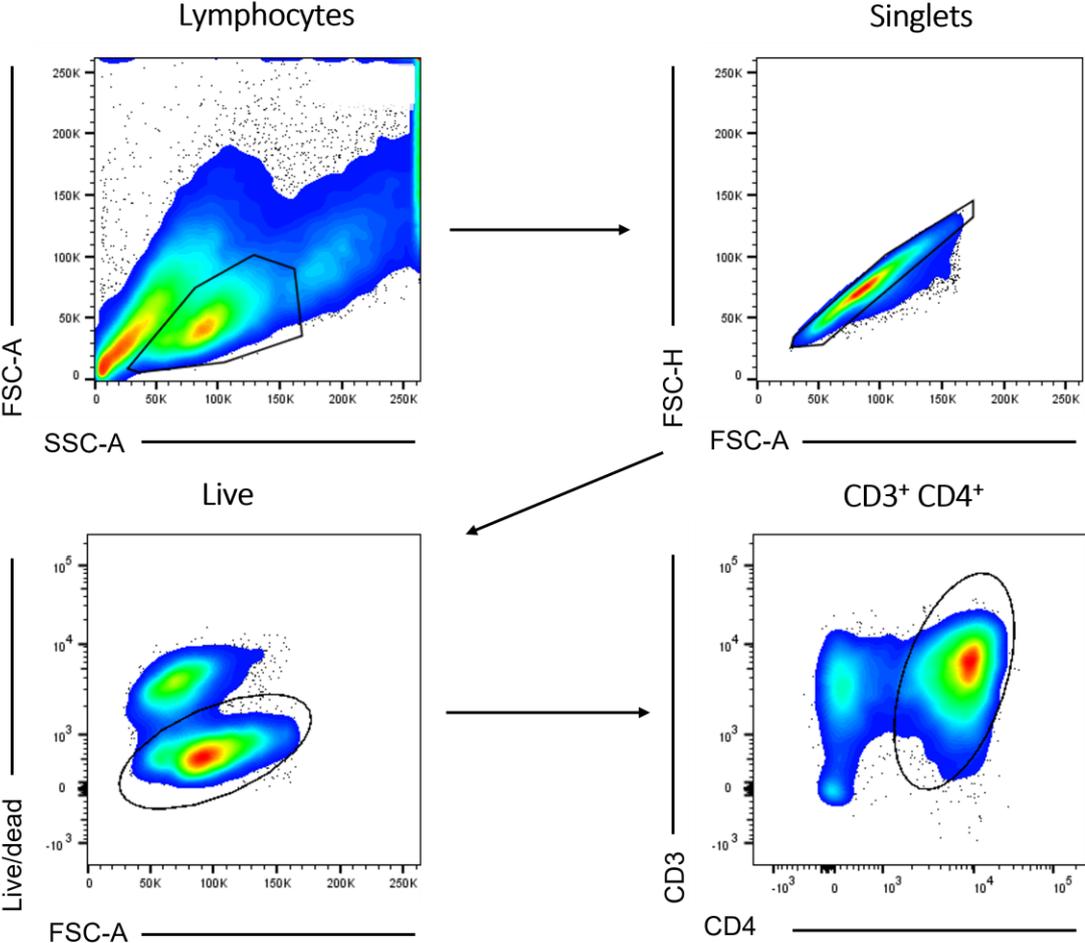


Figure 2.5: Gating strategy for flow cytometry

Cells were gated based on forward scatter and side scatter profiles of lymphocytes, followed by exclusion of doublets. Live cells were gated on by selecting cells that were negative for live/dead Zombie Aqua viability stain (Biolegend) before identifying CD3⁺CD4⁺ cells.

2.12 Statistical Analysis

For *in vivo* experiments where no preliminary data was available, pilot experiments were carried out using small n numbers using the resource equation (below). Following the generation of preliminary data, power calculations were performed using G*Power (<http://www.gpower.hhu.de/>) to calculate appropriate group sizes.

$$\text{Resource equation: } E = N - T;$$

where N = total number of animals, T = number of groups and E must be between 10 and 20.

Flow cytometry data was analysed using FlowJo Software Version 10 (FlowJo LLC, USA). Figures and statistical analysis were made using GraphPad Prism 9 (GraphPad Software Inc. La Jolla, USA). Studies comparing two groups that were normally distributed used a students t-test to determine statistical significance. For data that was not normally distributed, a Mann-Whitney U-test was performed. To test for statistical significance across multiple groups, a one-way ANOVA with Tukeys post test was used for normally distributed data. For comparing multiple groups across multiple timepoints or concentrations, a two-way ANOVA was used with a Sidak post-test. A $p < 0.05$ was considered statistically significant and graphs are shown as mean \pm standard error of the mean (SEM).

Chapter 3

Investigating galectin-3 inhibition in collagen induced arthritis

3.1 Introduction

Collagen-induced arthritis (CIA) is an excellent model to investigate the relationship between joint inflammation and cardiovascular co-morbidities because of its systemic nature. In addition, many of the pathophysiology and molecular mechanisms that underpin systemic inflammation in rheumatoid arthritis (RA) are also observed in CIA. For example, CIA and RA are polyarticular, possess comparable joint pathology (e.g., immune cell infiltration and pannus formation), and both display circulating markers (e.g., ACPAs, anti-CCPs, anti-type II collagen antibodies, and pro-inflammatory cytokines such as TNF α) (El Shikh et al. ; Förster et al. ; Schurgers et al. 2011; Jones et al. 2018). Furthermore, RA has a defined preclinical and clinical phase of disease (**Section 1.1**), and CIA is also considered to model both phases of RA. The “preclinical phase” is defined as the initial 20 days of the CIA protocol whereby mice develop a systemic immune response against collagen, but mice do not develop macroscopic arthritis. In our group, the “clinical phase” of CIA occurs after a second intradermal injection of collagen in CFA on Day 21 which boosts the immune response and results in the development of macroscopic arthritis (Brand et al. 2007). While cardiovascular disease and vascular inflammation have been studied during the clinical phase of CIA, the vasculature and early inflammatory mechanisms occurring in the preclinical phase of CIA are poorly understood.

The chronic articular inflammation in CIA leads to inflammation of the blood vessels and alterations to the function of these blood vessels (Reynolds et al. 2012; Palma Zochio Tozzato et al. 2016). Arthritis associated vascular inflammation and dysfunction is not unique to major blood vessels. In RA for example, inflammation of the blood vessels is characterised by heightened expression of angiogenic factors (e.g., VEGF) and changes in synovial blood vessel density (Paleolog 2009) and the circulating markers found in the blood of RA patients are also present in the joint. Cytokines such as TNF α , IL-6, and IL-17 play a role in endothelial cell activation in synovial blood vessels which promotes inflammation, leading to pannus formation (Urman et al. 2018). The synovium becomes hypoxic during RA which promotes angiogenic processes, resulting in a dysregulated process by which there is increased synovial blood vessels secreting pro-inflammatory cytokines and degrading proteases (e.g., MMP-9) leading to subsequent joint destruction (Paleolog 2002).

The mechanisms underlying vascular dysfunction in arthritis have yet to be elucidated but the CIA model has shown that the alterations to blood vessels may be due to enhanced

levels of inducible nitric oxide synthase (iNOS) which in turn results in decreased availability of nitric oxide (NO) (Palma Zochio Tozzato et al. 2016). Furthermore, MMP9 has been shown to play a role in defective vascular constriction responses in CIA (Reynolds et al. 2012). A rat model of adjuvant-induced arthritis with polyarthritis has shown that alterations to the vasculature may also be partly due to enhanced NADPH oxidase leading to the production of intracellular ROS (Haruna et al. 2006) and the sustained release of pro-inflammatory cytokines such as TNF α and IFN γ (Sprague and Khalil 2009). In CIA, these mechanisms are exhibited as decreased responsiveness of the thoracic aorta to vasoconstrictor serotonin (5HT), resulting in attenuated ability of the blood vessel to constrict. This has been reproducibly shown in our lab (Reynolds et al. 2012; Williams et al. 2016; Sime et al. 2017). The attenuated constriction response observed in CIA gives the model a functional readout of vascular inflammation, and it can be used to assess the effectiveness of pharmacological interventions that target the vasculature during inflammatory arthritis.

Galectin-3 has been identified as a prognostic marker of heart failure (Lok et al. 2010) and is elevated in many systemic inflammatory diseases as discussed in **Section 1.4.2**. Elevated galectin-3 is present in animal models of inflammation such as autoimmune experimental encephalitis (EAE) (Jiang et al. 2009), in fibrotic cardiomyopathy (Nguyen et al. 2018), and in CIA (Wang et al. 2010). Knockdown of the galectin-3 gene attenuated joint pathology in antigen induced arthritis (AIA) (Forsman et al. 2011) as measured by decreased synovitis, decreased bone erosion, and decreased levels of proinflammatory cytokines, TNF α and IL-6. Galectin-3 is expressed in vascular smooth muscle cells and has been identified as a potential modulator of pulmonary artery remodelling in a rat model of pulmonary hypertension (Barman et al. 2019) as it inhibited apoptosis, leading to fibrosis and arterial stiffening. Moreover, in patients with RA, raised serum levels of galectin-3 correlated with vascular stiffness, atherosclerosis and myocardial contractility (Anyfanti et al. 2018). Therefore, galectin-3 plays a critical role in RA-driven tissue damage and represents a potential target for intervention in inflammatory diseases. However, pharmacological inhibition of galectin-3 has not been tested in RA, or in RA associated comorbidities.

Galecto Biotech is a pharmaceutical company based in Copenhagen, Denmark and they specialise in the production of specific galectin-3 inhibitors. Galecto manufacture galectin-3 inhibitors that inhibit both intracellular and extracellular galectin-3. One of these inhibitors is GBO139, an inhaled small molecule inhibitor of galectin-3. GBO139 entered Phase IIb clinical trials for Idiopathic Pulmonary Fibrosis (IPF) in 2019. Galecto Biotech also have two

more galectin inhibitors, GB1211 and GB2064 in Phase I/IIa trials for NASH and myelofibrosis, respectively. Galecto Biotech provided us with a unique opportunity to use a galectin-3 inhibitor, GB1107, as a research tool to block galectin-3 function. Previous studies using GB1107 showed decreased tumour size and increased tumour M1 macrophage polarisation and CD8⁺ T cell infiltration in a mouse model of non-small cell lung cancer (Vuong et al. 2019). GB1107 also showed potency in inhibiting both intracellular and extracellular galectin-3 (Stegmayr et al. 2019). In this study by Stegmayr *et al*, GB1107 showed a strong affinity for galectin-3, and high permeability for crossing cell bilayers, both in the apical to basolateral direction and the basolateral to apical direction of a Caco-2 cell monolayer. These two studies showed that GB1107 has good bioavailability and efficacy in an *in vivo* and *in vitro* model.

Unpublished studies performed in our laboratory (Identification of targets for the prognosis and/or treatment of vascular pathology associated with inflammatory arthritis. Dr Katherine Sime. Ph.D. thesis. Cardiff University ORCA reference: <http://orca.cf.ac.uk/id/eprint/121975>) evaluated GB1107 blockade of galectin-3 function in the CIA model. Mice with CIA were administered 10 mg/kg GB1107 once daily from Day 21 to Day 30 of the CIA protocol resulting in:

- Delay of onset and attenuated arthritis in mice treated with GB1107, by measurement of paw diameters and clinical scores.
- Decreased circulating galectin-3 and decreased tissue levels of galectin-3 in the thoracic PVAT and thoracic aorta.
- Decreased osteoclast number in the hind paws of mice treated with GB1107.
- Decreased macrophage infiltration to the thoracic aorta during CIA.
- Partially restored constriction responses in mice treated with GB1107.
- Significant decrease in circulating pro-inflammatory cytokines IFN γ , GM-CSF, and IL-17A.

These results suggest novel roles for galectin-3 in arthritis progression and highlight the translational potential of targeting this pathway for patient treatment. These studies showed a reduction in disease activity by delayed onset and decreased joint pathology by a reduction in immune cell infiltration and partially restored vascular constriction responses. However, while these results indicate that galectin-3 may play a role in arthritis-associated

vascular inflammation, they did not show complete resolution of joint disease or full restoration of constriction responses. In this Thesis, GB1107 can be used as a tool to inhibit galectin-3 and understand its role in vascular inflammation. However, to do this, GB1107 must reach a systemic concentration high enough to fully inhibit galectin-3 in collagen induced arthritis.

Hypothesis and Aims

The overarching hypothesis for this Chapter is that galectin-3 is a critical mediator in vascular inflammation associated with inflammatory arthritis. To test this hypothesis, the following aims were set:

Aim 1: To investigate the function of galectin-3 in collagen induced arthritis, a polyarticular systemic chronic progressive disease.

Aim 2: To investigate the function of galectin-3 in the preclinical phase of inflammatory arthritis.

3.2 Results

3.2.1 Establishing the collagen induced arthritis model

Collagen induced arthritis was induced in 8-week-old male DBA/1 mice and reached an incidence of 80 – 100% (**Figure 3.1 A**). Macroscopic arthritis developed from Day 24 of the model and reached an incidence of 85% by termination on Day 29. This incidence is in line with previous studies conducted in the laboratory. Furthermore, the progression of arthritis was monitored from Day 21 to Day 29 by measurement of paw diameter, and each paw was scored based on swelling, and the number of joints affected. Paw diameter steadily increased from Day 25 to Day 29 with a baseline of 1.9 mm on Day 21 prior to the development of arthritis and 2.4 mm upon termination on Day 29 (**Figure 3.1 B**). The combined score of each paw gave a total score, termed as clinical score. The average clinical score upon termination was 6 (**Figure 3.1 C**), which is also in line with published data (Brand et al. 2007). The incidence and progression of arthritis, as determined by paw diameter and clinical score, followed the same time-course as published data as shown by increasing clinical score from Day 24 to termination on Day 29.

Aortic constriction responses to serotonin during CIA provide a good indicator of vascular dysfunction associated with inflammatory arthritis (Reynolds et al. 2012; Williams et al. 2016). Thoracic aortas from mice with CIA had significantly decreased constriction responses to 5HT (**Figure 3.1 D**). Healthy naïve mice (-CIA) had a maximum constriction response of 7.7 mN, whereas mice with CIA had a maximum response of 4.8 mN. The incidence, time-course, and presence of defective constriction responses was comparable with published data. This demonstrates technical proficiency for inducing the model. It also validated the model for investigating vascular dysfunction associated with inflammatory arthritis in my hands.

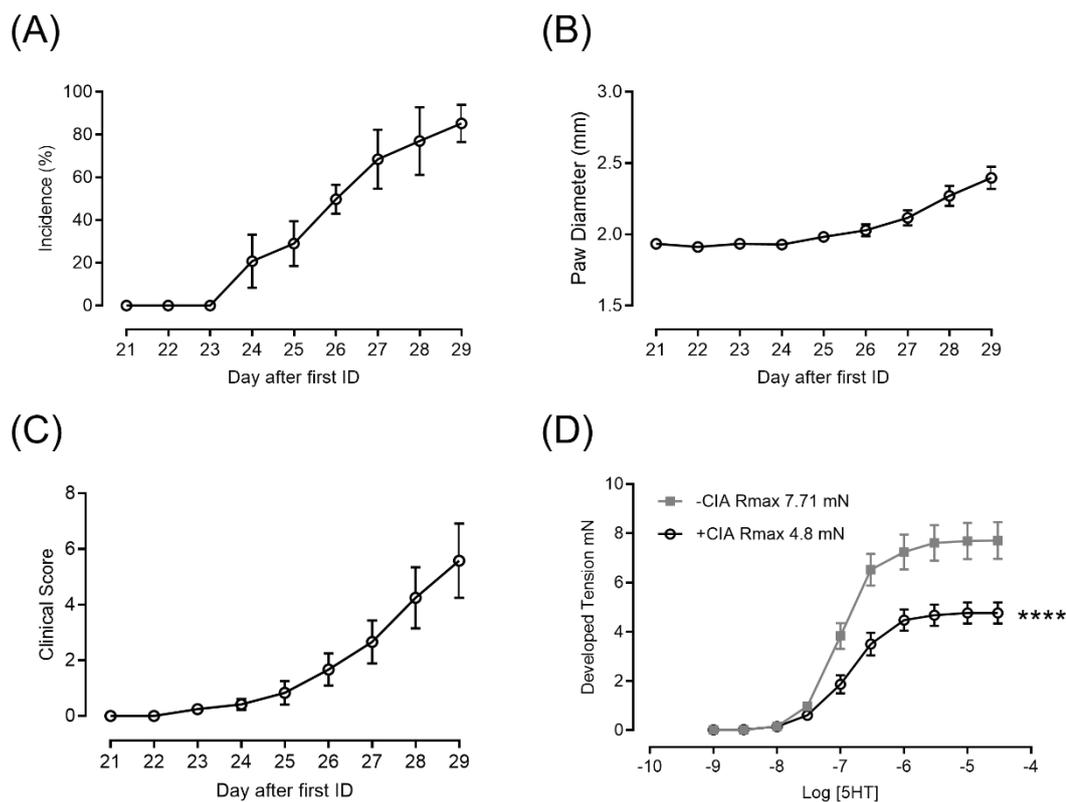


Figure 3.1: The establishment of collagen induced arthritis with the same incidence, timecourse and progression as previously published.

Collagen induced arthritis was established in male DBA/1 mice (n=12). (A) Incidence rates of 85% were observed in the model. (B) Average hind paw diameters were measured from Day 21-29. (C) The severity of swelling was measured and scored in each paw, which combined to form a clinical score. (D) Constriction responses are significantly lower in mice with CIA when compared to strain, age and sex-matched mice. Graphs are shown as mean \pm SEM. **** = $p < 0.0001$ as measured by 2-way ANOVA with Sidak post-test.

3.2.2 Systemic dysregulation of galectin-3 is required to study CIA-associated vascular dysfunction

Thoracic aortas from healthy mice were immersed in galectin-3 *ex vivo* for 2 hours prior to measuring 5HT induced constriction responses to determine if galectin-3, in direct contact with the blood vessel wall, influenced its ability to constrict. A single timepoint of 2 hours and a single concentration of 1 ng/mL was chosen as this was reported to induce marked changes in smooth muscle cell constriction responses using other cytokines (Giardina et al. 2002; Gillham et al. 2008). At this concentration, constriction responses (**Figure 3.2 A**) in rings from aortae incubated with galectin-3 (R_{max} of 9.6 mN) were comparable with control rings incubated in vehicle (0.1% BSA) for an equivalent time (R_{max} of 8.6 mN). Mindful of high priority on the 3Rs (reduction, replacement, and refinement) in biomedical research, the next step was to measure galectin-3 in the plasma of non-arthritic (normal) mice. The data would establish if the parameters for a near-physiological *ex-vivo* experimental system was possible and if the excessive use of mice for dose-ranging experiments was avoidable.

Galectin-3 is elevated in the synovial fibroblasts and the serum of patients with rheumatoid arthritis (Ohshima et al. 2003; Filer et al. 2009). As CIA was selected to investigate vascular inflammation and dysfunction associated with arthritis, galectin-3 was also measured in plasma samples from mice with CIA. Galectin-3 was quantified by ELISA. Levels were significantly increased in mice with CIA (150 ng/mL) versus non-arthritic mice (-CIA) that had levels of approximately 50 ng/mL (**Figure 3.2 B**). A significant elevation of galectin-3 was also shown in rat CIA (Shou et al. 2006).

Modelling and measuring the direct effect of a physiologically relevant concentration of galectin-3 during inflammatory arthritis was not achievable given that the concentration of galectin-3 could be up to 150 times greater than originally anticipated in an *ex vivo* system. The mechanisms by which galectin-3 directly, or indirectly, impacted upon vascular inflammation and function was therefore investigated *in vivo*. GB1107 was used as a tool to modulate galectin-3 *in vivo*. GB1107 is a specific orally active small molecule inhibitor of galectin-3. It had been used in the laboratory previously in a therapeutic capacity in CIA (**Section 3.1**), and had also been used in a mouse model of lung cancer (Vuong et al. 2019) to inhibit inflammatory pathological responses.

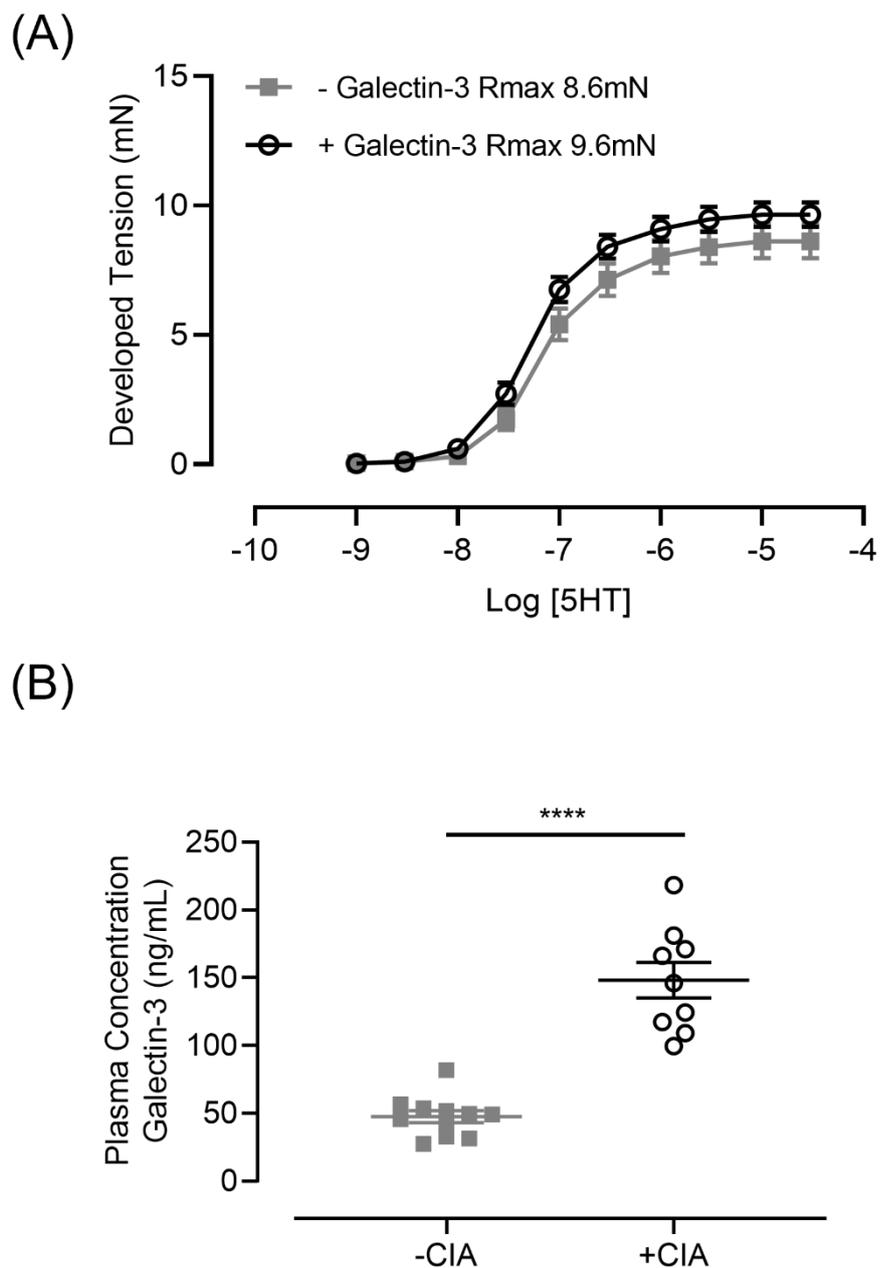


Figure 3.2: Galectin-3 does not play a direct role in vascular dysfunction in inflammatory arthritis

(A) Thoracic aortas from healthy male DBA/1 mice ($n=12$) were harvested and mounted on the myograph. Galectin-3 was added to the myograph bath for 2 hours prior to measuring 5HT induced constriction responses (B). Plasma from CIA mice ($n=9$) was harvested and a galectin-3 ELISA was performed. Significance was calculated by unpaired t-test where **** = $p < 0.0001$. Graphs are shown as mean \pm SEM.

3.2.3 Vehicle with better *in vivo* safety and solubility profile for GB1107 tested in CIA

Partial restoration of CIA-associated constriction responses was achieved previously in the laboratory using a single administration of 10 mg/kg GB1107 given orally, using the vehicle 10% solutol in PEG300. GB1107 was dissolved at its solubility limit in this vehicle for these experiments. However, constriction responses were not fully restored to the R_{max} values recorded for normal mice, nor did plasma galectin-3 return to the non-CIA level in these initial experiments. There was no scope for a dose escalation study using 10% solutol in PEG300.

Therefore, propylene glycol and HPMC was tested as an alternative vehicle. *In vivo* studies by Galecto Biotech showed this vehicle had better solubility and safety profiles than 10% solutol in PEG300. Propylene glycol and HPMC is a safe and well tolerated vehicle in pharmacological experiments using mice (Thackaberry et al. 2010). Studies using 10% propylene glycol, 0.5% HPMC as the vehicle for GB1107 inhibited lung adenocarcinoma when administered once a day (10 mg/kg) in CD1 mice (Vuong et al. 2019).

Mice were dosed once daily with 10 mg/kg GB1107 in 10% propylene glycol, 0.5% HPMC from Day 21 of the CIA induction protocol through to Day 30. The incidence of CIA was matched with expectations from previous experiments conducted by previous PhD students. Incidence of 85% was reached by Day 29 and achieved 100% by Day 30 (**Figure 3.3 A**). The data presented in **Figure 3.3** is representative of 2 independent experiments. One experiment was terminated on Day 30 and the other was terminated on Day 29. The end date was dictated by the severity limits specified in the project licence. All mice with CIA (HPMC-vehicle and GB1107 groups) lost weight which was significant compared to mice without CIA ($p < 0.01$, **Figure 3.3 B**). Paw diameter measurements, paw score and clinical score assessments were comparable in CIA mice given GB1107 versus vehicle (**Figure 3.3 C – E**).

Individual CIA experiments using small numbers of mice had not previously shown significant impact on macroscopic measures of arthritis severity. None-the-less, dramatic differences in vascular constriction responses (GB1107 versus vehicle) were consistently recorded. The primary outcome of this Chapter was functional, not pathological, so changes to vascular constriction responses were measured next.

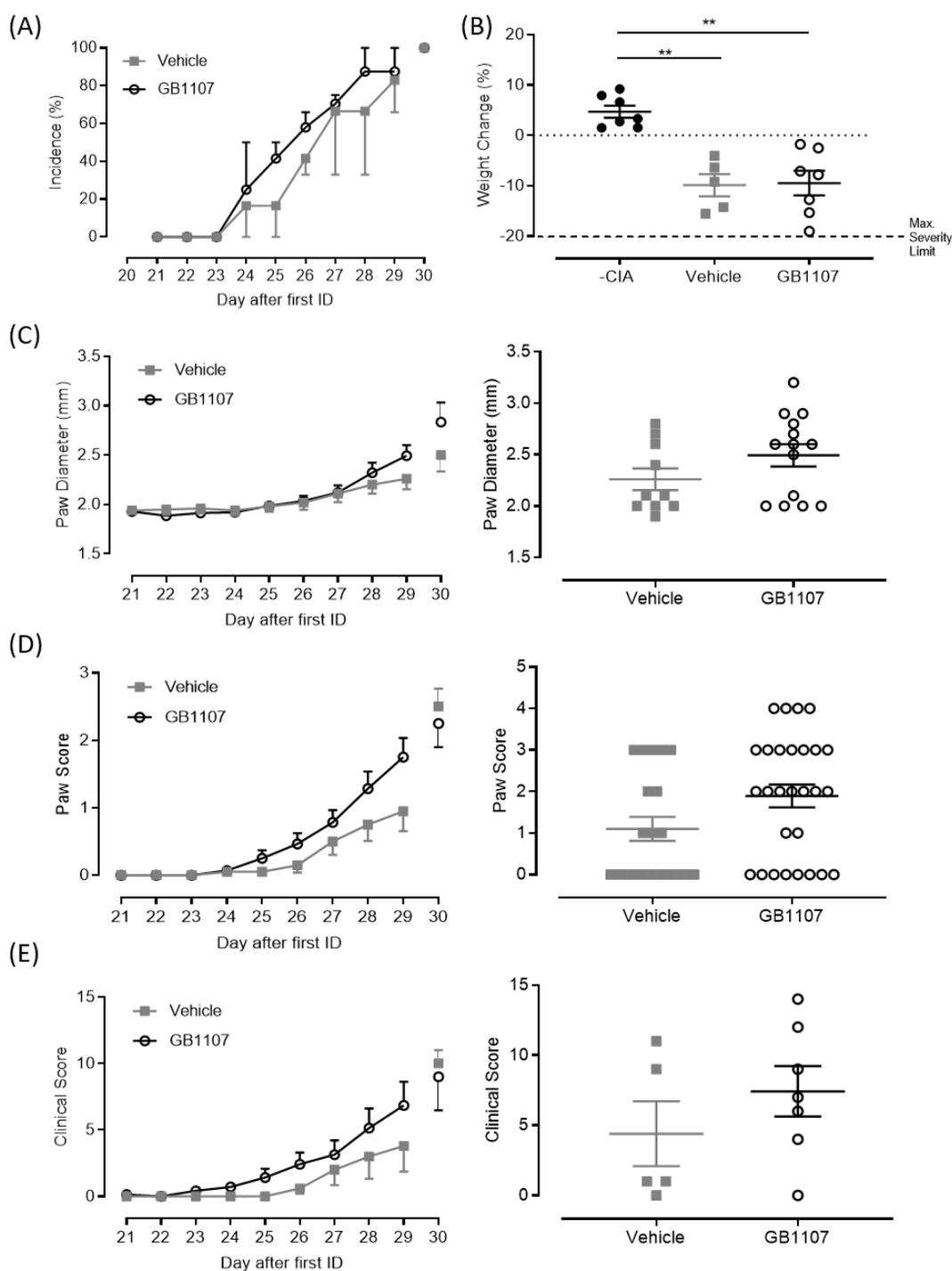


Figure 3.3: Clinical indices of arthritis were not affected by treatment with GB1107 in HPMC vehicle

Inhibition of galectin-3 with GB1107 during CIA (n=7) showed accelerated progression of arthritis when compared to vehicle treated group (n=5). (B) Percentage of weight change in mice with no CIA (n=6), vehicle (n=5) and GB1107 (n=7). **=p<0.01 as determined by Kruskal Wallis test with Dunn's post-test. (C) Paw diameters of both hind paws were measured daily until termination. (D) Paw score from all four paws from Day 21 - 30 (left) and at termination (right). (E) Clinical score is the sum of the four paw scores from each mouse. This data is indicative of 2 independent experiments, where one experiment was terminated on Day 29 and the other was terminated on Day 30. The single points on Day 30 represents data from 1 experiment. Graphs are shown as mean \pm SEM.

3.2.4 5HT induced constriction responses unaffected by GB1107 in CIA

Vascular constriction responses were measured by myography using aortic rings from mice with CIA that had been given GB1107 or HPMC-vehicle (see **Section 2.2** for methodology). Mice that had not been exposed to the CIA induction protocol (-CIA) provided a baseline (R_{max} of 7.7 mN) which CIA constriction responses were compared against. The mean R_{max} value for CIA groups was 5.2 mN and 3.9 mN for mice given HPMC-vehicle and GB1107 respectively (**Figure 3.4 A**). Constriction responses were significantly decreased between -CIA mice and CIA mice treated with GB1107 ($p < 0.05$). Therefore, GB1107 dissolved in HPMC-vehicle did not restore 5HT induced constriction responses. This was contrary to previous results that consistently showed restoration of constriction responses in mice with CIA given GB1107 in 10% solutol in PEG300. This restoration of aortic constriction responses was accompanied by a reproducible and significant reduction in plasma levels of galectin-3. It was for this reason that levels of galectin-3 were measured in CIA mice given HPMC-vehicle and GB1107 dissolved in HPMC-vehicle.

Circulating levels of galectin-3 were higher but not significantly different for the CIA group given HPMC-vehicle versus naïve -CIA mice. The levels of galectin-3 in plasma from the CIA group given GB1107 was significantly higher ($p < 0.01$) than the -CIA group (**Figure 3.4 B**). This contradicted previous findings from the laboratory that consistently showed a reduction in plasma levels of galectin-3 by GB1107 during CIA. Galectin-3 levels in the -CIA group was in the region of 50 ng/mL compared against 18.7 ± 1.6 ng/mL (mean \pm SEM) in previous experiments. Mice with CIA, treated with GB1107 and HPMC-vehicle group, recorded galectin-3 levels of between 75 and 200 ng/mL. This was substantially higher than previous experiments where levels of galectin-3 were 61.6 ± 6.8 ng/mL for mice with CIA that had been given 10% solutol in PEG300 as the vehicle control.

In conclusion, HPMC-vehicle was not a suitable carrier to conduct dose-ranging experiments with GB1107 in CIA. The loss of function of GB1107 suspended in HPMC-vehicle and the heightened levels of plasma galectin-3 did not warrant further investigation using this vehicle in CIA.

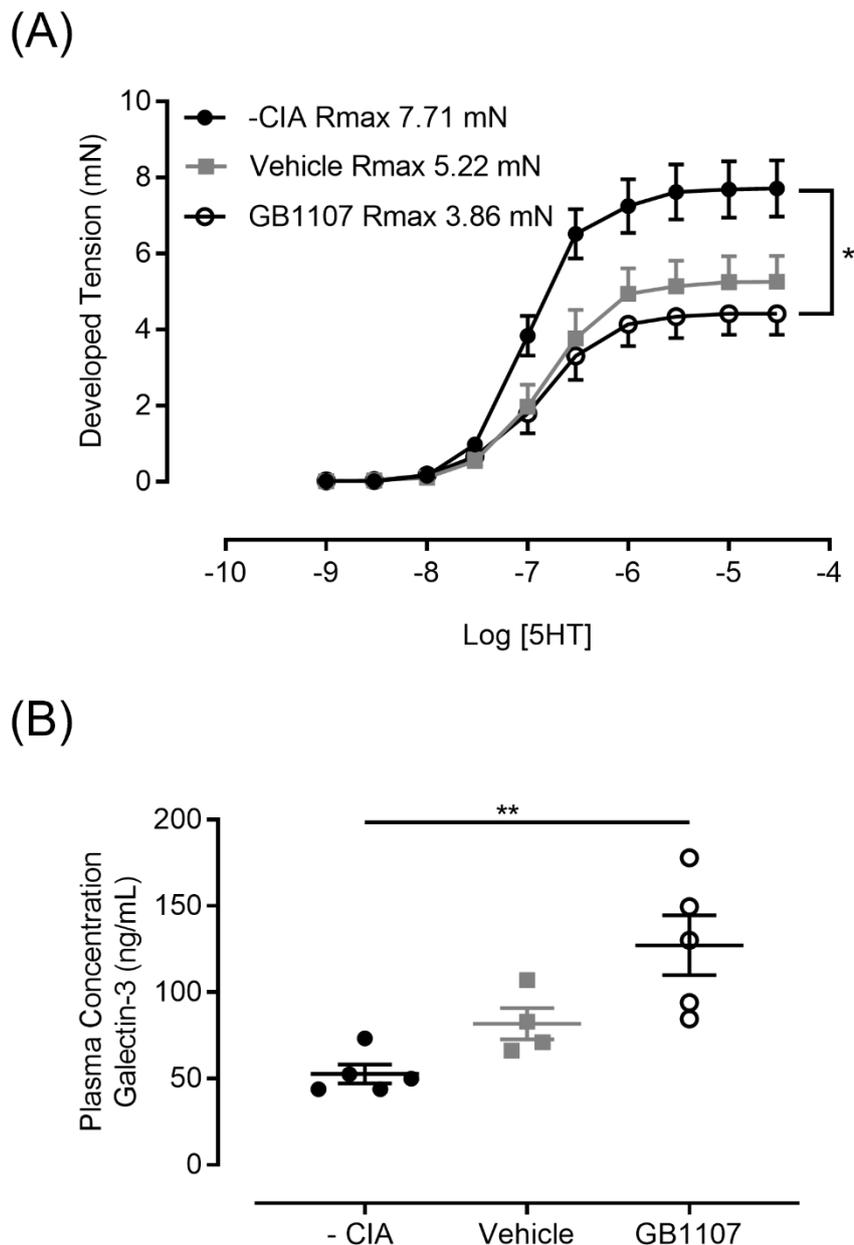


Figure 3.4: No change in vascular constriction responses or circulating galectin-3 in mice treated with GB1107 or vehicle

Myography is used to measure constriction responses of the thoracic aorta to 5HT. (A) Mice treated with GB1107 in HPMC vehicle ($n=7$) showed decreased constriction responses when compared to vehicle treated ($n=5$) and non-arthritic mice ($n=7$). Significance is calculated by two-way ANOVA with Sidak post-test where $*=p<0.05$. (B) Plasma from CIA mice was harvested and a galectin-3 ELISA was performed. Graphs are shown as mean \pm SEM and significance is calculated by one-way ANOVA with Tukey post test. $**=p<0.01$.

3.2.5 Reformulation of GB1107 in 10% solutol/PEG300 enabled oral administration galectin-3 inhibitor at 20 mg/kg

10% solutol in PEG300 was established as a suitable carrier for GB1107 in CIA. GB1107 (10 mg/kg) caused systemic levels of galectin-3 to drop significantly and induced partial restoration of vascular constriction responses in CIA when resuspended in 10% solutol in PEG300 (**Section 3.1**). In order to achieve full restoration of constriction responses, an initial oral administration of 20 mg/kg GB1107 was targeted - double the amount given in previous studies from the laboratory. A new formulation method was established which pushed GB1107 to its maximum solubility of 2 mg/mL in 10% solutol in PEG300 (see methodology in **Section 2.3.4**). In order to reduce the potential for choking, distress and/or discomfort caused to the mice by the procedure, the volume of solution given per administration was halved. This factor was also taken into consideration for dose escalation to 20 mg/kg of GB1107 and therefore the total was administered over 2 gavages rather than one bolus. Three treatment groups were assigned in these experiments; vehicle, GB1107 (10 mg/kg) and GB1107 (20 mg/kg), where the inhibitor was given once or twice each day respectively. Spreading the higher GB1107 dose over two administrations was also thought to improve the bioavailability of the inhibitor. Constriction responses to 5HT decreased with increased CIA severity (Reynolds et al. 2012). For this reason, two endpoints were selected, Day 27 and Day 30. Day 27 was chosen as this is the timepoint where 60-80% mice that received the CIA protocol had arthritis (**Section 3.2.1**), but CIA was macroscopically mild in nature. This gave maximum opportunity to see a change in galectin-3 levels, constriction responses, and arthritis measurements. Day 30 represented the chronic phase of CIA that would be used for comparison against the earlier study in this Chapter.

Mice showed the first macroscopic signs of arthritis on Day 25. CIA escalated rapidly in mice given vehicle and GB1107 (20 mg/kg); 100% incidence was reached by Day 28 in both groups. By Day 30, mice given GB1107 (10 mg/kg) also reached 100% incidence (**Figure 3.5 A**). All mice with CIA had significant weight loss when compared to -CIA controls ($p < 0.001$; **Figure 3.5 B**). Two mice from the GB1107 (10 mg/kg) and one from the GB1107 (20 mg/kg) groups reached the severity limit for weight loss stipulated in the project licence. Paw diameters, paw scores, and clinical scores steadily increased from Day 25 to the end of the experiment (**Figure 3.5 C – E**). No significant differences in arthritis severity was recorded between the 3 groups studied for any of the parameters measured.

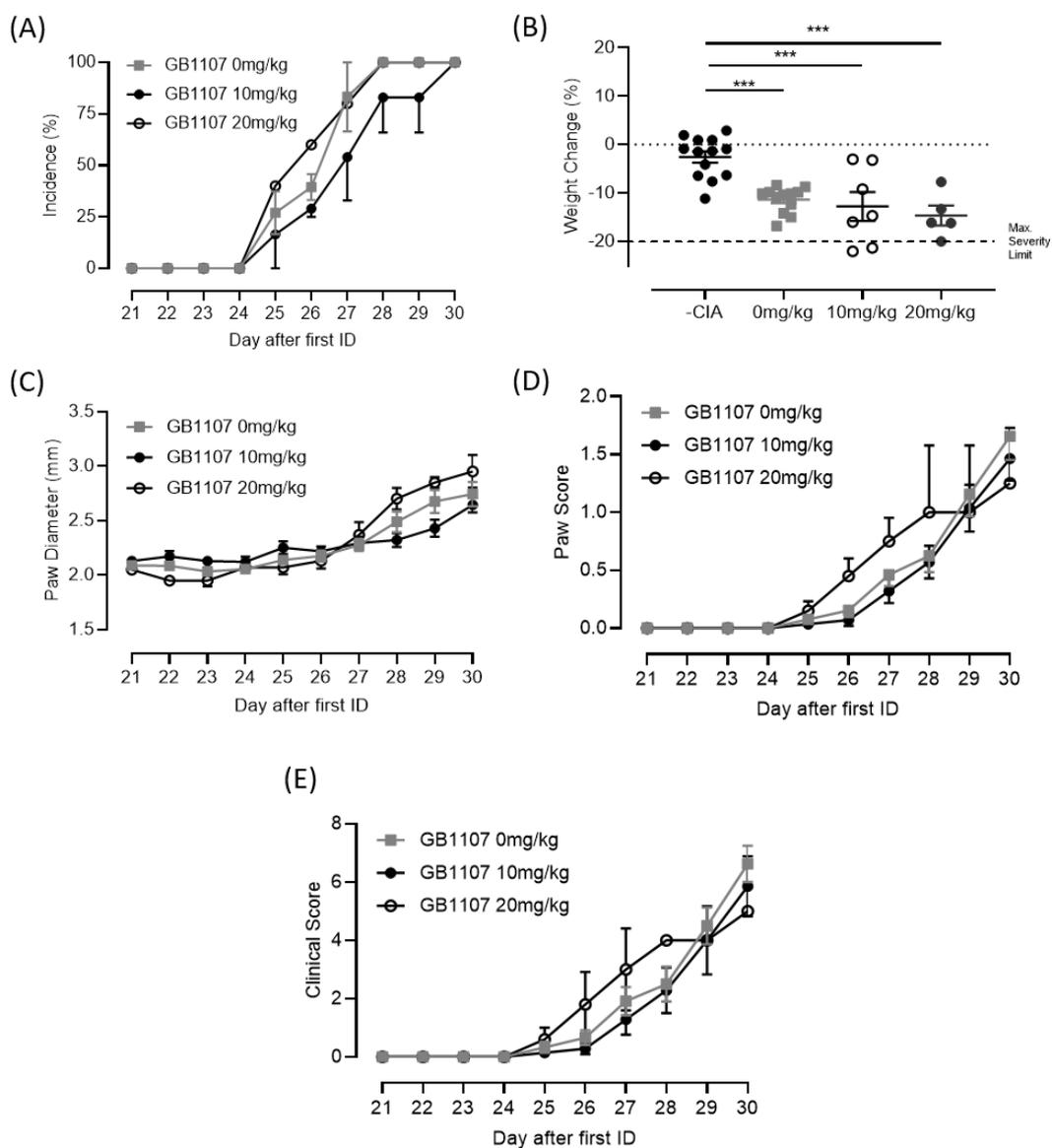


Figure 3.5: Treatment with GB1107 (10 mg/kg and 20 mg/kg) had no effect on clinical disease in CIA.

Inhibition of galectin-3 with 20 mg/kg/day GB1107 during CIA (n=5) showed accelerated progression of arthritis when compared to 10 mg/kg/day GB1107 (n=6) and vehicle treated group (n=12). (B) Percentage of weight change in mice with no CIA (n=13), vehicle (n=12), 10 mg/kg/day GB1107 (n=6) and 20 mg/kg/day GB1107 (n=5). (C) Paw diameters of both hind paws were measured daily until termination. (D) Paw score from all four paws from Day 21 - 30. (E) Clinical score is the sum of the four paw scores from each mouse. Data are shown as mean \pm SEM.

3.2.6.1 PVAT-associated and circulating levels of galectin-3 were unaffected by GB1107 (20 mg/kg) in CIA

GB1107 (10 mg/kg) had consistently reduced tissue expression of galectin-3 in the perivascular adipose tissue (PVAT) associated with the aorta during CIA (Identification of targets for the prognosis and/or treatment of vascular pathology associated with inflammatory arthritis. Ph.D. thesis. Cardiff University ORCA reference: <http://orca.cf.ac.uk/id/eprint/121975>). This histological change was evident in the absence of any change in the macroscopic progression of CIA.

FFPE sections through aortae harvested from vehicle and GB1107 (20 mg/kg) groups of mice with CIA were stained for galectin-3. Plasma from the same animals were also analysed by ELISA for galectin-3 to determine if increasing the dose of GB1107 to 20 mg/kg had altered either local and/or systemic levels of galectin-3. Galectin-3 expressing cells as a percentage of total cells was comparable in non-arthritic mice (-CIA) and mice with CIA, irrespective of treatment group (vehicle versus GB1107 (20 mg/kg) on Day 27 (**Figure 3.6 A, B**). Galectin-3 levels were significantly elevated in plasma from vehicle and GB1107 (20 mg/kg) groups when compared against -CIA non-arthritis controls ($p < 0.001$). No difference was measured between the treatment groups (**Figure 3.6 C**).

3.2.6.2 GB1107 reduced CD3 T-cell accumulation in PVAT

CIA features T cells as part of its pathology (Ehinger et al. 2001). Galectin-3 is expressed in T cells and can modulate their activation (Gilson et al. 2019). To learn more about the galectin-3 regulated inflammatory environment within PVAT during CIA it was important to establish if T cells were present in the tissue at such an early stage in arthritis progression. Immunohistochemistry for the T-cell marker CD3 revealed strong positive staining in PVAT. Furthermore, CIA tissue showed increased accumulation of T cells in the PVAT versus tissue from non-immunised mice without arthritis (-CIA). Treatment with GB1107 (20 mg/kg) significantly reduced the percentage of CD3 expressing cells in PVAT (**Figure 3.6 D, E**).

T-cells modulate vascular inflammation in a variety of ways – for example, by stimulating IFN- γ production, increasing vascular reactive oxygen species and vascular leukocyte infiltration - which in turn impact on the ability of the blood vessel to constrict and dilate. Constriction responses to 5HT were determined to gauge if the GB1107-associated decrease in CD3 T cells during CIA had any effect on the function of the thoracic aortas. On Day 27, there was no significant difference in constriction responses between the three groups analysed; -CIA (Rmax of 8.0 mN) and CIA/vehicle (Rmax of 6.6 mN) and CIA/GB1107

(R_{max} of 6.1 mN). Constriction responses in the -CIA group recorded the expected R_{max} values, in line with previous experiments, previous published data (Sime et al. 2017) and the results presented in this Chapter (**Figure 3.6 F**). Surprisingly, and for the first time ever, there was no significant CIA-associated reduction in constriction responses recorded using the established myography protocol.

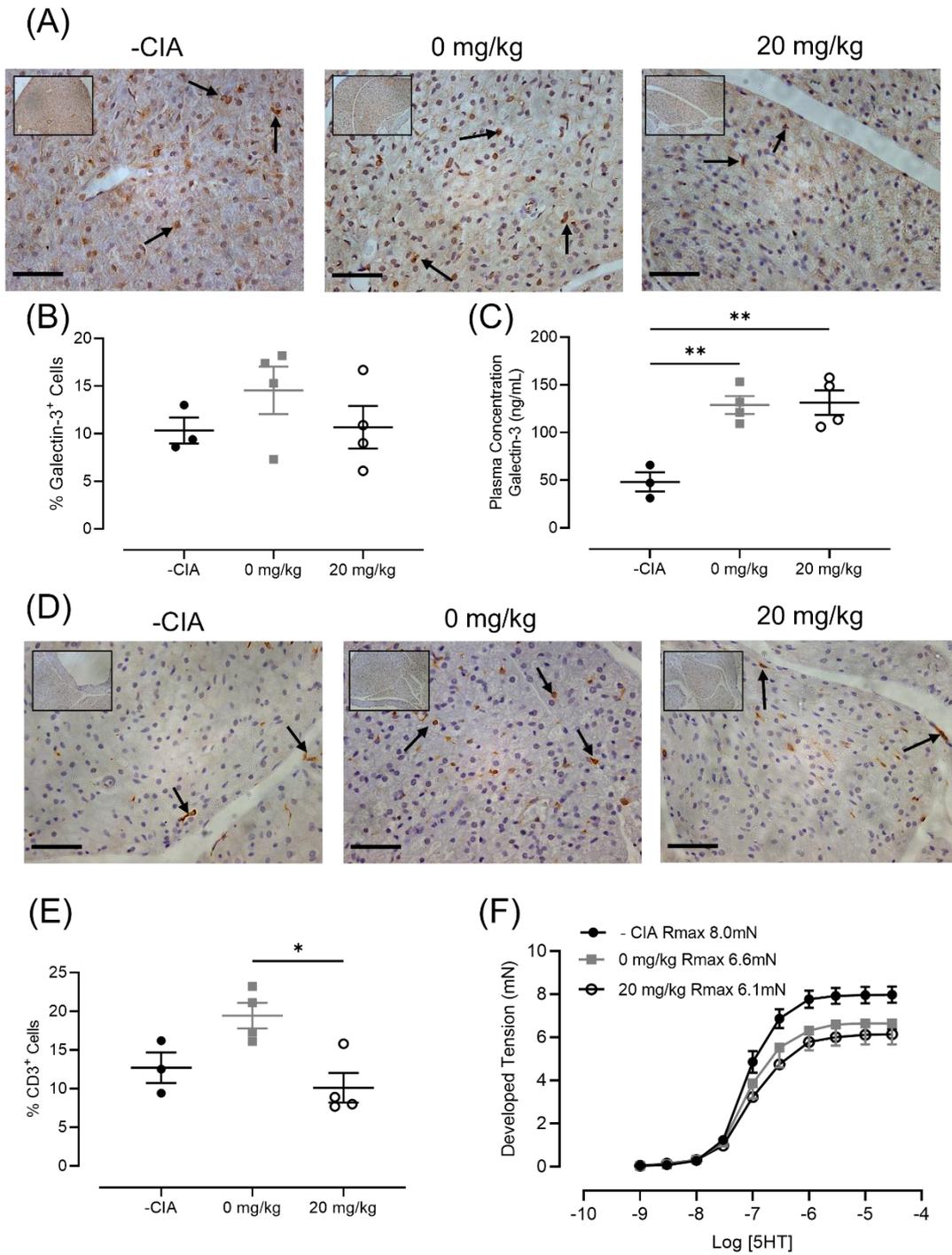


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Figure 3.6 No change in local and systemic galectin-3 levels at Day 27, but a reduction in T-cell accumulation following CIA induction in response to GB1107

(A, B) No difference in PVAT expression of galectin-3 was observed in mice treated with 20 mg/kg GB1107 in PEG300 vehicle (n=4) or vehicle only (n=4). Positive staining is denoted by black arrows and was quantified using ImageJ FIJI software. Scale bar is equivalent to 50 μ M. (C) There was no difference in circulating galectin-3 levels in mice treated with 20 mg/kg GB1107 when compared to mice treated with vehicle. (D, E) Mice treated with 20 mg/kg GB1107 had significantly decreased CD3⁺ T cells in the PVAT when compared to vehicle treated mice. (F) There was no difference in 5HT induced constriction responses in mice treated with 0 mg/kg or 20 mg/kg GB1107 when compared to -CIA controls. Graphs are shown as mean \pm SEM and significance is calculated by one-way ANOVA where *=p<0.05 and **=p<0.01.

3.2.7 T cell-associated cytokine IFN- γ raised on Day 27 of CIA

Given that T cell accumulation in the PVAT was reduced by GB1107 (20 mg/kg) at Day 27, T cell associated cytokines were measured by multiplex assay to probe for arthritis progression/resolution markers altered by GB1107.

Plasma levels of IFN γ , TNF α , IL-6, IL-17A, IL-13, and IL-22 were quantified. Mice given vehicle had significantly elevated IFN γ ($p < 0.05$; **Figure 3.7 A**) compared against mice without arthritis (-CIA group). The CIA group given GB1107 (20 mg/kg) recorded reduced levels of IFN γ versus the CIA group given vehicle. There was no significant change in any of other cytokines (CIA versus -CIA) measured (**Figure 3.7 B-F**).

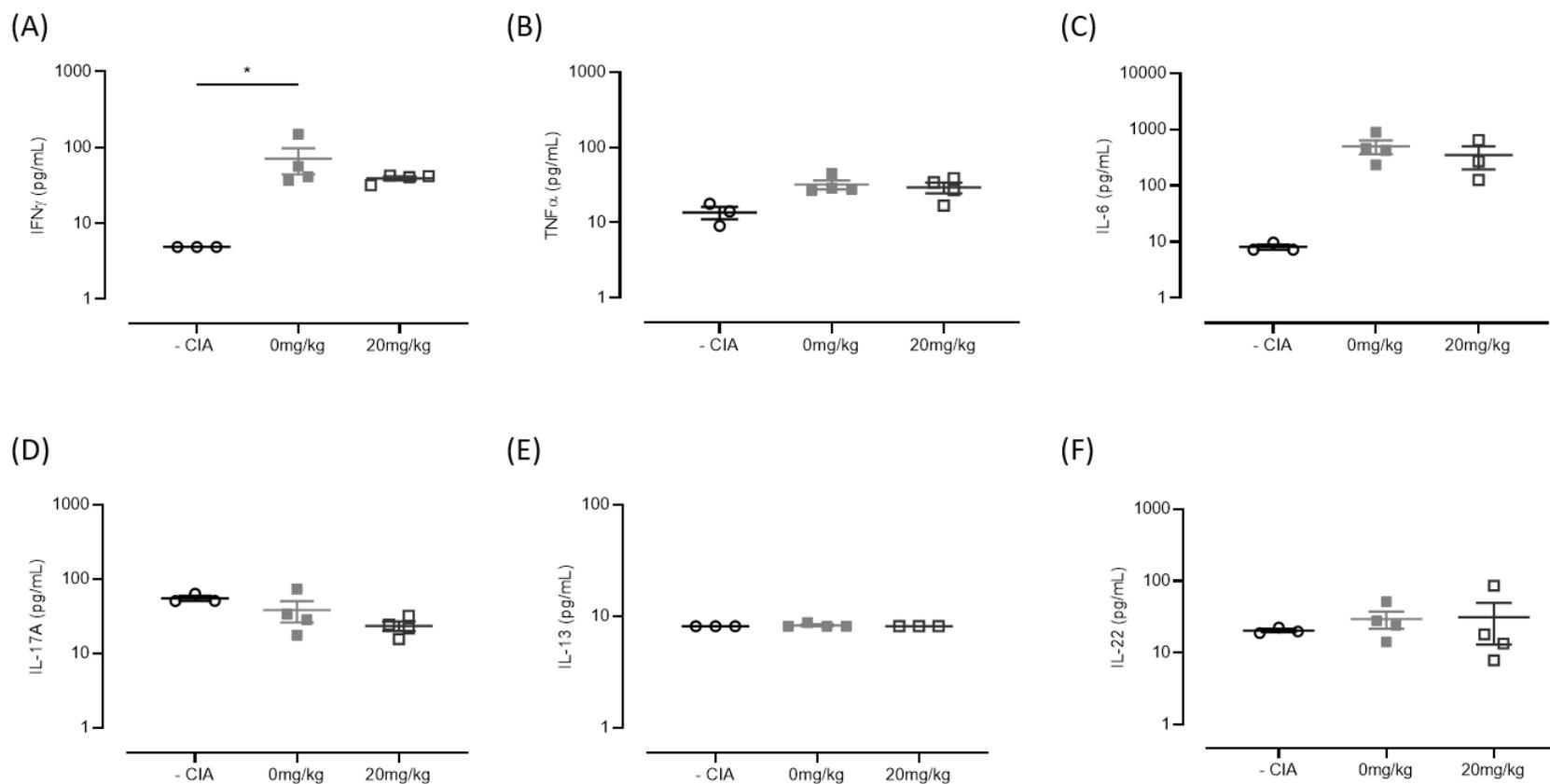


Figure 3.7 Systemic levels of proinflammatory cytokines in mice with CIA treated with GB1107

To determine if GB1107 elicited any effect on systemic levels of pro-inflammatory cytokines at Day 27, plasma was harvested from mice and a LEGENDplex multiplex assay was performed. (A) IFN γ . (B) TNF α . (C) IL-6. (D) IL-17A. (E) IL-13. (F) IL-22. Data are shown as mean \pm SEM and significance is calculated by one-way ANOVA where * = $p < 0.05$.

3.2.8 No difference in vascular constriction recorded between mice with and without CIA at Day 30.

Contrary to previous findings in this thesis (**Figure 3.1**) and published data (Williams et al. 2016; Sime et al. 2017) there was no significant difference in constriction responses recorded between -CIA and CIA at Day 30. This was unexpected and unprecedented given the severity of the CIA. Day 30 assessments with GB1107 (10 mg/kg) served as a direct comparator to earlier experiments in this Chapter and previous experiments conducted in the lab using this GB1107/vehicle combination. There were no significant differences in Rmax across the groups at Day 30 (**Figure 3.8 A**). The result was a major concern because it provided the first evidence that the CIA model was not fit for purpose for investigating restoration of constriction responses by GB1107, in this group of animals at least.

For completeness and to compare against the Day 27 data collated for these experiments, galectin-3 was measured in plasma samples by ELISA. Galectin-3 levels were significantly elevated in all treatment groups at Day 30 when compared to treatment naïve mice without arthritis (**Figure 3.8 B**). There was no significant difference in galectin-3 levels when plasma from vehicle, GB1107 (10 mg/kg) and GB1107 (20 mg/kg) were compared.

Sections from formalin fixed paraffin embedded (FFPE) blocks of thoracic PVAT from mice with CIA (vehicle and GB1107 groups) and mice without arthritis (-CIA) were stained for galectin-3. There was no difference in the percentage of galectin-3 positive cells in mice treated with vehicle or GB1107 (10 mg/kg) when compared to -CIA controls (**Figure 3.8 C, D**). These findings together with observations from Day 27 signified that the model was drifting away from previously established galectin-3 dependence for functional and inflammatory pathological readouts in the thoracic aorta during CIA. By Day 30 there was no difference in CD3 T cells in the PVAT between the -CIA control group and +CIA groups (**Figure 3.8 E, F**). Whilst levels of CD3 positive T cells were comparable (10% of total cells) in the -CIA group on Day 27 and Day 30, the percentage of CD3 positive T cells in mice with CIA (vehicle and GB1107) was restored to the same basal level by Day 30.

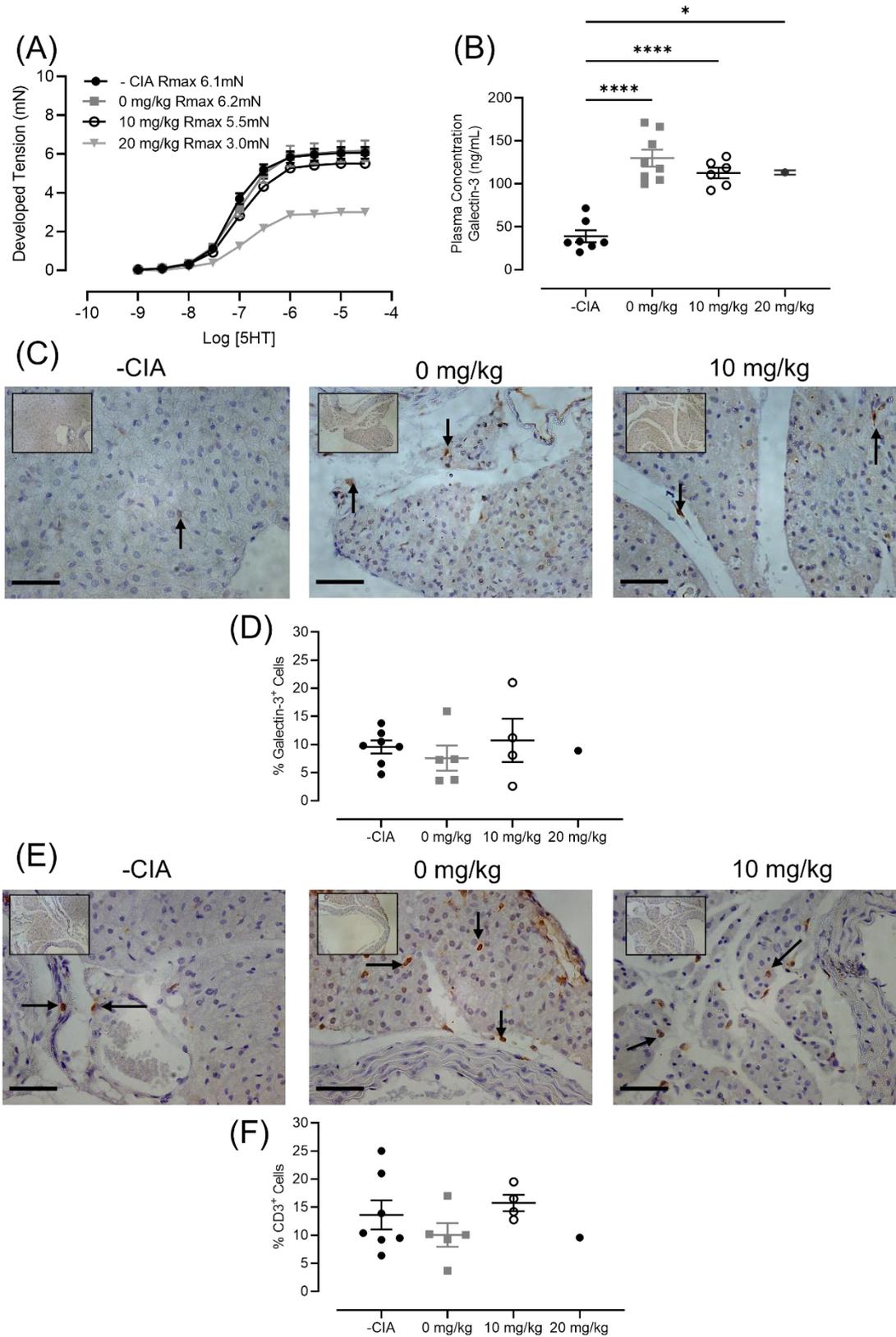


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Figure 3.8 No effect of GB1107 on constriction responses, systemic and local galectin-3 levels or T cell accumulation at Day 30 of the CIA model.

(A) 5HT induced constriction responses in mice treated with vehicle (n=8), 10 mg/kg (n=6), 20 mg/kg (n=1) GB1107 and compared to -CIA (n=9) mice. (B) Plasma was harvested from mice at Day 30 and galectin-3 levels were quantified by ELISA. Significance is calculated by one-way ANOVA. * = $p < 0.05$; **** = $p < 0.0001$. (C – F) FFPE sections of PVAT were stained for galectin-3 and CD3. Positive staining is denoted by black arrows and was quantified using ImageJ FIJI software. Scale bar is equivalent to 50 μ M. Data are shown as mean \pm SEM.

3.2.9 Constriction responses in thoracic aorta reduced at Day 21 of CIA

Based on the results of CIA experiments terminated on Day 27 and Day 30 of CIA it was clear that the model was different from established phenotype we'd used to build the hypothesis for this Chapter both in terms of its vascular function and its dependence on galectin-3. The cause of the changing pattern of PVAT-associated expression of galectin-3 and circulating levels of galectin-3 (-CIA versus CIA) was unclear. Elevated baseline (-CIA) galectin-3 levels might accelerate the progression rate in these studies versus the studies conducted in the laboratory in previous years. Here CIA progressed from initiation to a severe phenotype in a timeframe of 48 – 72 hours. In previous studies progression was slower, spanning 4 - 5 days, and was controllable by GB1107. It was time to re-evaluate the current status of the CIA model.

The first step was to establish galectin-3 levels in age matched immunised versus non-immunised DBA/1 mice without arthritis. This would determine if the raised plasma levels of galectin-3 observed in CIA and previously attributed to arthritis development was an artefact of the immunisation protocol. Galectin-3 levels at Day 21 of CIA were quantified and compared against non-immunised, age-matched mice. There were no significant difference in circulating galectin-3 levels between non-immunised and immunised mice (**Figure 3.9 A**). A mean level of 43.4 ng/mL was measured in non-immunised mice versus 50.8 ng/mL in immunised mice without macroscopic signs of arthritis. This finding confirmed that the arthritis associated with CIA, rather than the immunisation protocol, caused the elevation in circulating galectin-3 observed in this Chapter. The levels of galectin-3 recorded on Day 21 of CIA was comparable with that of severe CIA from previous studies by Dr Sime. This raises a possibility that an environmental factor(s) may be contributing to raised levels of galectin-3 in DBA/1 mice.

Cardiovascular disease can precede joint disease in rheumatoid arthritis patients, indicating the breach of tolerance and autoimmunity activity is occurring in the vasculature before symptoms develop (Foster et al. 2012). Taking this into account, 5HT induced constriction responses were measured at Day 21 of the CIA. This provided new data that would determine if the CIA model mirrored the vasculopathy associated with the preclinical phase of RA when the immune system is primed but macroscopic arthritis is absent.

Mice were immunised with type II collagen on Day 0 (+ immunised) and were culled on Day 21 of CIA induction protocol. 5HT induced constriction responses were measured in rings from the thoracic aorta by myography. Non-immunised (-immunised) age-matched mice

were used for comparison. Non-immunised mice had a R_{max} of 9.9 mN. Immunised mice had significantly decreased constriction responses ($p < 0.0001$) with a R_{max} of 7.0 mN (**Figure 3.9 B**). This was the first-time that constriction responses were recorded on Day 21 of the CIA model. The difference in R_{max} (+immunised versus -immunised groups) was striking (2.9 mN) and a larger difference than the differences between -CIA and +CIA mice on Day 30.

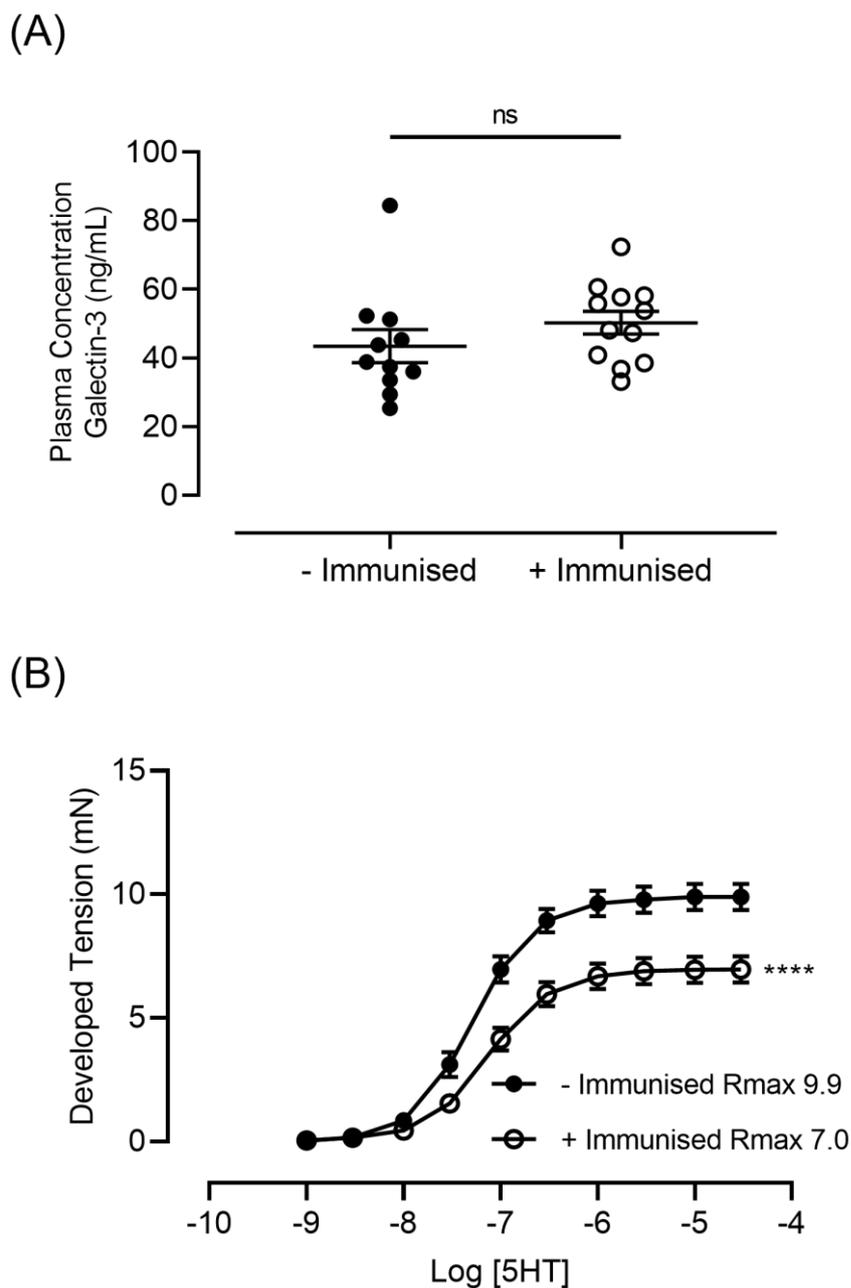


Figure 3.9: No change in circulating galectin-3 levels in immunised mice, but significantly decreased 5HT induced constriction responses.

Mice were immunised with collagen in CFA (n=12) and culled on Day 21 of the CIA protocol. (A) Plasma was harvested from mice and circulating galectin-3 levels were quantified. Graphs are shown as mean \pm SEM. (B) Immunised mice have significantly decreased constriction responses compared to non-immunised controls (n=11). Data is shown as mean \pm SEM and significance was calculated by 2-way ANOVA with Sidak post-test. ****= $p < 0.0001$.

3.2.10 Significantly elevated galectin-3 and CD3 T cells in immunised mice at Day 21 of the CIA protocol

Inflammation in the vasculature can result in the production of cytokines, growth factors and mediators that cause immune cell infiltration, which act on the blood vessel in a paracrine manner directly to promote chronic inflammatory phenotype within the tissue (Sullivan et al. 2000). Thoracic aortae and PVAT were stained for galectin-3 by immunohistochemistry to determine if galectin-3 had a role in modulating localised tissue inflammation in the preclinical phase of CIA. Thoracic aortae from immunised mice had significantly higher ($p < 0.05$) numbers of galectin-3 expressing cells in the associated PVAT when compared to tissues from non-immunised mice (**Figure 3.10 A**). The average percentage of cells staining positive for galectin-3 in non-immunised mice was 8% compared to 16% in immunised mice. Aortae were also stained for CD3 as a marker for T cells. There was a significant elevation of CD3 T cells ($p < 0.01$) in the PVAT from immunised mice (average of 21%) versus 10% in the non-immunised group (**Figure 3.10 B**).

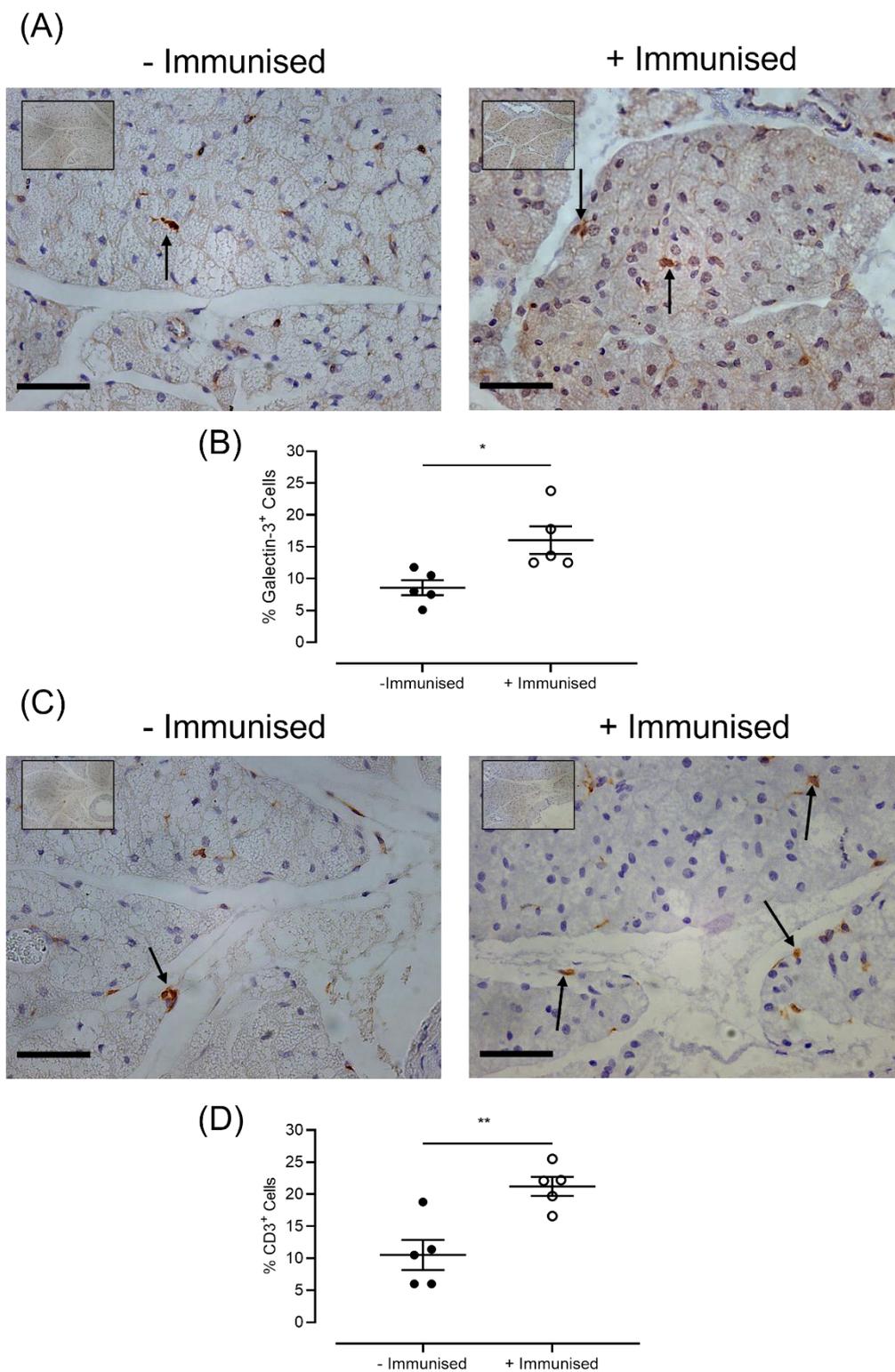


Figure 3.10 Significantly elevated galectin-3 and CD3 cells in PVAT of immunised mice at Day 21 of the CIA model.

FFPE sections of aorta and surrounding PVAT from immunised (n=5) and non-immunised (n=5) mice were stained for galectin-3 and CD3 T cells. Positive cells are denoted by black arrows were quantified by ImageJ FIJI analysis. Scale bars 50 μ M. Data are shown as mean \pm SEM and significance is calculated by t-test where * = $p < 0.05$; ** = $p < 0.01$.

3.2.11 Elevated T cell-associated cytokines in mice immunised with Type II collagen

T cell cytokines were measured by multiplex assay on plasma from immunised and age-matched non-immunised mice. The Th1 cytokine IFN γ was significantly elevated in immunised mice ($p < 0.001$; **Figure 3.11 A**). The pro-inflammatory cytokines TNF α and IL-6 were also elevated in immunised mice (**Figure 3.11 B, C**) indicating that although macroscopic arthritis has not yet occurred, systemic inflammation is present. The systemic inflammation that was independent of galectin 3 may also contribute to impaired vascular constriction responses in immunised mice. Additionally, although Th17 related cytokines were not increased at Day 27 of the model, IL-22 was elevated at Day 21 (**Figure 3.11 F**). This indicated that a Th17 response may be an early inflammatory pathway involved in CIA and that there is definitive T cell dysregulation in CIA.

This Chapter showed that CIA in DBA/1 mice, in its present phenotype, is not fit for purpose to determine galectin-3 mediated mechanisms implicated in vascular inflammation and function during the chronic, progressive phase of the model. The window of opportunity to study early vascular inflammation regulated by galectin-3 may be narrow and should be re-evaluated. The role of GB1107 in correcting the vascular dysfunction associated with inflammatory arthritis should be studied in an alternative, less aggressive model. To my knowledge, vasculopathy and associated vascular dysfunction has not been characterised in another inflammatory arthritis model. In light of this, **Chapter 5** of this Thesis will investigate if the antigen-induced arthritis model is appropriate to study the effect of galectin-3 on arthritis-associated vascular constrictions responses.

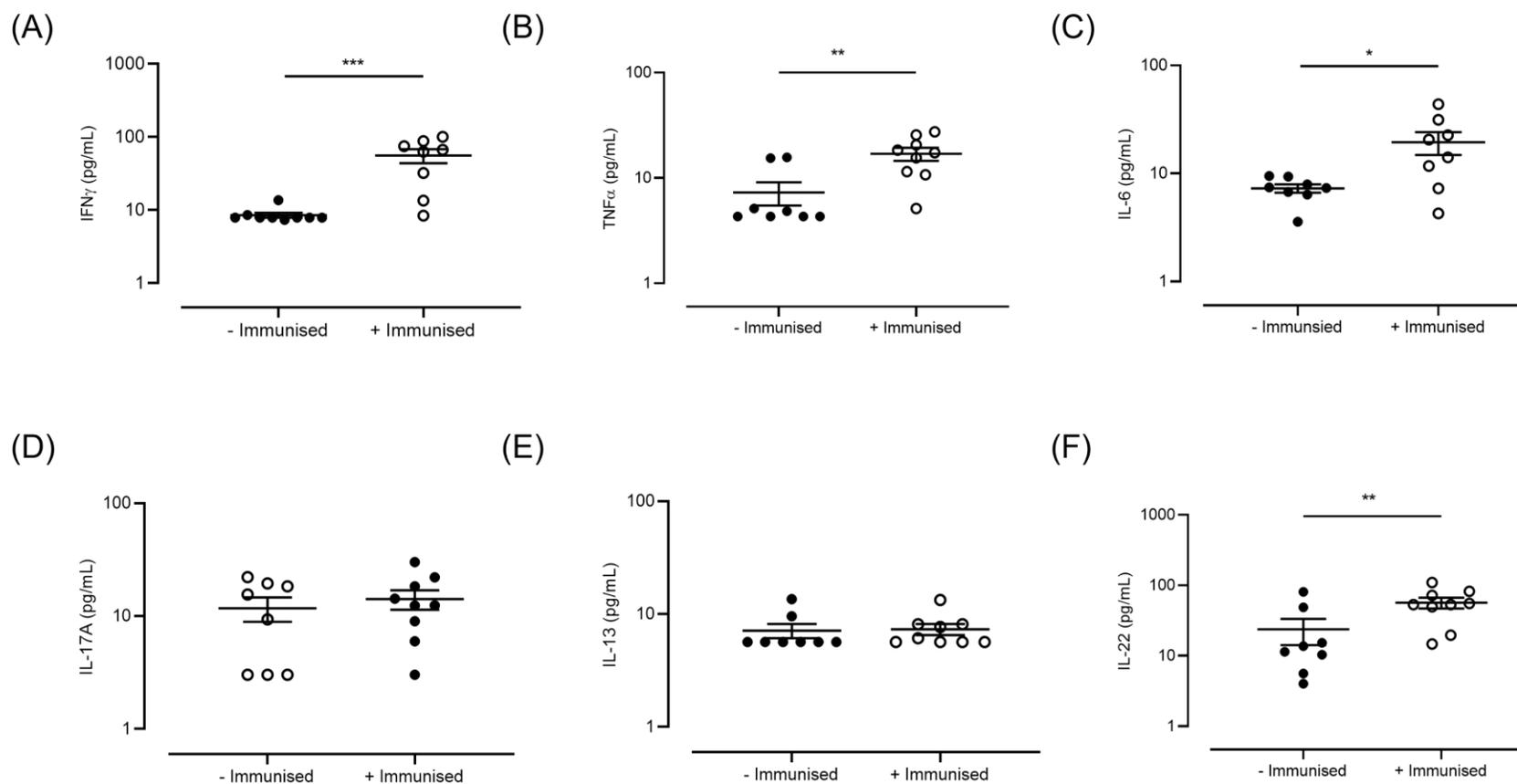


Figure 3.11 Systemic levels of cytokines in collagen immunised mice, prior to the onset of macroscopic arthritis.

To quantify T cell and inflammatory cytokines in immunised mice, a LEGENDplex multiplex assay was performed on plasma. (A) IFN γ . (B) TNF α . (C) IL-6. (D) IL-17A. (E) IL-13. (F) IL-22. Graphs are shown as mean \pm SEM and significance is calculated by non-parametric t-test. * = p<0.05; ** = p<0.01; *** = p<0.001.

3.3 Discussion

Bacterial infections and the local housing environment impact the CIA model

The aim of this Chapter was to test the hypothesis that galectin-3 is a critical mediator of vascular inflammation in arthritis. It is widely stated in the literature that galectin-3 is elevated during systemic inflammation, and knockout galectin-3 mice have shown alleviated disease pathology in antigen-induced arthritis models, (Forsman et al. 2011) experimental autoimmune encephalitis (EAE) (Jiang et al. 2009), and Con-A induced hepatitis (Radosavljevic et al. 2012). The presence of defective vascular constriction responses in the CIA model gives a functional phenotype in which to measure vascular inflammation and the effect of pharmacological inhibition on vascular inflammation of various therapeutic targets, including galectin-3. The identification of galectin-3 as a potential driver of systemic inflammatory disease; the presence of an ability to measure vascular inflammation; and elevated galectin-3 levels in CIA to allow for the assessment of galectin-3 inhibition makes CIA an appropriate model to study the role of galectin-3 in inflammatory arthritis. Experiments to investigate the role of galectin-3 in vascular inflammation utilised a novel galectin-3 inhibitor, GB1107. Initial GB1107 experiments used propylene glycol in HPMC as a vehicle. However, efficacy of GB1107 was not observed in these experiments. It was noted that GB1107 did not remain in solution for long periods of time during the administration of GB1107 to the mice. The poor efficacy observed may therefore partly been due to GB1107 dropping out of solution shortly after entering the stomach, meaning poor absorption to the bloodstream. Another possible reason is also due to the environment of the animal housing facility at Cardiff University. The GB1107 in HPMC vehicle data presented in this Chapter (**Figures 3.3 and 3.4**) was combined data from two independent experiments conducted over a 3 - 4-month period. During the second experiment, we were notified of a *Pasteurella* bacterial infection outbreak in the housing rooms in the animal facility. Mice were placed on broad-spectrum antibiotic enrofloxacin (also known as Baytril) antibiotic treatment from Day 1 to Day 7 of the protocol. Incidence was slightly lower than anticipated in this experiment, while still within the expected range for the model, with a total incidence of 80% upon termination. This finding is consistent with a study conducted by Rogier *et al* showing that changes to the intestinal microbiome during the preclinical phase of CIA attenuates arthritis. The use of broad-spectrum antibiotics during the pre-clinical phase of CIA (prior to the booster injection on Day 21) resulted in almost complete loss of any bacteria in the Bacteroidetes phylum in the intestine (Rogier et al. 2017). However, in another study published by Dorozynska *et al*,

treatment with enrofloxacin prior to and during CIA shown to exacerbate the severity of arthritis, by depletion of natural gut flora (Dorożyńska et al. 2014). The differences observed between Rogier and Dorozynska's papers may be explained in part by Dorozynska's study treating CIA mice with enrofloxacin during the pre-clinical and clinical phases of arthritis. However, the mice in the experiments presented in this Chapter were only treated with enrofloxacin in the preclinical phase of CIA. It is possible that the exacerbation of arthritis in Dorozynska's study occurred during the arthritic phase of CIA, and that may elicit different effects on the immune response against collagen.

Nonetheless, while antibiotic treatment affected the incidence of arthritis, the poor efficacy of GB1107 was observed in both experiments, where only one experiment was affected by an in-house bacterial infection. The incidence and parameters of arthritis measurement for the individual experiments can be seen in **Supplemental Figures 1 and 2** to show that regardless of the infection state of the housing unit, there was still a lack of efficacy of GB1107 in HPMC vehicle.

The timepoints and dosing in these experiments were the same as in previous experiments where efficacy of GB1107 was observed. The only variant in these experiments was prior experiments conducted in the lab and described in **Section 3.1** used a different vehicle for GB1107 than the current experiments. Therefore, the logical explanation was to use the vehicle that has previously shown efficacy – 10% solutol in PEG300. Solubility tests were conducted to ensure maximum solubility of GB1107 but with minimal invasiveness in the mice. DBA/1 mice are smaller than typical C57Bl/6 wild type mice and weigh 18 – 22 grams in adulthood. Therefore, oral gavage is a highly invasive procedure in these mice and due to their size, the stomach can only hold small volumes of liquid at any one time period. The solubility tests were performed to ensure the mice received a dose that was in a therapeutic range, but also an appropriate volume for them to tolerate. GB1107 made into suspension at a concentration of 2 mg/mL remained stable in solution at a physiologically relevant temperature of 37°C, and so mice could be dosed up to 10 mg/kg per dosing. The dose range experiments in this Chapter used a single daily dose of GB1107 at 10 mg/kg or a twice daily dose of 10 mg/kg, equating to a total daily dose of 20 mg/kg. In all experiments, incidence occurred at Day 25 and 100% incidence was typically reached by Day 27 - 28. Following from Day 28, the arthritis observed progressively worsened with larger paw diameters and increased paw and clinical scores, which is in keeping with previous studies (Sime et al. 2017). Therefore, to allow for the maximum therapeutic effect to be seen, some mice were terminated on Day 27, when arthritis had just reached 100% incidence

and was mild (paw score <5), and others were treated until Day 30 and terminated, as in other CIA experiments. Mice terminated on Day 30 was used as a comparator to previous experiments conducted in the lab. Of note, two mice were also dosed with 20 mg/kg GB1107 until Day 30 to determine if the enhanced dosage would achieve full galectin-3 inhibition when arthritis had progressed from mild to moderate/severe. However, one mouse was culled during the experiment due to health issues unrelated to the model, therefore resulting in one data point in **Figure 3.7**. The data shown in **Figure 3.5** followed the same group of animals in the same experiment, with different endpoints on Day 27 and Day 30. However, there was no change between vehicle, 10 mg/kg, or 20 mg/kg GB1107 in arthritis parameters, constriction responses, or circulating galectin-3 levels was observed at either endpoint.

There is no effect of GB1107 on constriction responses, at 10 mg/kg or 20 mg/kg, which confirms that there was no efficacy in GB1107 at either dose. Moreover, of note was the poor constriction response in the naïve -CIA group. This group consistently showed lower than average constriction responses across 3 independent experiments for naïve -CIA mice. Any technical or equipment related issues was ruled out by performing myography on C57Bl/6 mice and other batches of DBA/1 mice, and both groups showed normal and expected constriction responses to 5HT. Therefore, it can be argued that this is an experiment specific issue. The animal facility had parasitic Pinworm infection outbreaks over the course of these studies. Mice were tested for Pinworm for the duration of one experiment, and the subsequent results returned clear and no pinworm was detected in that batch of mice. However, constriction responses from the naïve -CIA mice in those experiments were still lower than average. Concordant with this, baseline circulating galectin-3 levels were ~50ng/mL which is in line with CIA experiments over the course of the experiments in this Thesis but is twice that of galectin-3 levels in previous years of the model. There have been reports within the users of the animal facility at Cardiff University that other inflammation studies have also been affected by the naïve control mice having a higher-than-average baseline inflammation. It has been hypothesised that the diet of the mice has a high fat content, which may be leading to low grade inflammation and alterations to the microbiome. Therefore one can suggest that this can have a significant impact on the immune response and severity of the disease models and this may be the reason for the lower than average constriction responses in the -CIA mice, but definitive studies were not carried out at the time of writing. However, this hypothesis may not fully explain the normal constriction responses observed in naïve non-immunised DBA/1 mice

culled at Day 21 in the CIA protocol, who are on the same diet as Day 27 and Day 30 naïve - CIA mice.

Therapeutically targeting galectin-3 in CIA elicits a T cell dependent response in the inflamed vasculature

While there were differences observed in systemic levels of inflammation, tissue levels of inflammation were also investigated to determine if GB1107 affected the local inflammatory environment. The absence of any difference in tissue expression of galectin-3 and CD3 T cells at any dosage of GB1107 at Day 30 shows that the inflammation has reached a point where it is too severe and has passed the point where the role of galectin-3 in inflammation can be investigated by use of GB1107. While there were no differences in systemic inflammation across groups on Day 27, the significant decrease in tissue expression of CD3 T cells and a trend towards the return of galectin-3 expression to baseline indicated that inflammation could be regulated at this timepoint. While significant differences in galectin-3 tissue expression was not achieved across groups, there was a trend towards increased galectin-3 levels in the 0 mg/kg group and a trend toward decreased galectin-3 expression in the 20 mg/kg dosage group. However, n numbers were low (n=4 per group) and this experiment would need to be repeated to determine if the trends observed were true differences in expression. The significant decrease in CD3 T cells in mice dosed with 20 mg/kg GB1107 indicates that galectin-3 may act by regulation of T cell infiltration to tissues. Galectin-3 has been recognised as a negative regulator of T cell activation in CD4⁺ T cells (Chen et al. 2009) and so perhaps has a role in inflammation by modulation of T cell expression and function. The quantification of circulating cytokines at Day 27 indicated that GB1107 did not affect systemic inflammation. Levels of IFN γ , TNF α , and IL-6 increased in CIA mice, and importantly their levels were unchanged by treatment with GB1107. CIA is considered a Th1 dominant disease and is associated with high production of IFN γ (Zhang 2007). This indicates that the macroscopic phase of arthritis may be Th1 T-cell driven. Both TNF α and IL-6 are drivers of inflammation and although not significant, are elevated at Day 27 of the model. TNF α plays a key role in CIA and anti-TNF treatments have shown to significantly ameliorate joint disease (Williams et al. 1992; Notley et al. 2008). Furthermore, IL-6 is required for the induction of CIA (Alonzi et al. 1998) and inhibition of IL-6 and TNF α are first line treatments for RA patients (Ma and Xu 2013; Ogata et al. 2019). Therefore, the trend towards heightened levels of these cytokines is as expected, and lack of significance is likely due to low n numbers. IL-13, a negative regulator of IL-17A secretion from Th17 cells (Newcomb et al. 2012), and IL-22, a Th17 cell cytokine

(Liang et al. 2006), levels did not change upon induction of arthritis. Of note, IL-17A levels were higher in -CIA mice than in mice with CIA. IL-17A functions in initiating a host immune response to bacterial and fungal infections (Jin and Dong 2013). Given the repeated infection outbreaks in the animal house, it is possible that -CIA mice had the early stages of infection upon termination. However, it is difficult to say without a definitive confirmed test.

Regardless, the lower than average constriction responses and higher baseline galectin-3 levels in -CIA mice at Day 27 and Day 30 led to changing the timepoint at which to test the hypothesis that galectin-3 mediates vascular inflammation in inflammatory arthritis. At Day 30 of the CIA model, inflammation is progressive, and it is therefore challenging to study the regulation of galectin-3. Furthermore, while local tissue expression had differences between groups at an earlier endpoint of Day 27, there were still no systemic changes in macroscopic arthritis, constriction responses, or circulating cytokines between treatment groups, resulting in Day 27 being an inappropriate timepoint to investigate the role of galectin-3 on vascular inflammation.

Tissue expression of galectin-3 and T cells are elevated in preclinical stages of disease and may contribute towards defective constriction responses

Vascular inflammation can precede joint pathology and so the timepoint to test the hypothesis shifted to Day 21 of the CIA model. Day 21 was chosen as the timepoint as it is considered the baseline of disease, as no macroscopic arthritis has developed but the mice are immunologically primed, therefore mirroring the preclinical phase of human disease. During the preclinical phase of rheumatoid arthritis, proteins are citrullinated which leads to activation of T cells and production of antibodies by B cells, leading to subclinical inflammation (Holers 2013). However, to my knowledge, vascular inflammation in the preclinical phase of disease has not been studied in models of inflammatory arthritis. Although there was no difference in circulating galectin-3 at Day 21, constriction responses were significantly decreased in immunised mice. This finding informs us of two points: 1) initiation of dosing mice with GB1107 at Day 21 is an appropriate timepoint as galectin-3 levels are not already raised and 2) defective constriction responses occur independently of a direct effect of galectin-3. However, galectin-3 may be having an indirect effect by driving inflammatory processes in immune cells. Galectin-3 knockout (*Lgals3*^{-/-}) mice have shown to have decreased circulating T and B cells during helminth infection (Breuilh et al. 2007). Galectin-3 is highly expressed in macrophages and has shown to be essential for the

promotion of a pro-fibrotic phenotype (Henderson et al. 2008). Galectin-3 is also expressed locally in atherosclerotic plaques and is considered as an “amplifier” of vascular inflammation due to promoting monocyte chemoattraction (Papaspriidonos et al. 2008). While galectin-3 has been widely recognised as being activated and secreted by macrophages, it has also been recognised to be expressed in activated CD4⁺ and CD8⁺ T cells. T cells, namely CD4 T-helper cells further propagate inflammation by differentiating to pro-inflammatory T cell subsets and facilitating the release of pro-inflammatory cytokines to the local environment. The significant increase in tissue expression of T cells at Day 21 of the model mirrors preclinical RA (de Hair et al. 2014). Moreover, increased galectin-3 expression in immunised mice indicates that galectin-3 may have a role in preclinical inflammation. This could be investigated in future work by the use of *Lgals3*^{-/-} mice in the CIA model and measurement of vascular constriction responses to obtain a defined role of galectin-3 in this disease setting. Alternatively, galectin-3 may be promoting inflammation indirectly via T cells. Notably, the concurrent heightened galectin-3 and CD3 T cell expression in the PVAT could indicate a potential relationship between galectin-3 and T cells in early inflammation. Moreover, significant elevation of IFN γ in immunised mice further suggests that CIA is a Th1 dominant disease. Although TNF α and IL-6 levels were not significantly increased in mice at Day 27, they were significantly raised in immunised mice at Day 21, indicating that Day 21 is a good model of preclinical RA, and that inflammation precedes the development of macroscopic arthritis. Furthermore, circulating cytokines quantified in preclinical RA patients also have elevated IFN γ , TNF α and IL-6, further highlighting the relevance of Day 21 as a model of preclinical RA (Kokkonen et al. 2010). Preclinical RA patients also have elevated IL-2, which is elevated in immunised mice (**Supplemental Figure 4**). There was no difference in circulating IL-17A or its regulator, IL-13, but there was a significant increase in Th17 associated cytokine, IL-22 in immunised mice. *IL22*^{-/-} mice are less susceptible to the development of CIA and IL-22 is known to promote osteoclastogenesis in CIA (Geboes et al. 2009). Therefore, although there was no difference in circulating IL-22 levels across groups at Day 27, it is possible that Th17 related cytokines contribute early stage of inflammation in CIA. **Chapter 4** of this Thesis will investigate the role of galectin-3 in these T cells. Nonetheless, as galectin-3 levels are unchanged between immunised and non-immunised mice at Day 21, investigating the function of galectin-3 in vascular inflammation during the preclinical phase of arthritis is not possible in the CIA model.

This Chapter identifies, for the first time, a defective constriction response in the preclinical phase of inflammatory arthritis, coupled with increased expression of galectin-3 and CD3 T cells in the inflamed vasculature. Furthermore, elevated circulating T cell related cytokines in the preclinical phase of arthritis indicates that T cells and galectin-3 may play a role in early disease mechanisms that fuel pathology. However, targeting galectin-3 in the CIA model is challenging due to a shift in the phenotype of the model resulting in an acute onset and progression. This presents as a narrow window to measure the therapeutic effect of galectin-3 inhibition on vascular inflammation. While it was not possible to investigate the role of galectin-3 on vascular inflammation using the CIA model, studies have shown attenuated arthritis in *Lgals3*^{-/-} mice using the antigen-induced arthritis (AIA) model (Forsman et al. 2011). Therefore, the role of galectin-3 on vascular function during inflammatory arthritis could be investigated in an *in vivo* setting, that is primarily CD4⁺ T cell driven (Wong et al.) by the use of the AIA model of inflammatory arthritis and will be investigated in **Chapter 5** of this Thesis.

Chapter 4

Evaluating the role of galectin-3 in T Cells

4.1 Introduction

Galectin-3 is expressed in numerous immune and stromal cell types and has varied functions including cell adhesion, chemoattraction and apoptosis. The expression of galectin-3 changes according to the immune cell type and activation status of the cell. Its effects on disease pathology are dependent upon the tissue site and the cell type it is expressed in or on (Dumic et al. 2006). Galectin-3 is synthesized in the cytoplasm. It is found in the nuclear compartment, on the cell surface and in the extracellular environment (Fowles et al. 1995). Galectin-3 is expressed in macrophages, neutrophils, fibroblasts and T cells.

This Chapter evaluated the role of galectin-3 in T cells. Published literature revealed that galectin-3 orchestrates multiple and often opposing effects on T cells. The role of galectin-3 on T cell function was dependent upon where it was expressed in, on or around the cell. For example, endogenous galectin-3 is essential for IL-2 dependent cell growth. Indeed, primary T cells cell growth was impaired by downregulation of endogenous galectin-3 (Joo et al. 2001). Galectin-3 is only expressed in activated T cells, and it is found in both intracellular and extracellular compartments. Intracellular galectin-3 inhibited T cell apoptosis (Yang et al. 1996) and TCR activation (Chen et al. 2009). Contrary to this, extracellular galectin-3 promoted apoptosis of T cells (Fukumori et al. 2003). Several mechanisms were proposed for the regulation of T cell apoptosis by galectin-3. These include galectin-3 acting as a ligand for CD7 and CD29 receptors in Jurkat cells (**Table 1.3**), resulting in cytochrome c release and caspase-3 activation via mitochondrial apoptosis pathways (Yang et al. 1996; Fukumori et al. 2003). Cross talk mechanisms between intracellular and extracellular galectin-3 to maintain a balance in apoptosis of T cells was also reported (Nakahara et al. 2005).

The role of galectin-3 in controlling the lifecycle and fate of T-cells is not limited to its ability to promote and suppress apoptosis. Cell death was increased in an immortalized line of human T lymphocytes (Jurkat cells) by blocking galectin-3 expression (Yang et al. 1996). Furthermore, cell proliferation was decreased in primary T cells by decreasing the level of endogenous galectin-3 in culture using galectin-3 antisense oligonucleotides (Joo et al. 2001).

Galectin-3 has diverse functions related to the regulation of the inflammatory response by T cells. It enhances cell adhesion by forming oligomers and bridging ligands for receptors (Ochieng et al. 2002). Galectin-3 also influences the balance between Th1/Th2

differentiation and regulates their effector responses (Breuilh et al. 2007). Maintaining the correct balance between Th1 “cellular immunity” and Th2 “humoral immunity” is vital for a healthy immune system. Disruption of Th1/Th2 homeostasis by a dominant Th1 response causes autoimmunity whereas a dominant Th2 response causes allergy and airway hypersensitivity.

The generation of galectin-3 knockout mice (*Lgals3^{-/-}*) provided excellent tools that enabled the delineation of specific T-cell-associated functions that promoted pathology in several experimental models. For example, galectin-3 deficiency decreased circulating IL-4 and increased IFN γ in a mouse model of ovalbumin-induced asthma (Zuberi et al. 2004). In a model of fungal infection, intracellular galectin-3 indirectly modulated Th17 cell responses through control of cytokine production by dendritic cells. Activated galectin-3^{-/-} dendritic cells showed higher IL-23 secretion which supported Th17 effector responses (Fermin Lee et al. 2013). In mouse models of experimental autoimmune encephalomyelitis (EAE), galectin-3 deficiency lessened IL-17 levels and increased the number of FoxP3 regulatory T cells in the circulation (Jiang et al. 2009).

The literature suggests that the role of galectin-3 in T cells is context dependent and can vary depending on the status of immune activation or disease state in which it is acting. A systematic understanding of how galectin-3 contributes to T-cell associated inflammation is still lacking. Furthermore, there are few studies that investigate the therapeutic potential of suppressing galectin-3 to modulate T cell survival and effector functions. This Chapter will delineate galectin-3 dependent mechanisms that regulate T cell responses, including expansion profiles, proliferation, apoptosis and cytokine output.

Hypothesis and Aims

The overarching hypothesis of this Chapter is that galectin-3 modulates T cell phenotypes and their associated biology. To test this hypothesis, the following aims were set:

Aim 1: To characterise galectin-3 expression in CD4 T cells.

Aim 2: To evaluate the effect of galectin-3 on CD4 T cell proliferation, differentiation and survival through utilising GB1107 as a tool to dissect galectin-3 dependent cellular mechanisms.

Aim 3: To combine use of newly developed T cell bioassays and *Lgals3*^{-/-} T cells to investigate the specificity of GB1107 for galectin-3.

4.2 Results

4.2.1 Galectin-3 is upregulated in CD4 T cells during experimental inflammatory arthritis

To establish if there was a relationship between galectin-3 expression and the induction of a T cell-dependent mouse model of arthritis (mBSA antigen-induced arthritis; AIA), RNA-sequencing datasets generated in our group were interrogated. First, RNA sequencing of whole synovial tissue recovered from mice at Day 10 of AIA (**Section 2.3.6**) revealed that *Lgals3* (protein coding gene for galectin-3) transcripts were significantly higher when compared to healthy control synovial tissue from naïve mice (**Figure 4.1 A**). This increased *Lgals3* expression is consistent with the induction of galectin-3 by various leukocyte populations upon immune activation and likely reflects the increased recruitment of these cells to the inflamed joint during arthritis (Sano et al. 2000; van den Berg et al. 2007). To more specifically investigate *Lgals3* expression in CD4 T cells during AIA, expression was also evaluated in splenic and joint-infiltrating CD4 T cells. Ten days after systemic challenge with mBSA/CFA, *Lgals3* was significantly upregulated in effector CD4 T cells (CD4⁺CD25⁻CD44^{hi}CD62L^{lo}) recovered from the spleen when compared with naïve CD4 T cells (CD4⁺CD25⁻CD44^{lo}CD62L^{hi}) and was amongst the most highly differentially expressed genes (top 31 genes; p.adj ≤ 0.05, log2FC ≥ 1) (**Figure 4.1 B**). Consistent with this observation, *Lgals3* was also among the most highly differentially expressed genes (top 20 genes; p.adj ≤ 0.05, log2FC ≥ 1) when comparing joint-infiltrating CD4 T cells from mice at Day 10 of AIA with naïve splenic CD4 T cells (**Figure 4.1 C**). Together, the above data show that *Lgals3* is induced in CD4 T cells *in vivo* upon induction of a T cell-dependent model of inflammatory arthritis and may therefore play a role in CD4 T cell effector function and the progression of AIA.

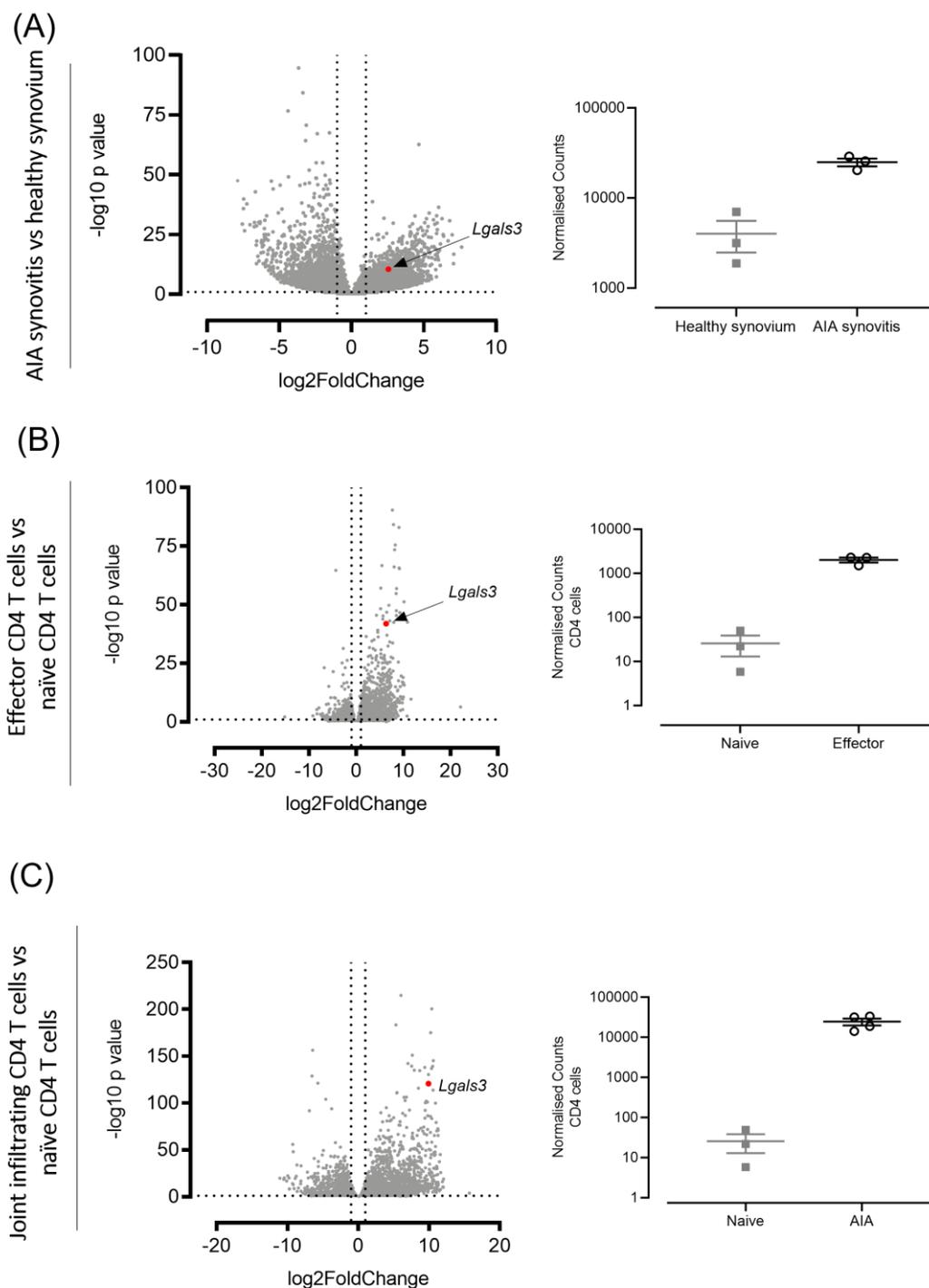


Figure 4.1: The expression of *Lgals3* is upregulated during AIA

(A-C) Volcano plots of protein-coding genes from RNA-sequencing analysis performed in wild-type mice during AIA. Dotted lines identify significant genes ($p_{\text{adj}} \leq 0.05$, $\log_2 \text{FC} \geq 1$). (A) Synovium was recovered from the inflamed joints of mice with AIA at Day 10 and compared to healthy mice. RNA-sequencing was performed on whole synovial tissue. (B) RNA-sequencing of effector CD4 cells ($\text{CD4}^+ \text{CD25}^- \text{CD44}^{\text{hi}} \text{CD62L}^{\text{lo}}$) compared to naïve CD4 cells ($\text{CD4}^+ \text{CD25}^- \text{CD44}^{\text{lo}} \text{CD62L}^{\text{hi}}$) isolated from the spleens of mice 10 days after challenge with mBSA/CFA. (C) RNA-sequencing of CD4 T cells isolated from the synovium of mice with AIA compared to naïve splenic CD4 T cells. Data are shown as mean \pm SEM. AIA, RNA-sequencing and bioinformatic pipelines performed by D. Hill with

bioinformatic support from the SIURI, Cardiff University. Interrogation of the available datasets for Lgals3 expression performed by A. Morrin.

4.2.2 Galectin-3 is located intracellularly and extracellularly in CD4 T cells

Galectin-3 is expressed in the cytosol of T cells (Chen et al. 2009). As an initial step, culture conditions were established to test galectin-3 detection in activated CD4 T cells by flow cytometry, consistent with its reported expression in the literature and supported by my analysis of RNA-sequencing datasets presented in **Figure 4.1** (Joo et al. 2001; Hsu et al. 2009). Splenic CD4 T cells were isolated from DBA/1 mice, activated by anti-CD3/CD28 co-stimulation and followed by galectin-3 detection performed by flow cytometry. To quantify galectin-3 expression, cells were stimulated with PMA and ionomycin in the presence of monensin (**Section 2.11.1**). Flow cytometry can be used to discriminate between intracellular and extracellular proteins (Adan et al. 2017). Therefore, studies first determined whether galectin-3 could be detected in CD4 T cells and if so, whether it was located intracellularly or extracellularly. To help discriminate cellular location, cells were either stained for cell surface galectin-3 alongside CD3 and CD4, or first stained for the cell surface markers CD3 and CD4 before permeabilisation to allow intracellular detection of galectin-3 (**Section 2.11.1**).

Following both cell surface and intracellular staining, 85% of anti-CD3/CD28 activated cells were galectin-3 positive (**Figure 4.2 A**). Detection of galectin-3-positive cells was highest when extracellular and intracellular staining was performed together, suggesting that galectin-3 was more prominently localised intracellularly (~50% of cell staining accounted for by intracellular detection, ~25% of positive staining by extracellular detection) (**Figure 4.2 B**).

These early pilot experiments were performed using activated CD4 T cells isolated from DBA/1 mice. In order to carry out more robust phenotypic analyses of galectin-3 in CD4 T cells, subsequent experiments used CD4 T cells from C57Bl/6 mice which are more conventionally used to study CD4 T cells *in vitro* and would also allow comparisons involving galectin-3-deficient (*Lgals3^{-/-}*) mice available on the C57Bl/6 background.

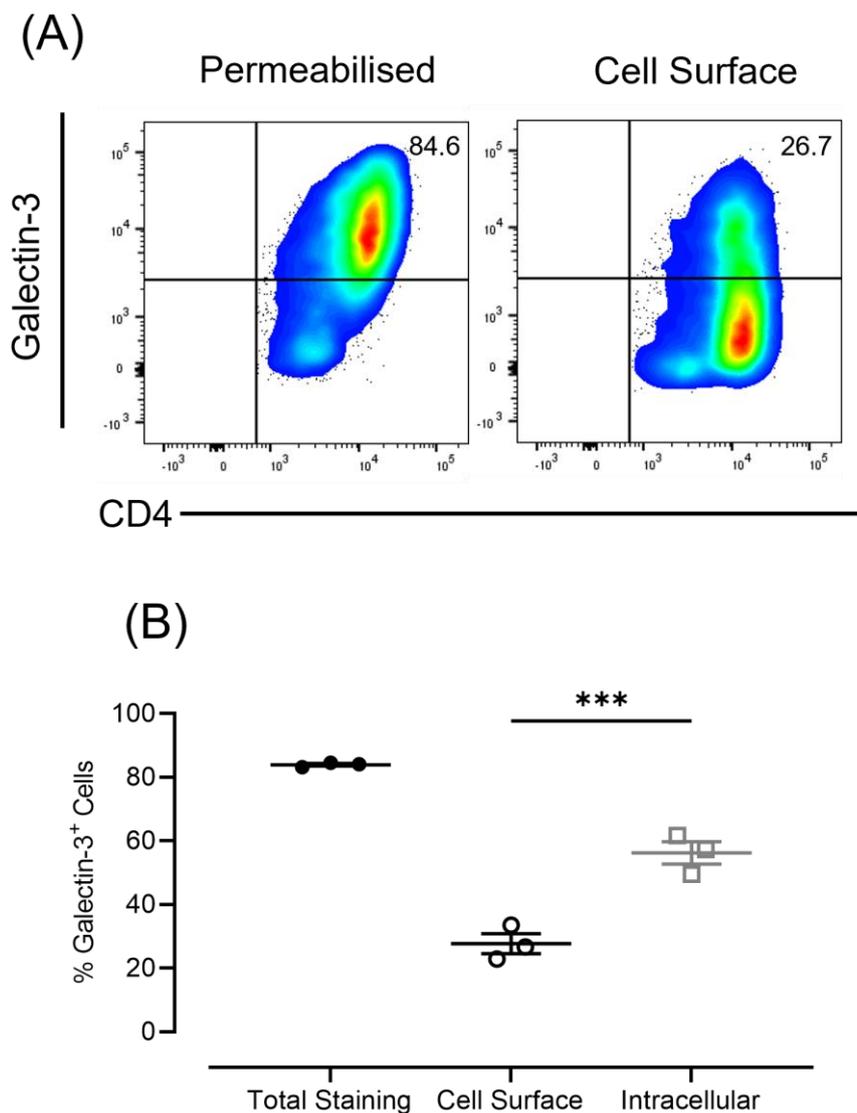


Figure 4.2: CD4 cells express intracellular and extracellular galectin-3

Splenic CD4 T cells from DBA/1 male mice were isolated by negative MACS separation and cultured in the presence of anti-CD3 and anti-CD28 for 96 hours. Cells were stimulated with PMA, ionomycin and monensin for the final 4 hours prior to antibody staining for the cell surface markers CD3 and CD4 and galectin-3. For intracellular staining, cell membranes were permeabilised before staining for galectin-3. (A) Representative FACS plots of cell surface staining and permeabilised staining. (B) Proportion of intracellular galectin-3 detection was calculated by subtracting cell surface staining from total staining. Significance was calculated by one-way ANOVA with Tukey post-test. *** = $p < 0.001$. Data are representative of 1 experiment and is shown as mean \pm SEM.

4.2.3 Permeabilisation of resting CD4 T cells results in galectin-3 detection

A study by Joo *et al* showed that resting CD4 T cells do not express galectin-3 (Joo et al. 2001). The data presented in **Figure 4.2** showed that a high percentage of activated CD4 cells expressed galectin-3. However, these initial experiments that aimed to optimise galectin-3 detection by flow cytometry did not include resting CD4 cells as negative controls. Taking this into account, and to appropriately control this experiment, splenic CD4 cells were isolated from C57Bl/6 mice and immediately stained for intracellular and extracellular galectin-3 (termed non-cultured cells). Additionally, to determine if cells spontaneously express galectin-3 while resting in culture (i.e., cultured in RPMI media without addition of anti-CD3/CD28), cells were plated and left to rest at 37°C for 4 hours (the same length of time as cells stimulated with PMA and ionomycin in the presence of monensin). Cells were stained for CD3, CD4 and galectin-3 and analysed by flow cytometry. Negligible detection of surface galectin-3 was observed in non-cultured or resting CD4 T cells or in resting CD4 T cells stimulated with PMA, ionomycin in the presence of monensin (**Figure 4.3 A - B**). Unexpectedly, upon permeabilization of cells under the same conditions, a high proportion of CD4 cells showed robust intracellular galectin-3 staining (**Figure 4.3 A - B**). Previous studies suggest that resting CD4 T cells do not express intracellular galectin-3 (Joo et al. 2001). Moreover, my RNA-sequencing analysis of CD4 T cells during AIA similarly suggests that *Lgals3* is highly upregulated in effector CD4 T cells following activation (**Figure 4.1**). Therefore, detection of galectin-3 by flow cytometry in permeabilised cells either suggests that resting CD4 T cells contain a reservoir of intracellular galectin-3, or that detection of galectin-3 in these cells is an artefact associated with the permeabilisation protocol. As intracellular staining was an important aspect of subsequent experiments, where detection of CD4 T cells subsets (e.g., by the signature cytokines IFN γ and IL-17) was important, detection of *Lgals3* transcripts by qPCR was tested as a more reliable way to track expression (**Figure 4.3 D**). Here, activation of CD4 T cells with a combination of PMA, ionomycin and monensin resulted in a significant increase in *Lgals3* expression detected by qPCR and was therefore adopted as the method to track galectin-3 expression *in vitro*.

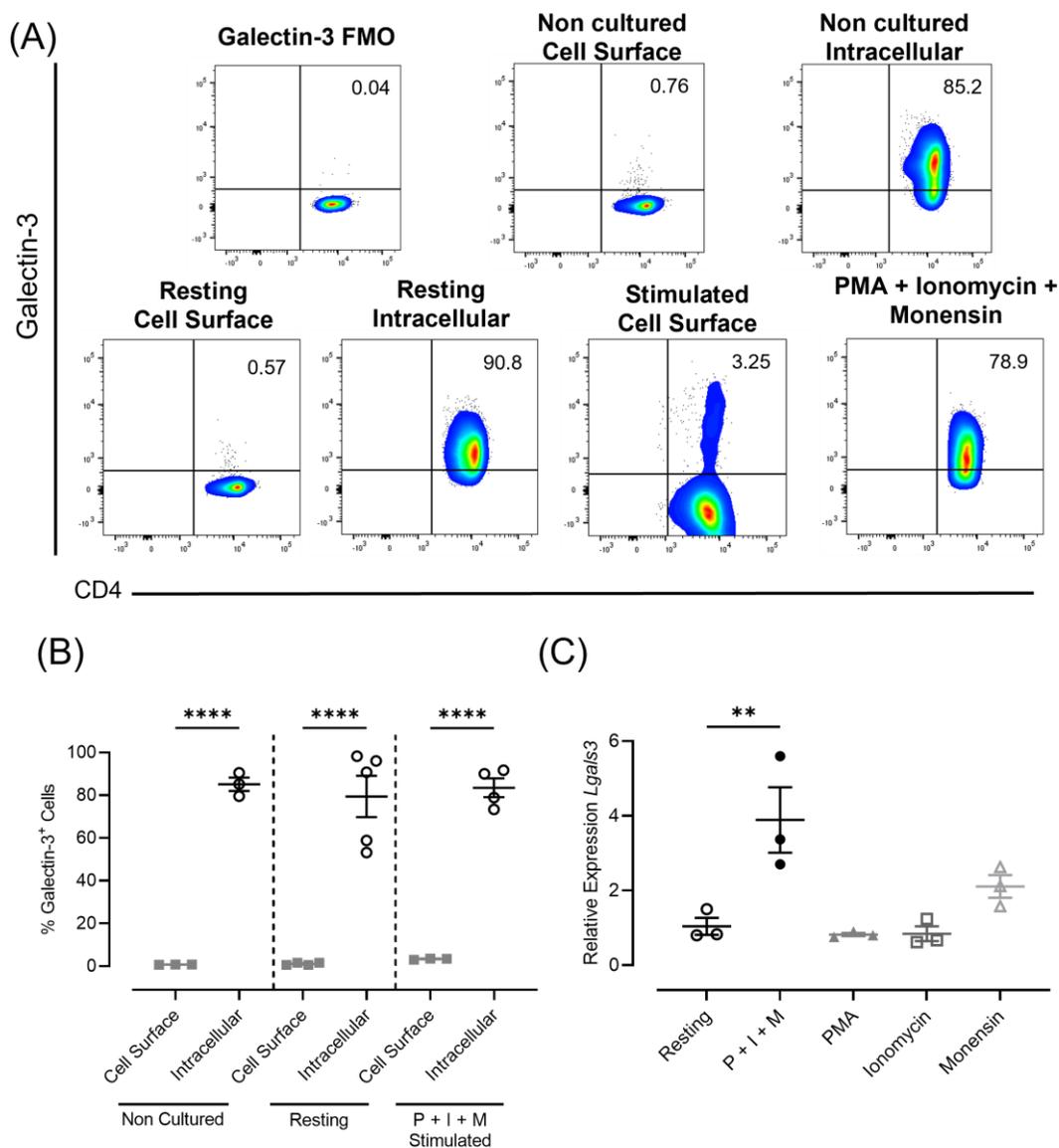


Figure 4.3: Stimulation of resting CD4 cells with PMA, ionomycin and monensin increases galectin-3 levels.

(A) Representative FACS plots of galectin-3 expression in response to stimulants. FMO controls were used to aid the gating strategy. The number in the CD4⁺ galectin-3⁺ quadrant relates to the percentage of galectin-3⁺ cells. (B) Splenic CD4⁺ cells were isolated from WT C57Bl/6 mice and were cultured in the presence of PMA (P) or ionomycin (I) or monensin (M) for 4 hours. Graphs are representative of 2 independent experiments. Significance was calculated using a one-way ANOVA with Sidak post-test. (C) qPCR for *Lgals3* showing relative expression normalised to resting cells harvested at the same timepoint. Relative expression was quantified using the Delta-Delta Ct method and normalised to housekeeping gene *Actb*. Significance was calculated using a one-way ANOVA with Tukey post-test. Data are representative of 1 experiment. ** = $p < 0.01$, **** = $p < 0.0001$. Error bars are shown as mean \pm SEM.

4.2.4 *Lgals3* is highly expressed in CD4 T cells under Th17-polarising conditions

Although it is known that galectin-3 is expressed in CD4 cells, the next objective of this Chapter was to quantify galectin-3 on various CD4 T cell subsets. To determine this, CD4 T cells were cultured for 48 hours in conditions (**Section 2.9.3**) to promote the differentiation of Th1, Th2 and Th17 cells, followed by qPCR detection of *Lgals3*. There was a significant increase in *Lgals3* mRNA in Th17-polarising while conditions (**Figure 4.4 A**), indicating that galectin-3 is strikingly expressed in these cells. While there was a slight increase in *Lgals3* mRNA in Th1-polarising conditions compared to Th0, this was not significant. Although previous studies have shown that galectin-3 deficiency results in decreased IL-17 production from splenic cells (Forsman et al. 2011), and that *Lgals3* is identified in a transcriptional signature in pathogenic Th17 cells (Lee et al. 2012), few have investigated directly targeting galectin-3 action in CD4 T cells as a way to modulate Th17 effector responses and associated pathology. To this end, galectin-3 gene and protein expression was characterised during T cell activation and differentiation.

First, the temporal regulation of galectin-3 expression by qPCR throughout the time course of the Th17 cell differentiation was considered. For this, CD4 cells were cultured under Th17 polarising conditions and mRNA was harvested after 24, 48, 72 and 96 hours of culture. *Lgals3* expression was significantly elevated at 24 and 48 hours and returned to baseline by 72 hours (**Figure 4.4 B**). Therefore, *Lgals3* expression is induced early during activation of CD4 T cells under Th17 differentiating conditions, and 24 – 48 hours post activation is an appropriate timepoint to track *Lgals3* expression by qPCR.

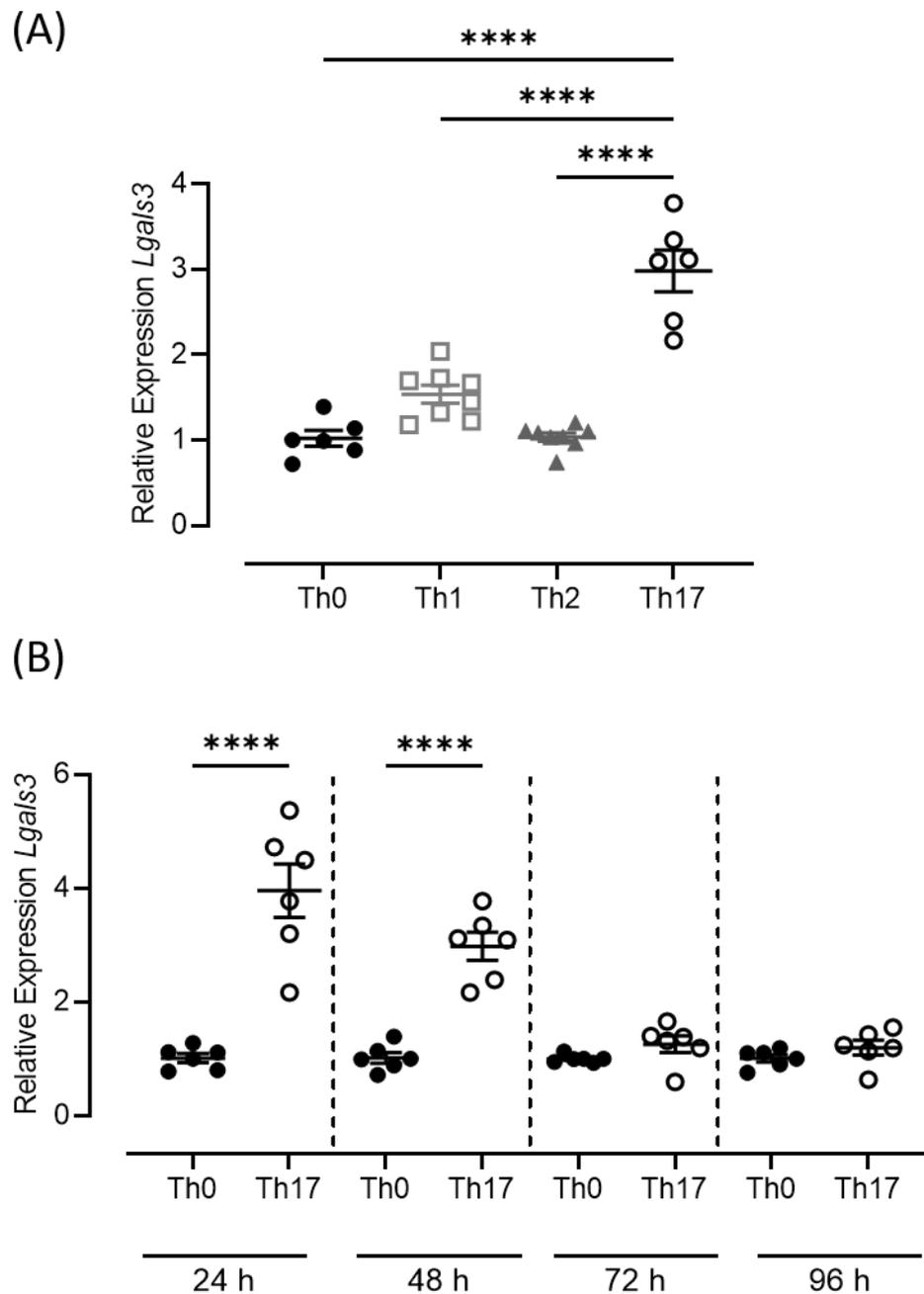


Figure 4.4: *Lgals3* expression is induced in CD4 T cells under Th17-polarising conditions

CD4 T cells were cultured in Th1, Th2 and Th17 differentiating conditions. Th0 conditions represent cells activated with anti-CD3/CD28 in RPMI media without exogenous cytokines. (A) qPCR for *Lgals3* was performed after 48 hours of culture. Data are representative of 2 independent experiments. Significance was calculated by one-way ANOVA with Tukey post-test. (B) qPCR for *Lgals3* at 24, 48, 72 and 96 hours of culture. Data are representative of 2 independent experiments. *Lgals3* expression is presented as relative expression to a Th0 sample at the same time point. Significance was calculated by two-way ANOVA with Tukey post-test where **** = $p < 0.0001$. Data are shown as mean \pm SEM.

4.2.5 Galectin-3 is secreted by Th17 cells in a time dependent manner

To determine if the increase in *Lgals3* expression in Th17 cell cultures resulted in increased soluble galectin-3 protein in the culture media, a galectin-3 ELISA was performed on the supernatants from T cells cultured under all polarising conditions at 24, 48, 72 and 96 hours. Galectin-3 levels increased in cell culture supernatants under Th0 and Th17 conditions during the experimental time-course (**Figure 4.5**). While there was no differences in galectin-3 secretion between Th17 and Th0 cells at 24 and 48 hours, a statistically significant difference was seen at 72 and 96 hours. Galectin-3 concentration was also higher in Th17-polarising conditions compared to Th1 and Th2 conditions at these time points. Thus, the temporal increase in *Lgals3* gene expression at 24 and 48 hours was followed by a concomitant increase in protein expression. Galectin-3 was not secreted from Th1 or Th2 cells, which is consistent with the finding that *Lgals3* transcripts was uniquely elevated in Th17 cells (**Figure 4.4**). The selective expression of galectin-3 at the gene and protein level in CD4 T cells cultured under Th17 polarising conditions suggests a potential role for galectin-3 in regulating the expansion, survival, proliferation and/or maintenance of Th17 effector functions.

4.2.6 A small molecule inhibitor of galectin-3 (GB1107) inhibits *Il17a* expression during Th17 differentiation

The heightened expression of *Lgals3* and soluble galectin-3 in Th17 cells suggests a role for galectin-3 in Th17 cell regulation and provided an assay system to investigate inhibitors of galectin-3 in clinical development. The small molecule inhibitor, GB1107, was used to investigate potential galectin-3 dependent mechanisms involved in Th17 cell regulation. Initially, a pilot study to investigate the effects of galectin-3 inhibition on *Il17a* expression was conducted, where 20 μ M GB1107 was added to Th17 differentiation cultures and the expression of the Th17 signature cytokine, IL-17A, determined by qPCR.

A pilot study suggested that 20 μ M GB1107 effectively inhibited *Il17a* expression in CD4 T cells (**Figure 4.6**). However a low recovery of RNA (<10 ng/ μ L; data not shown) from cultures containing 20 μ M GB1107 was noted. To determine if GB1107 selectively inhibited *Il17a* expression or affected the viability of CD4 T cells that could account for low RNA yields, a dose titration of GB1107 was performed. Cell viability and the effect of GB1107 on

T cell differentiation under various Th-polarising conditions was assessed by flow cytometry.

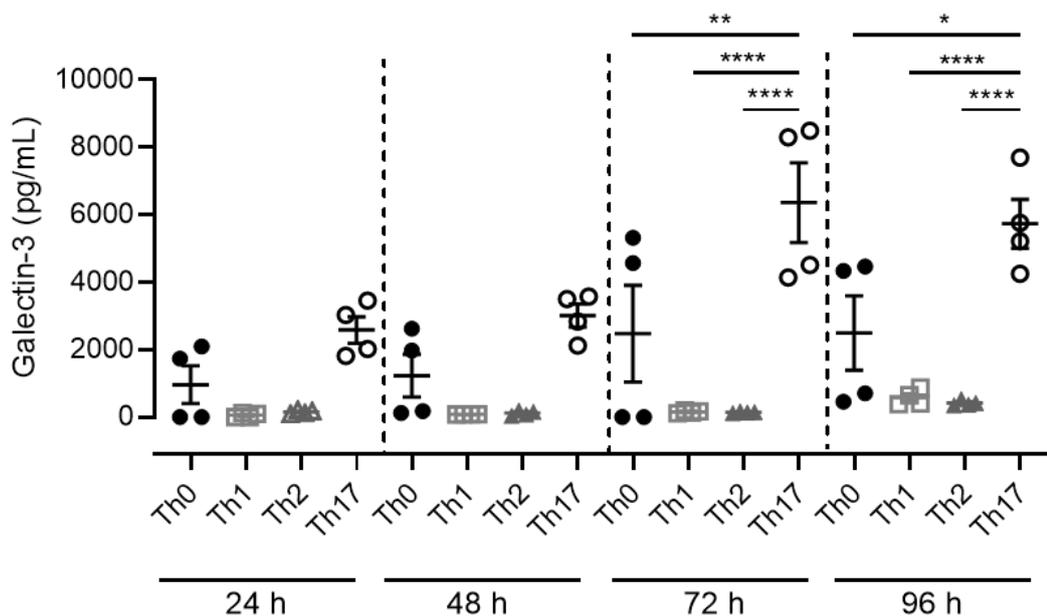


Figure 4.5: Galectin-3 is secreted by Th17 cells

Supernatants from T cells cultured for 24, 48, 72 and 96 hours under the indicated polarising conditions. Soluble galectin-3 levels were measured by ELISA. Data are representative of 2 independent experiments. Significance was calculated by two-way ANOVA with Tukey post-test. ** = $p < 0.001$, **** = $p < 0.0001$. Data are presented as mean \pm SEM.

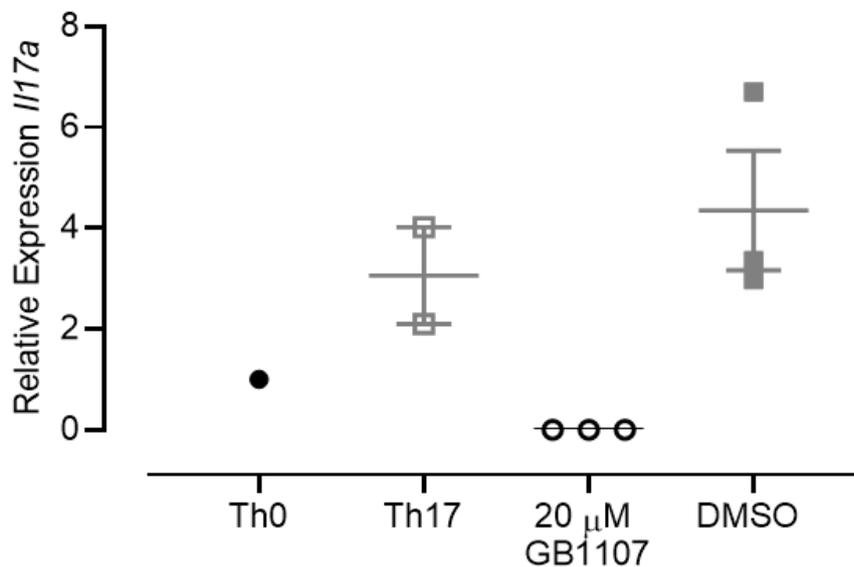


Figure 4.6: Loss of *IL17a* expression in Th17 cell cultures treated with 20 μ M GB1107

CD4 cells were cultured in Th17 differentiating conditions, and in the presence of 20 μ M GB1107 or DMSO vehicle control for 48 hours. qPCR for *IL17a* was performed using TaqMan reagents. "Th17" is indicative of cells in Th17 culture conditions but not treated with GB1107 or DMSO. Data are representative of 1 pilot experiment and are shown as mean \pm SEM.

4.2.7 Th17 cells remain viable in concentrations up to 20 μ M GB1107

Before investigating the effects of GB1107 on Th17 cells, a titration of GB1107 was performed to determine a working concentration range. A concentration range from 1 μ M to 200 μ M GB1107 was included in Th17 cultures and cells were stained with Zombie Aqua stain to determine viability. Zombie Aqua binds to intracellular proteins in cells that have compromised and/or permeabilised membranes as a result of cell death. Therefore, cells that stain positive for Zombie Aqua are considered dead. High concentrations of GB1107 resulted in loss of cell viability, as evidenced by a 70% decrease in percentage of live cells (**Figure 4.7 A,B**).

DMSO was the vehicle control for GB1107, which is known to be toxic to T cells at higher concentrations. In my studies, significant toxicity was only observed at concentrations of >1% v/v (**Figure 4.7 B, C**), which was consistent with previous studies (Timm et al. 2013). Therefore, GB1107 was prepared in DMSO at the highest concentration possible where solubility was not adversely affected (1000 μ M) and further diluted to the final concentrations listed in **Figure 4.7 B, C** to ensure the lowest amount of DMSO possible in cultures.

To allow the effect of galectin-3 inhibition on T cell differentiation to be studied, a working range of GB1107 that did not result in toxicity was selected to be used for future assays. As the viable concentration range of GB1107 was determined to be within 2 μ M and 20 μ M (allowing for potential partial toxicity at 15 μ M and 20 μ M), the next step was to observe the effect of GB1107 treatment on Th17 expansion.

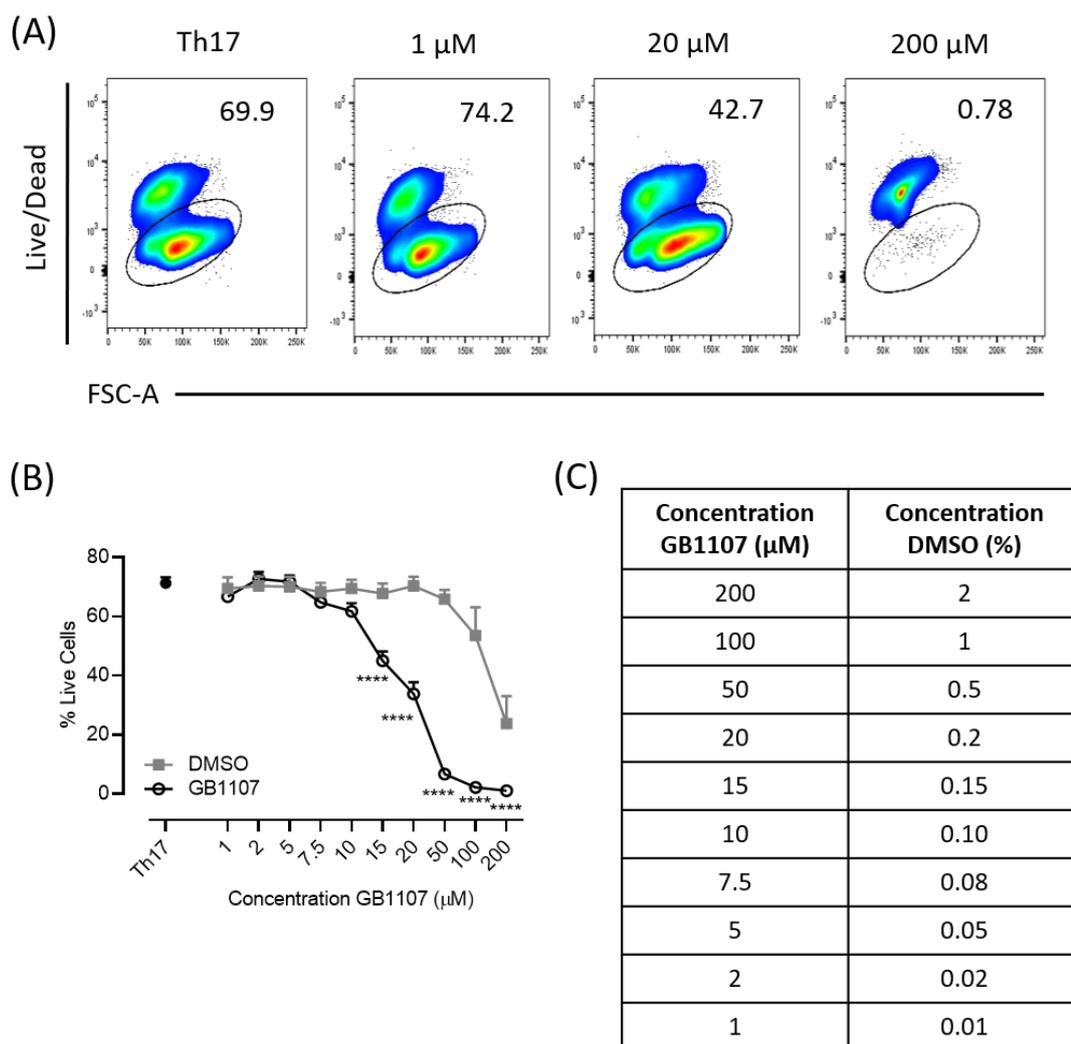


Figure 4.7: Concentrations of GB1107 above 50 μM results in reduced viability of CD4 cells

CD4 cells were cultured in Th17 differentiating conditions and in the presence of increasing concentrations of GB1107 for 96 hours. Samples were stained for live cells prior to fixing and acquisition on BD FACS Canto. (A) Representative flow plots at increasing concentrations of GB1107. Numbers indicate the percentage of lymphocytes in the live cell gate. (B) Graph showing % viable cells at different concentrations of GB1107 and equivalent amount of the DMSO vehicle. (C) The concentration of DMSO (v/v) for each concentration of GB1107. Data are representative of 5 independent experiments.

4.2.8 GB1107 inhibits Th17 expansion in a concentration dependent manner

To evaluate the effect of GB1107 treatment on Th17 cell expansion, CD4 T cells were cultured under Th17-polarising conditions with and without addition of GB1107 (1 μ M – 20 μ M) to the culture media. To track Th17 differentiation, the percentage of cells expressing the signature Th17 cytokine, IL-17A, was determined by flow cytometry. GB1107 treatment resulted in a potent and dose-dependent reduction in the development of IL-17A-producing cells (**Figure 4.8 A, B**) with a maximum percentage inhibition of 75% in GB1107 treated cells (**Figure 4.8 C**).

To measure if GB1107 had any effect on total CD4 cell number, cell counting beads were added to the cells immediately prior to performing flow cytometry. Although there is a decrease in total CD4 cell number in the presence of GB1107 or DMSO, these cell numbers are not significantly decreased as the concentration of GB1107 increases (**Figure 4.8 D**).

To test whether the concentration dependent inhibition of Th17 expansion by GB1107 was selective for Th17 cells, next experiments investigated the effect of GB1107 under other Th-polarising conditions.

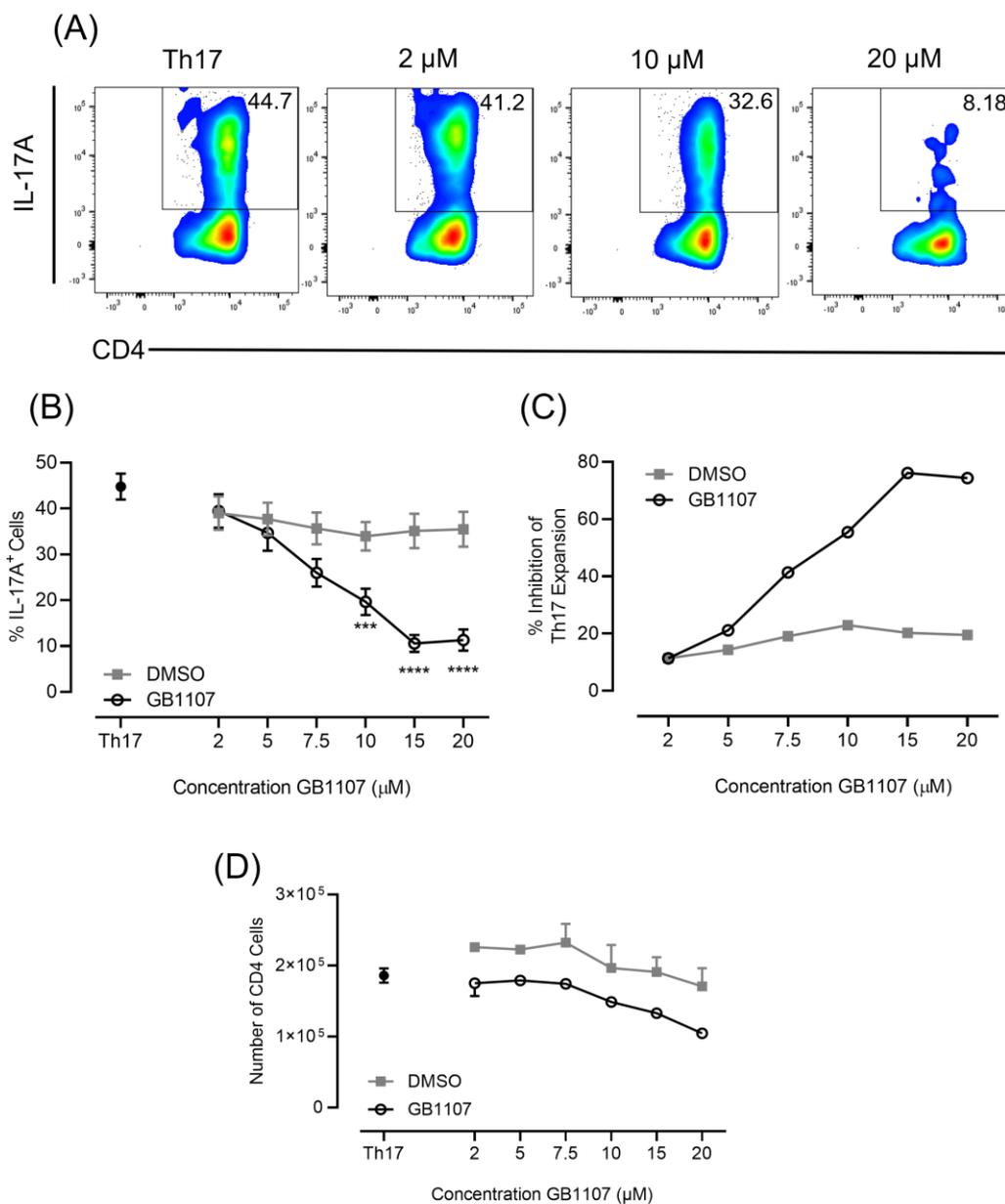


Figure 4.8: GB1107 inhibits Th17 expansion

CD4 cells were cultured in Th17 differentiating conditions and in the presence of increasing concentrations of GB1107 for 96 hours. Samples were stained for live cells, surface markers CD3 and CD4, and intracellular IL-17A by flow cytometry. (A) Representative flow cytometry plots at increasing concentrations of GB1107. Plots are gated on CD4⁺CD3⁺ cells. Number denotes the percentage of IL-17⁺ cells. (B-C) Graphs showing % of CD4 T cells producing IL-17 (B) and % inhibition of Th17 cells (C) in response to GB1107 concentrations. Data are representative of 9 independent experiments run in duplicate. Significance was calculated by 2-way ANOVA with Sidak post-test. (D) Cell counting beads were used to determine absolute cell number CD4 cells. *** = $p < 0.001$, **** = $p < 0.0001$ and data are shown as mean \pm SEM.

4.2.9 GB1107 does not have a direct effect on Th1 cell differentiation

To determine if the effect of GB1107 on T helper cell differentiation was selective for the inhibition of Th17 cells, CD4 T cells were cultured in Th1 differentiating conditions in the presence of GB1107 and stained for the signature Th1 cytokine, IFN γ . Approximately 20% of CD4 cells stained positive for IFN γ , evidencing a robust differentiation of Th1 cells in our cultures (**Figure 4.9 A**). Treatment with GB1107 did not significantly affect the viability of CD4 T cells (**Figure 4.9 B**). Furthermore, consistent with the selective expression of galectin-3 under Th17-polarising conditions (see **Figures 4.4, 4.5**), inhibition of galectin-3 by GB1107 treatment did not result in a loss of Th1 differentiation in our culture system (**Figure 4.9 A, C**). Thus far, the data supported a role for galectin-3 in Th17 cell differentiation or survival, where the small molecule inhibitor GB1107 promotes selective inhibition of Th17 cell differentiation. As selective inhibition of Th17 differentiation by targeting galectin-3 has potential therapeutic applications, it was also important to determine whether GB1107 affects the differentiation of other T helper cell subsets. For example, previous reports show that galectin-3 activity influences the balance between Th1 and Th2 cell expansion (Breuilh et al. 2007).

4.2.10 GB1107 significantly increases Th2 cell expansion

Th2 cells promote inflammation during allergy response, but can be protective against helminth infections and during inflammatory arthritis (Chen et al. 2016). Galectin-3 expression is not significantly increased in CD4 T cells cultured under Th2 differentiating conditions (**Figure 4.4 A**) but has shown to play a role in regulating the relationship between Th1 and Th2 responses in inflammation. Galectin-3 deficiency has shown to drive development of Th1 type response during *Schistosoma mansonii* infection and decreased Th2 responses during asthma models, highlighting the potential roles of galectin-3 in Th1-Th2 balance (Ruas et al. 2009). Under Th2 polarising conditions, approximately 12% of CD4 T cells produced the Th2 cytokine IL-4 (**Figure 4.1 A**). To determine if GB1107 had an effect on Th2 expansion, CD4 cells were cultured in Th2 differentiating conditions and IL-4 expression was quantified by flow cytometry. GB1107 did not negatively impact on CD4 cell viability in Th2 polarising conditions (**Figure 4.10 B**). Interestingly, GB1107 significantly increased IL-4 expression with the percentage of IL-4 expressing cells increasing from 11% to 40% at 20 μ M GB1107 (**Figure 4.10 C**). This finding indicates that the targeting of galectin-3 may allow for the inhibition of pathogenic Th17 cell responses, while favouring

Th2 cell expansion. This may be beneficial in certain autoimmune diseases such as rheumatoid arthritis, where Th17 cells responses are pathogenic while Th2-type responses have been associated with resolution of early joint inflammation (Skapenko et al. 2005; Chen et al. 2019).

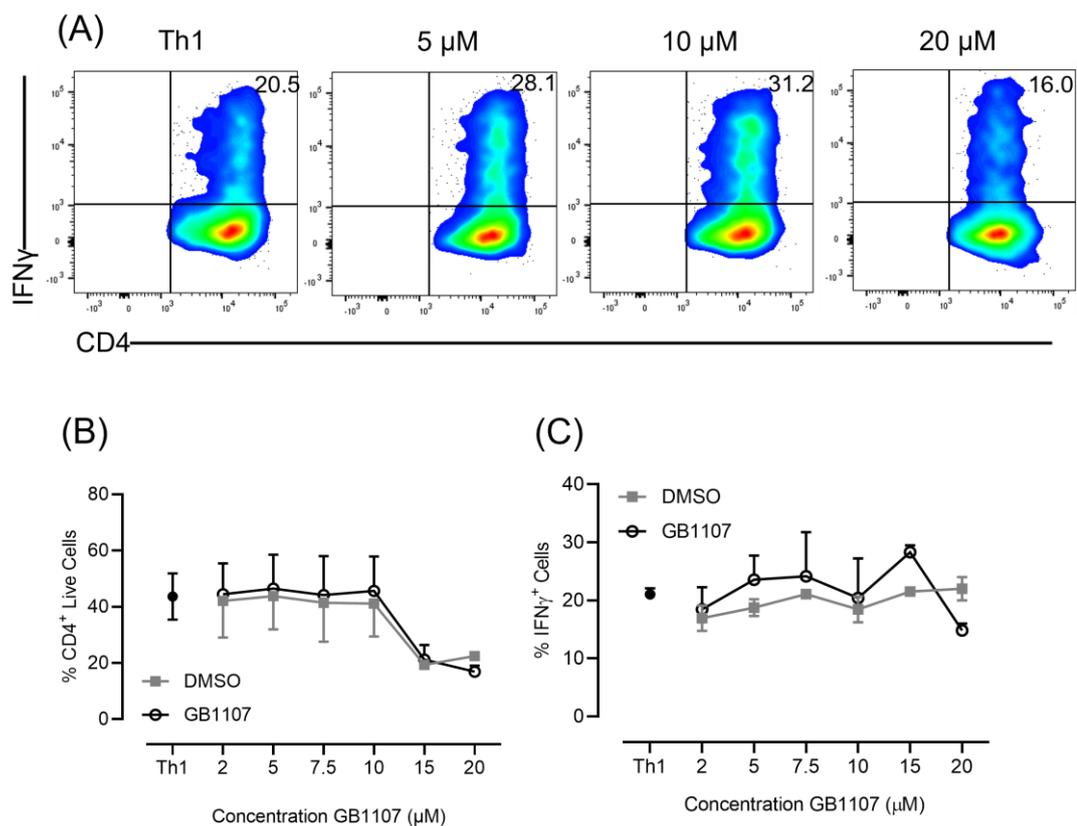


Figure 4.9: GB1107 does not affect Th1 cell expansion

CD4 cells were cultured in Th1 differentiating conditions and in the presence of increasing concentrations of GB1107 for 96 hours. Cells were stained for live cells, cell surface CD3 and CD4, and intracellular IFN γ by flow cytometry (A) Representative density plots showing IFN γ in live CD3 $^+$ CD4 $^+$ gated cells. (B) Graph showing percentage of viable cells in the presence of GB1107 or DMSO, as determined by negative ZombieAqua staining. (C) Percentage of IFN γ expressing cells when cultured in the presence of GB1107 versus DMSO. Data are shown as mean \pm SEM and is representative of 2 independent experiments at concentrations 1 to 10 μ M and n = 1 at 15 – 20 μ M..

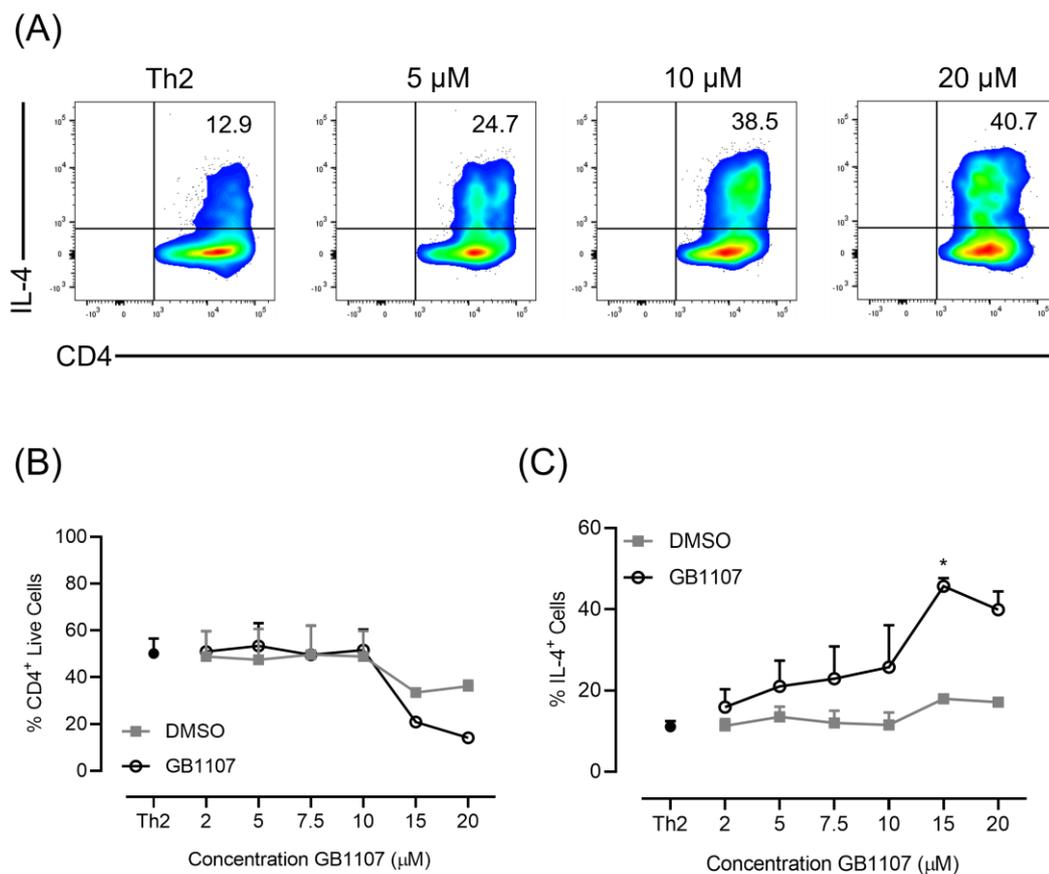


Figure 4.10: GB1107 significantly increases Th2 expansion

CD4 cells were cultured in Th2 differentiating conditions and in the presence of increasing concentrations of GB1107 for 96 hours. Cells were stained for live cells, cell surface CD3 and CD4, and the Th2 cytokine IL-4. (A) Representative flow cytometry plots of Th2 expansion. (B) Graphs showing cell viability, as determined by negative ZombieAqua staining. (C) Percentage of IL-4 expressing cells in the presence of GB1107 or DMSO. Significance is calculated by 2-way ANOVA with Sidak post-test. Data are representative of 2 independent experiments at concentrations 1 to 10 μ M and $n = 1$ at 15 – 20 μ M. * $p < 0.05$. Error bars are shown as mean \pm SEM.

4.2.11 GB1107 significantly increases Treg expansion

Th17 and Treg cells are often reciprocally regulated (Omenetti and Pizarro 2015). To determine if GB1107 influenced regulatory responses through control of FoxP3-positive Treg cells, CD4 cells were cultured in T-regulatory (Treg) differentiating conditions in the presence of GB1107 and stained for the Treg master transcriptional regulator, FoxP3. Here, Treg polarising conditions resulted in approximately 55% of CD4 T cells expressing FoxP3 (**Figure 4.11 A**). GB1107 did not affect overall CD4 cell viability in cells in Treg polarising conditions (**Figure 4.11 B**) and a modest but significant increase in the proportion of FoxP3-positive cells was observed in the presence of GB1107, with 67% of cells staining positive for FoxP3 at the highest dose of GB1107 (**Figure 4.11 A, C**).

The experiments presented above suggest that GB1107 may have application in autoimmune conditions where Th17 cells are associated with pathology, and where the expansion of populations that favour resolution of inflammation such as Treg and Th2 cells may be beneficial. In this regard, galectin-3 deficiency in EAE decreases IL-17 levels, increases the number of FoxP3 cells in the CNS and attenuates the severity of disease (Jiang et al. 2009). The remaining experiments presented in this Chapter were aimed at gaining mechanistic insight into how targeting galectin-3 using GB1107 may mediate these effects.

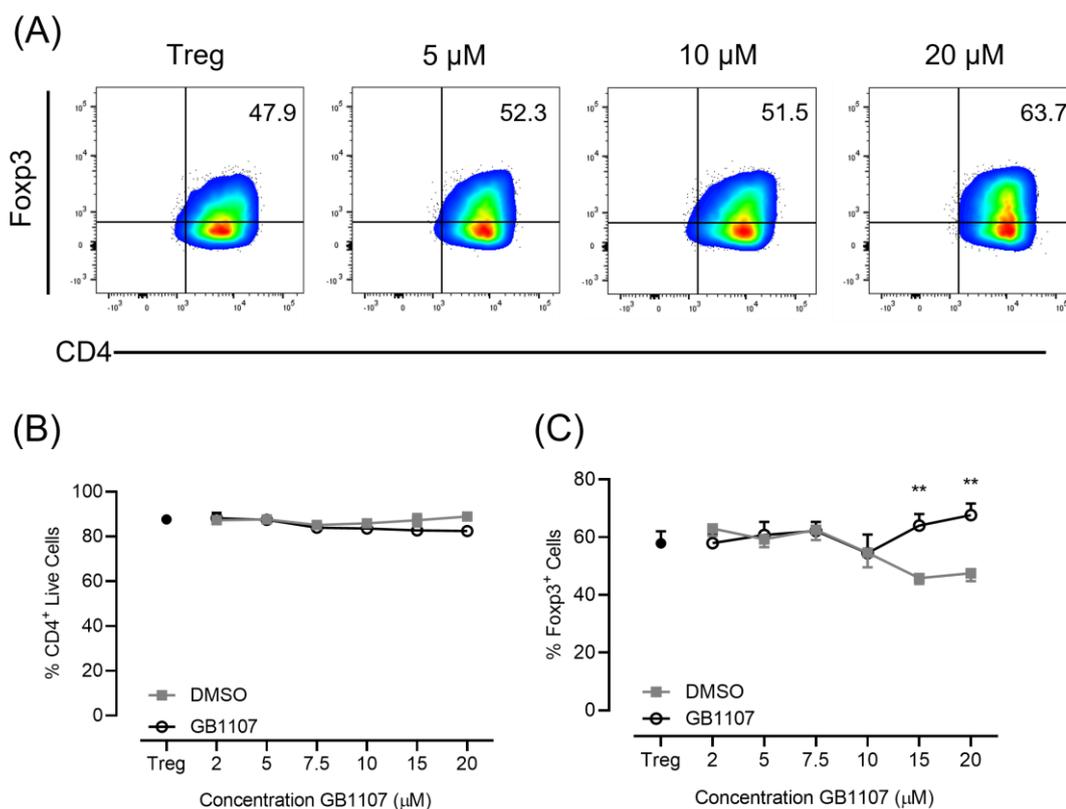


Figure 4.11: GB1107 enhances Treg expansion

CD4 cells were cultured in Treg differentiating conditions and in the presence of increasing concentrations of GB1107 for 96 hours. Cells were stained for live cells, surface markers CD3 and CD4, transcription factor marker FoxP3. Samples were fixed and run on BD FACS Canto. (A) Representative flow cytometry plots of Treg expansion. (B) Graph showing cell viability of CD4 cells as determined by negative ZombieAqua staining. (C) Graph showing percentage FoxP3 expressing cells when cultured in the presence of GB1107 or DMSO. Significance is calculated by 2-way ANOVA with Sidak post-test and $**=p<0.01$. Data are shown as mean \pm SEM and is representative of 2 independent experiments.

4.2.12 Inhibition of Th17 cell differentiation by GB1107 promotes a modest reciprocal increase in IFN γ production and FoxP3 expression

CD4 effector cells such as Th17 cells have a degree of plasticity and display a reciprocal relationship with Th1 and Treg cells (Zhao et al. 2010; Omenetti and Pizarro 2015). Th17 cells in the presence of IL-12, IL-23 and IL-1 β can co-express the Th17 master transcriptional regulator ROR γ t and the Th1-associated cytokine IFN γ . Furthermore, stimulation of Th17 cells with IL-12 can support the differentiation of cells to a more stable Th1 phenotype (**Figure 4.12 A**). This is true in conditions such as rheumatoid arthritis and colitis, where acquisition of IFN γ secretion by Th17 cells contributes to their pathogenicity (Harbour et al. 2015; Kotake et al. 2017). My studies show that GB1107 does not influence the differentiation of CD4 T cells under Th1 conditions (**Figure 4.9**). However, to determine if inhibition of IL-17 expression by GB1107 under Th17-polarising conditions resulted in a reciprocal increase in IFN γ expression, cells were co-stained for IFN γ and IL-17 secreting cells. As expected, GB1107 inhibited the proportion of cells secreting IL-17 (**Figure 4.12 B, C**). Interestingly, as IL-17A expression decreased, GB1107 caused a modest but significant increase in IFN γ expression at higher concentrations (**Figure 4.12 C, D**), with the percentage of IFN γ expressing cells increasing from 2% to 6% in Th17 cells treated with 20 μ M GB1107.

Both Th17 and Treg cells require TGF β for development and, similar to Th1 cells, Th17 and Treg cells also display a reciprocal relationship (Kleinewietfeld and Hafler 2013). While the absence of IL-6 favours the development of Treg cells, its presence inhibits FoxP3 expression and instead promotes ROR γ t induction and the differentiation of Th17 cells (Pasare and Medzhitov 2003; Bettelli et al. 2006) (**Figure 4.13 A**). In autoimmunity the balance between these cells is often disturbed and identifying mechanisms that favour the development of regulatory-type responses over Th17-type responses has therapeutic potential (Lee 2018). To determine if suppression of Th17 development as a result of galectin-3 inhibition caused a deviation towards Treg differentiation, cells were cultured in Th17 differentiating conditions in the presence of GB1107 and were stained for Treg marker, FoxP3. Consistent with my previous data, Th17 expansion was inhibited by GB1107 (**Figure 4.13 B, C**). While there was a trend toward increased FoxP3 expression (from 0.5% to 4% of CD4 T cells) following GB1107 treatment, this did not reach statistical significance (**Figure 4.13 D**).

Together, the above data suggest that GB1107 acts as a potent inhibitor of Th17 cells differentiation, which allows a modest expansion of Th1 and Treg cells in our cultures that likely reflects the reciprocal inhibition observed between Th17 cells and these other T helper populations.

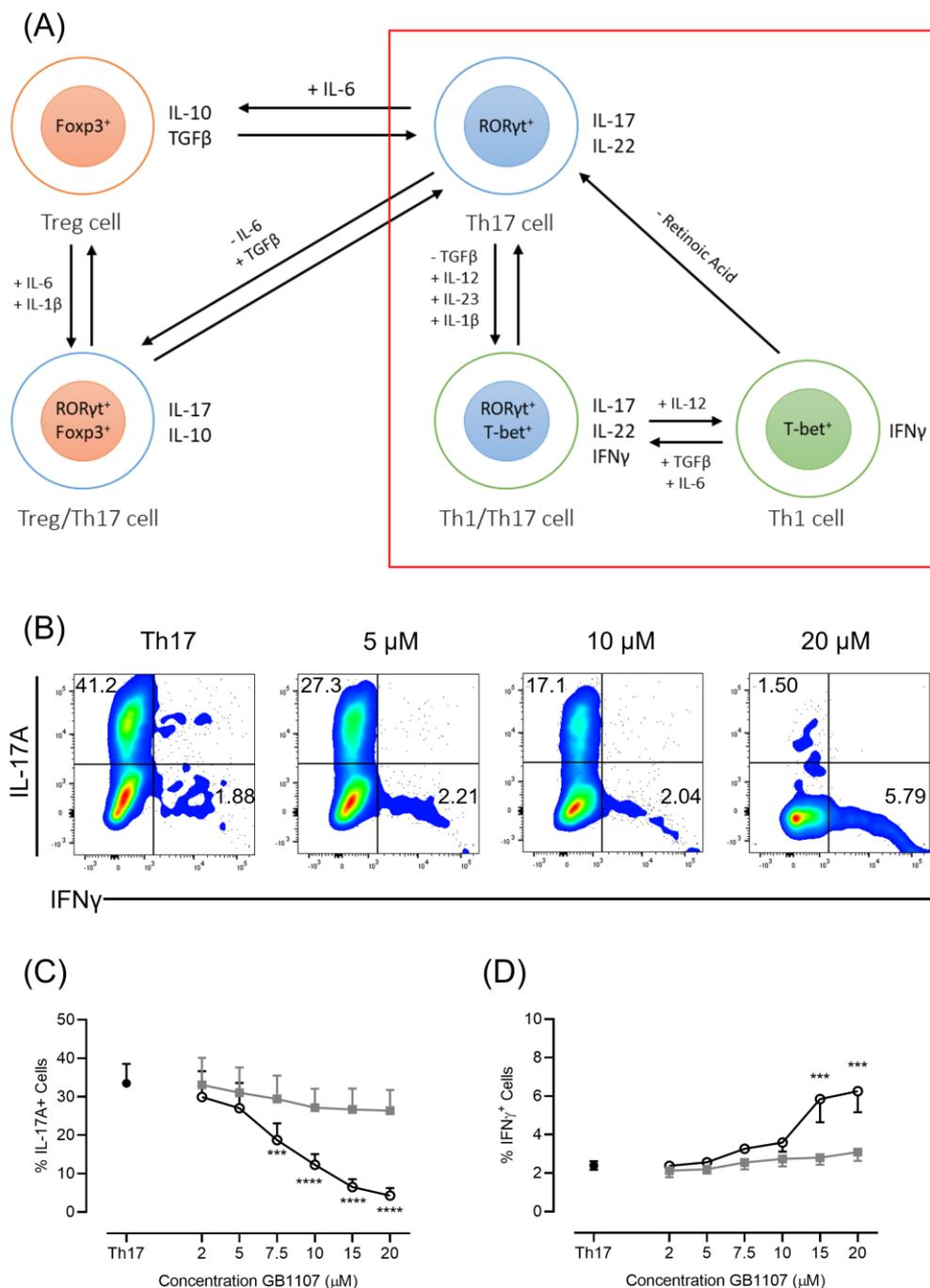


Figure 4.12: GB1107 causes a significant increase in IFN γ expressing cells under Th17 cell cultures

CD4 cells were cultured in Th17 differentiating conditions and in the presence of increasing concentrations of GB1107 for 96 hours. Cells were stained for live cells, surface markers CD3 and CD4, intracellular Th17 marker IL-17A and Th1 marker IFN γ . Samples were fixed and run on BD FACS Canto. (A) Overview of the reciprocal relationship between Th17 and Th1 cells. Figure adapted from Omenetti and Pizarro, 2015. (B) Representative flow cytometry plots of IL-17A and IFN γ expression. The numbers in each quadrant are the percentage of cells in the gate. (C) Graphs showing % IL-17A producing cells in the presence of GB1107. (D) Graph showing percentage IFN γ expressing cells in the presence of GB1107 or DMSO. Significance was calculated by 2-way ANOVA with Sidak post-test. Data are representative of 4 independent experiments. All graphs are shown as mean \pm SEM.

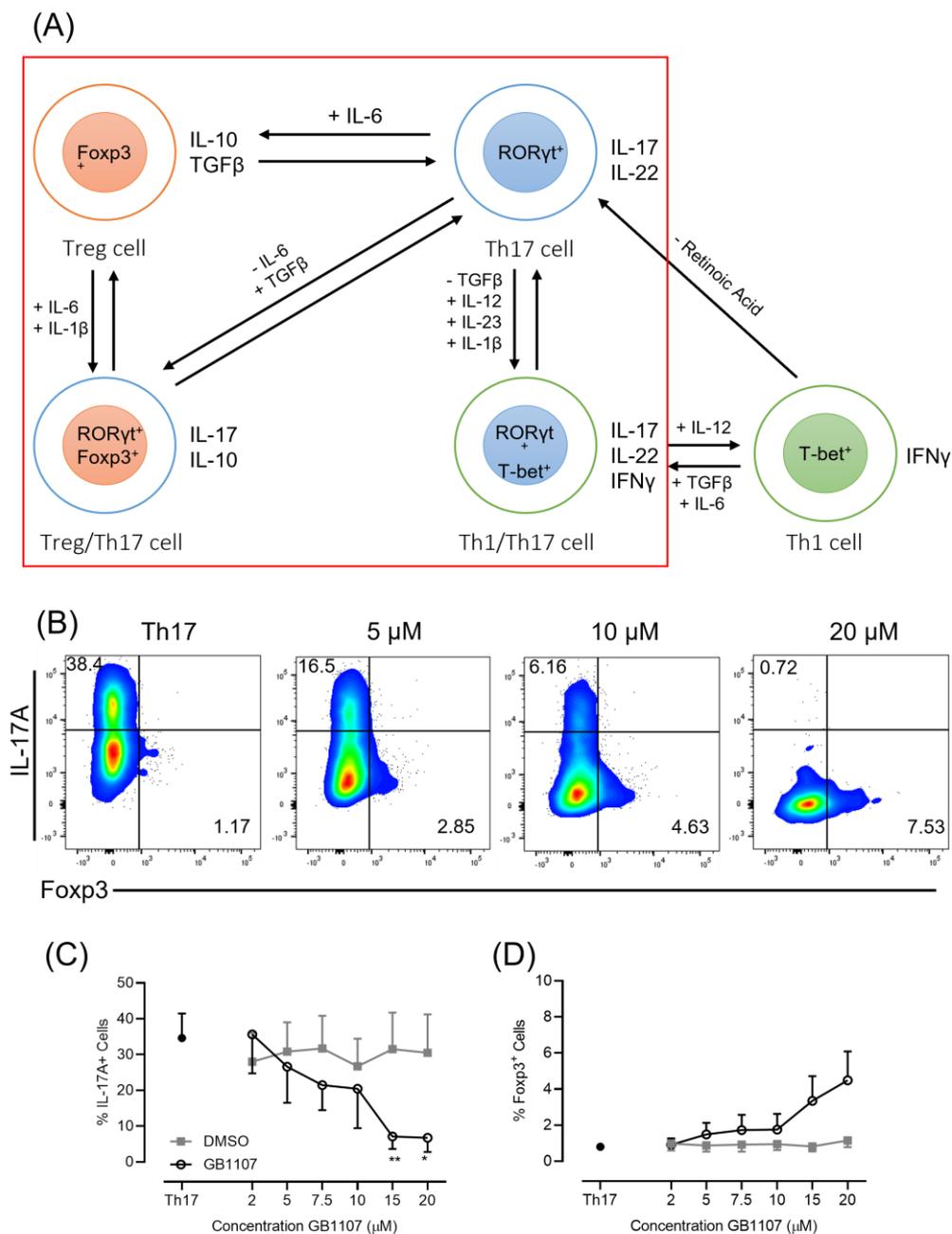


Figure 4.13 No change in FoxP3 expression in cells treated with GB1107

CD4 cells were cultured in Th17 differentiating conditions and in the presence of increasing concentrations of GB1107 for 96 hours. Cells were stained for live cells, surface markers CD3 and CD4 intracellular Th17 cytokine; IL-17A and Treg transcriptional regulator; FoxP3 by flow cytometry. (A) Overview of the reciprocal relationship between Th17 and Treg cells. Figure is adapted from Omenetti and Pizarro, 2015. (B) Representative flow cytometry plots. Numbers in each quadrant are the percentage of cells in that gate. (C) Graph showing percentage of IL-17A expressing cells in the presence of GB1107 or DMSO. Significance was calculated by 2-way ANOVA with Sidak post-test. (D) Percentage FoxP3⁺ cells in the presence of GB1107 or DMSO. Data are representative of 3 independent experiments. All graphs are shown as mean \pm SEM.

4.2.13 GB1107 inhibits the proliferation of CD4 cells under Th17-polarising conditions

The mechanisms underlying the inhibitory effect of GB1107 on Th17 expansion remain to be fully elucidated. To address this, a series of carboxyfluorescein fluorescent (CFSE) dilution assays were performed to assess cell proliferation. CFSE dye acts by covalently binding to intracellular molecules in lymphocytes, and when the cells undergo division the amount of CFSE in each cell depletes by half, allowing for each round of cell division to be quantified (Quah and Parish 2010). CD4 T cells were labelled with CFSE before culture in Th17 differentiating conditions in the presence and absence of GB1107. As observed before, there was a reduction in Th17 expansion in the presence of GB1107 (**Figure 4.14 A**). However, when CFSE profiles were examined, GB1107 also inhibited proliferation in a concentration dependent manner (**Figure 4.14 A, B**), based on the proportion of proliferating cells and the number of rounds of cell proliferation observed in the presence of GB1107 when compared to cells in Th17 polarising conditions in the absence of any treatment.

A second method to assess cellular proliferation in response to GB1107 was also employed. Th17 cell cultures were stained for the proliferation marker Ki67. Ki67 is a widely used marker of cell proliferation. It is a nuclear protein that is expressed during all active phases of the cell cycle but is not expressed in resting cells (i.e., cells that are in a G0 state). Ki67 has roles in various stages of the cell cycle including promoting normal cellular distribution of heterochromatin antigens and prevention of aggregation of mitotic chromosomes (Sun and Kaufman 2018). Given these roles, Ki67 is expressed in every cell undergoing mitosis and is therefore used as a marker of proliferation. Cells were gated based on lymphocytes, single cells and Ki67⁺ cells (**Supplemental Figure 5**) as per the literature (Soares et al. 2010). There was a significant decrease in Ki67-positive cells in the presence of 15 μ M and 20 μ M GB1107 (**Figure 4.14 D, E**). Together, the above data suggests that GB1107 acts to inhibit the proliferation of CD4 T cells under Th17-polarising conditions.

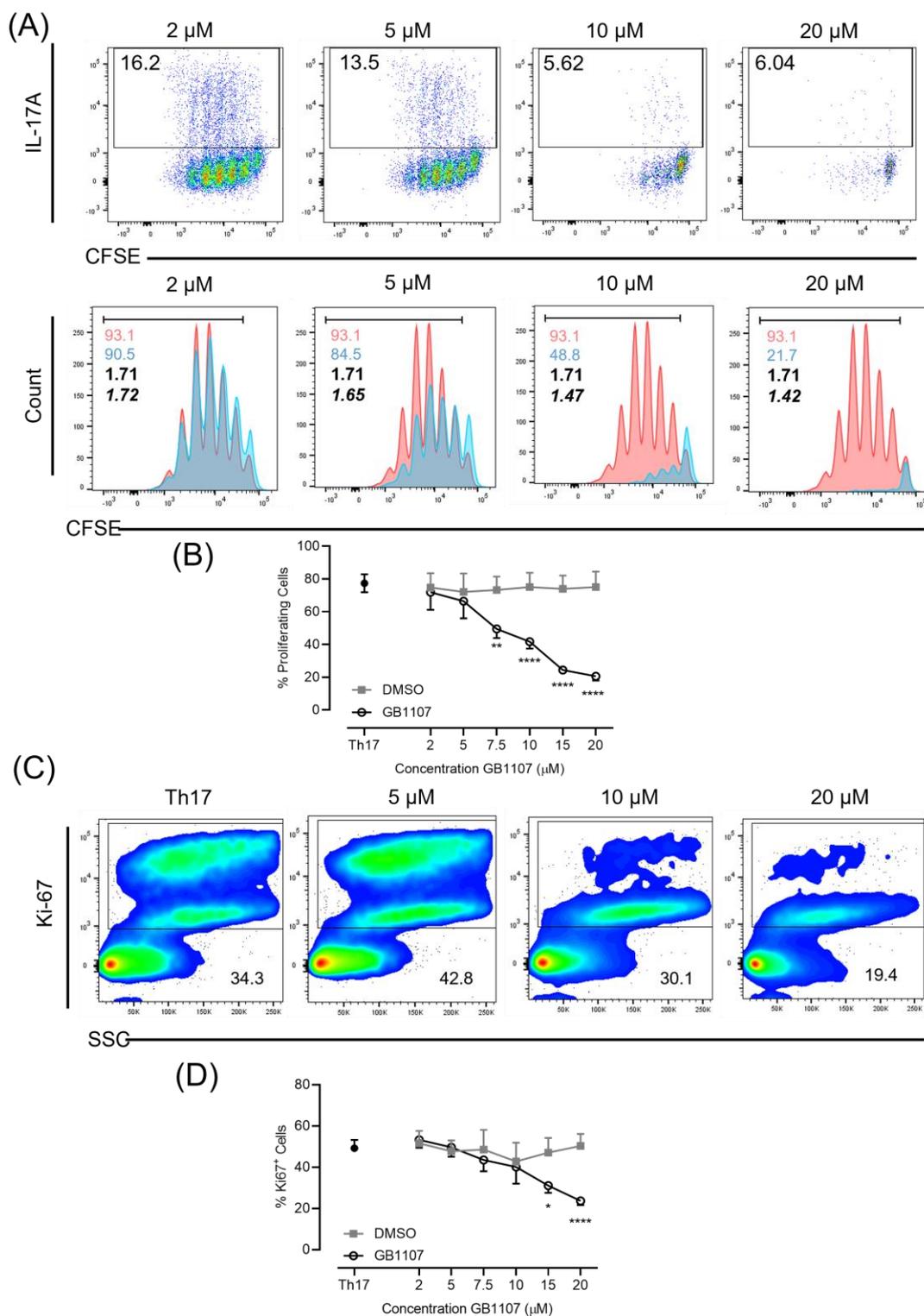


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Figure 4.14: GB1107 inhibits proliferation of Th17 cells

CD4 cells were first stained with CFSE to measure proliferation before culturing in Th17 differentiating conditions and in the presence of increasing concentrations of GB1107 for 96 hours. (A) Cells were stained with IL-17A to confirm expansion. Large number in each gate denotes the percentages of IL-17A⁺ cells at each concentration of GB1107. Histograms show CFSE dilution profiles in the absence (pink) or presence (blue) of GB1107, with corresponding numbers indicating the percentage of proliferating cells. Bold numbers indicate proliferation index for dividing cells in Th17 differentiating conditions, with bold italicised numbers indicating the proliferating index for cells in Th17 differentiating conditions cultured in the presence of GB1107. (B) Graph depicting rounds of proliferation cells undergo in the presence or absence of GB1107. Significance is calculated by 2-way ANOVA with Sidak post-test where **= $p < 0.001$; ****= $p < 0.0001$. Data are shown as mean \pm SEM and are representative of 2 independent experiments. (C) Representative FACS plots and (D) graph of %Ki67⁺ cells at the indicated concentrations of GB1107. The large number in each FACS plot denotes the percentage of cells in the Ki67 gate. Data are shown as mean \pm SEM and are representative of 3 independent experiments. Significance was calculated by a 2-way ANOVA with Sidak post-test where *= $p < 0.05$ and ****= $p < 0.0001$.

4.2.14 High concentrations of GB1107 results in CD4 T cell apoptosis

Proliferation and apoptosis are intrinsically linked and are required for homeostasis to limit the growth of cells with oncogenic mutation (Guo and Hay 1999). To determine if the reduction in proliferation in cells treated with GB1107 was associated with an increase in apoptosis, Annexin V staining and analysis was performed by flow cytometry. Annexin V is a member of the annexin family of membrane proteins and are commonly used for the identification of cells undergoing apoptosis (Mirsaeidi et al. 2016). Annexin V binding to phosphatidylserines exposed on the extracellular side of the plasma membrane. As phosphatidylserine is only located on the outer edge of the plasma membrane during apoptosis, detection of apoptotic cells by Annexin V binding provides in a sensitive method to detect these cells. Approximately 20% of Th17 cells stained positively for Annexin V. However, this increased to approximately 70% of cells staining positively for Annexin V in the presence of 15 μ M and 20 μ M GB1107 (**Figure 4.15 A, B**). Gating was based on CD3⁺CD4⁺ populations, however gating on CD3⁺CD4⁺IL-17A⁺AnnexinV⁺ populations yielded the same trend of results (data is not shown). As discussed in **Section 4.2.7** (see also **Figure 4.6**), Zombie Aqua staining revealed a partial loss of cell viability at 15 and 20 μ M concentrations of GB1107. Here, the increase in Annexin V staining is consistent with a reduced cell viability observed at higher concentrations of GB1107. Therefore, for subsequent studies investigating the effect of GB1107 on Th17 cell inhibition a concentration range of 0 -10 μ M was used.

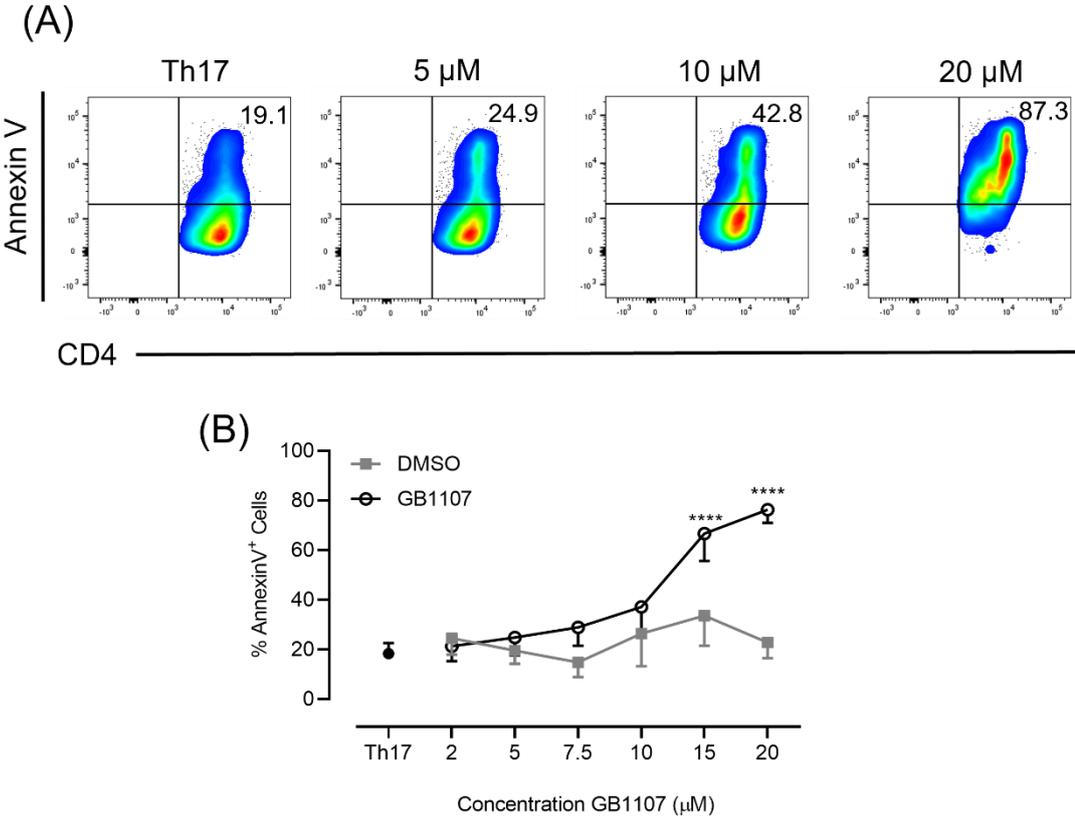


Figure 4.15 GB1107 enhances apoptosis of Th17 cells

CD4 cells were cultured in Th17 differentiating conditions in the presence of GB1107. Cells were stained and gated on lymphocytes, single cells, live cells, CD3⁺CD4⁺, CD4⁺AnnexinV⁺. (A) Representative FACS plots of Annexin V staining. (B) Percentage AnnexinV⁺ cells in the presence of GB1107 or DMSO. Significance was calculated by 2-way ANOVA with Sidak post-test. Data are shown as mean ± SEM and is representative of 3 independent experiments.

4.2.15 GB1107 reduces the secretion of T cell associated cytokines

Th17 cells display a wide range of effector characteristics that includes the secretion of cytokines beyond the signature cytokine, IL-17A. For example, Th17 cells also secrete IL-17F, IL-22, IFN γ , TNF and IL-10 (Liang et al. 2006; Kato et al. 2013; Wu et al. 2018). As GB1107 was inhibiting the proliferation and survival of Th17 cells, it was proposed that GB1107 would reduce the secretion of Th17 effector cytokines, rather than selectively inhibit IL-17. Therefore a multiplex cytokine assay was performed on the supernatant of Th17 cells cultured in the presence and absence of GB1107. Consistent with my analysis of IL-17A by flow cytometry, IL-17A levels were high (at the top of the standard curve of the assay) in supernatants of cells cultured under Th17-polarising conditions and was reduced in the presence of GB1107 (**Figure 4.16 A**). Moreover, another member of the IL-17 cytokine family, IL-17F, showed a comparable reduction to IL-17A and was reduced in the presence of GB1107, from 10,000 pg/mL to 300 pg/mL (**Figure 4.16 B**). IL-17F is one of the founding cytokines assigned as a Th17-type cytokine and shares 55% homology with IL-17A. It is induced under Th17 differentiation but has shown to be non-essential in the progression of EAE and inflammatory arthritis (Ishigame 2009). GB1107 also caused a concentration dependent reduction in the secretion of IL-22 (**Figure 4.16 C**), a pro-inflammatory cytokine secreted by Th17 cells and Th22 cells that promotes the expression of antimicrobial peptides involved in host defence (Rutz et al. 2013). IL-22 further propagates inflammation by increasing the proportion of Th17 cells in inflamed joints, while increasing Th1 cells in the draining lymph nodes of mice with CIA (Justa et al. 2014). GB1107 also reduced the levels of IFN γ and TNF α , which can also be secreted by Th17 cells (Stumhofer et al. 2006) (**Figure 4.16 D - E**). Interestingly, GB1107 also decreased the levels of the regulatory cytokine, IL-10, in Th17 cell cultures (**Figure 4.16 F**) from 250 pg/mL to 150 pg/mL when treated with 10 μ M GB1107. IL-10 functions in Th17 cells to maintain the balance between appropriate activation of immune cells and excessive production of inflammatory cytokines and can negatively regulate the pathogenic potential of Th17 cells (Guo 2016).

The studies herein outline a role for a small molecule inhibitor designed to target galectin-3, GB1107, as a suppressor of Th17 cell expansion. Small molecule inhibitors can display off-target effects, which could potentially account for the inhibitory effect observed on Th17 cell differentiation. Investigations involving CD4 T cells sourced from galectin-3-deficient (*Lgals3*^{-/-}) mice provided a unique opportunity to investigate the requirement of

endogenous galectin-3 in Th17 cell differentiation and to test whether GB1107 inhibition of Th17 cell effector function was mediated via galectin-3.

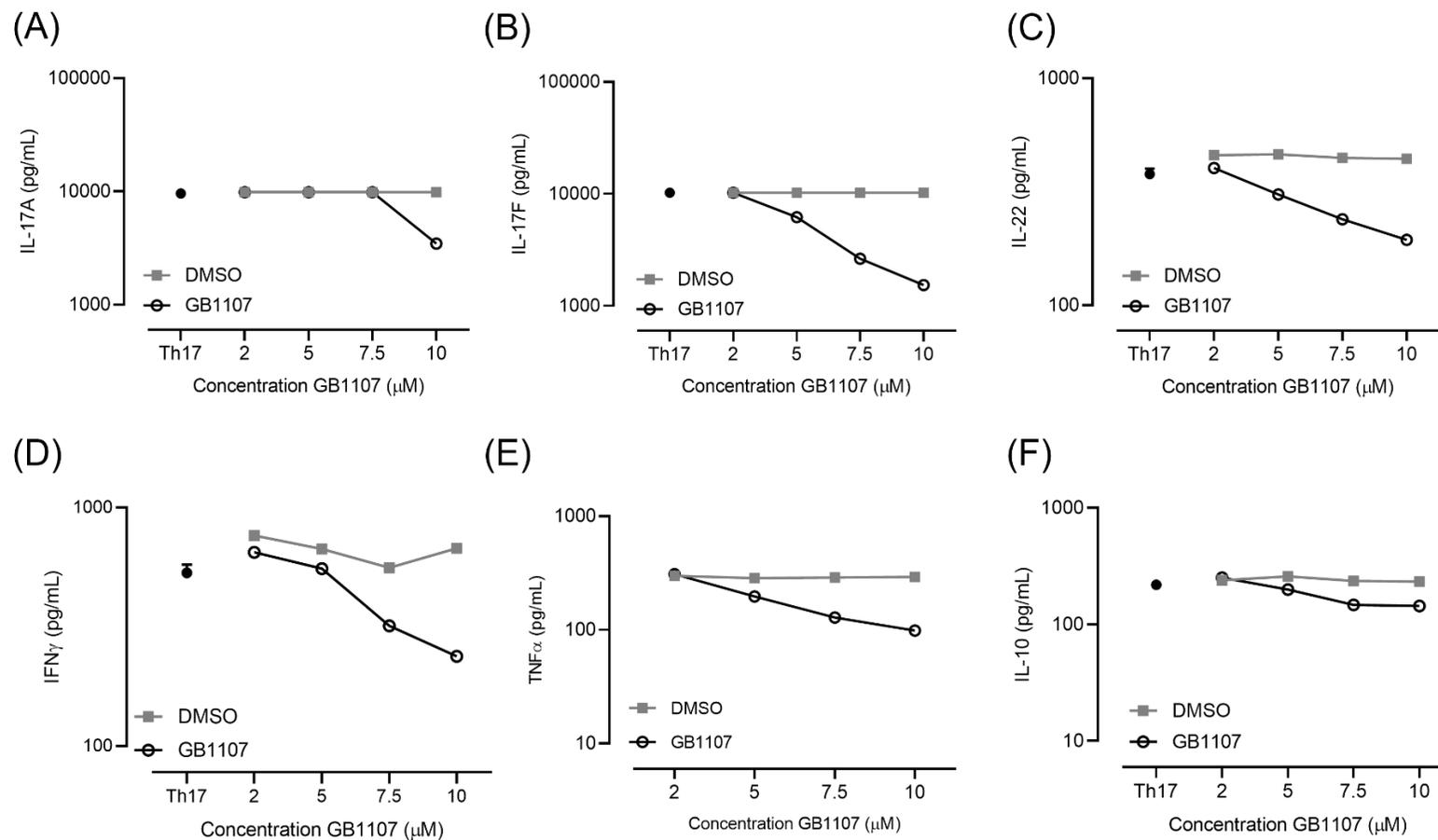


Figure 4.16: Levels of cytokine output from Th17 cell cultures in the presence of GB1107

LEGENDplex multiplex assay showing the concentration of the indicated cytokines (A-F) in culture supernatants of CD4 T cells activated under Th17-polarising conditions with and without GB1107 for 96 hours. (A) IL-17A. (B) IL-17F. (C) IL-22. (D) IFN γ . (E) TNF α . (F) IL-10. Data are representative of 1 experiment run in duplicate.

4.2.16 GB1107 does not influence galectin-3 expression at the gene or protein level

GB1107 has strong affinity for galectin-3 and acts by inhibiting both intracellular and extracellular galectin-3 (Stegmayr et al. 2019), but little is known whether GB1107 alters galectin-3 expression at a genetic level. To determine if GB1107 inhibits galectin-3-regulated responses, studies were performed to determine whether the inhibitor influenced *Lgals3* expression. CD4 cells were cultured in Th17 differentiating conditions with GB1107 at 7.5 μ M and 10 μ M for 24 hours. These concentrations were chosen as they display a significant reduction in Th17 expansion without affecting cell viability (**Figure 4.8 and Figure 4.7**) and this timepoint was chosen as *Lgals3* expression is significantly increased in CD4 cells in Th17 polarising conditions at 24 hours (see **Figure 4.4**). Galectin-3 expression was measured by qPCR of *Lgals3*, as expression could not be reliably measured by flow cytometry (see **Figure 4.3**).

Il17a expression in Th17 cells was significantly heightened at 24 hours. Furthermore, expression of *Rorc*, the master transcriptional regulator for Th17 cells, was also significantly upregulated in Th17 cell cultures at 24 hours (**Figure 4.17 A**), confirming that the cultures expanded appropriately to a Th17 phenotype. *Lgals3* levels were significantly increased in Th17 cells, which is in keeping with data observed in **Figure 4.4 A**. However, this expression was not changed when cells were treated with GB1107 (**Figure 4.17 B**). This finding informs us that GB1107 does not influence the transcriptional control of *Lgals3*. Furthermore, GB1107 treatment did not alter the levels of galectin-3 detected by ELISA in culture supernatants (**Figure 4.17 C**). While these findings suggest that GB1107 does not inhibit galectin-3 at the mRNA level, the limited time course investigated here is a limitation. Further investigations across a time-course would allow me to determine whether 24 hours of culture was indeed the optimum time frame to determine if GB1107 acts via targeting *Lgals3* expression and would therefore confirm that the action of GB1107 is not mediated by direct inhibition of *Lgals3* expression.

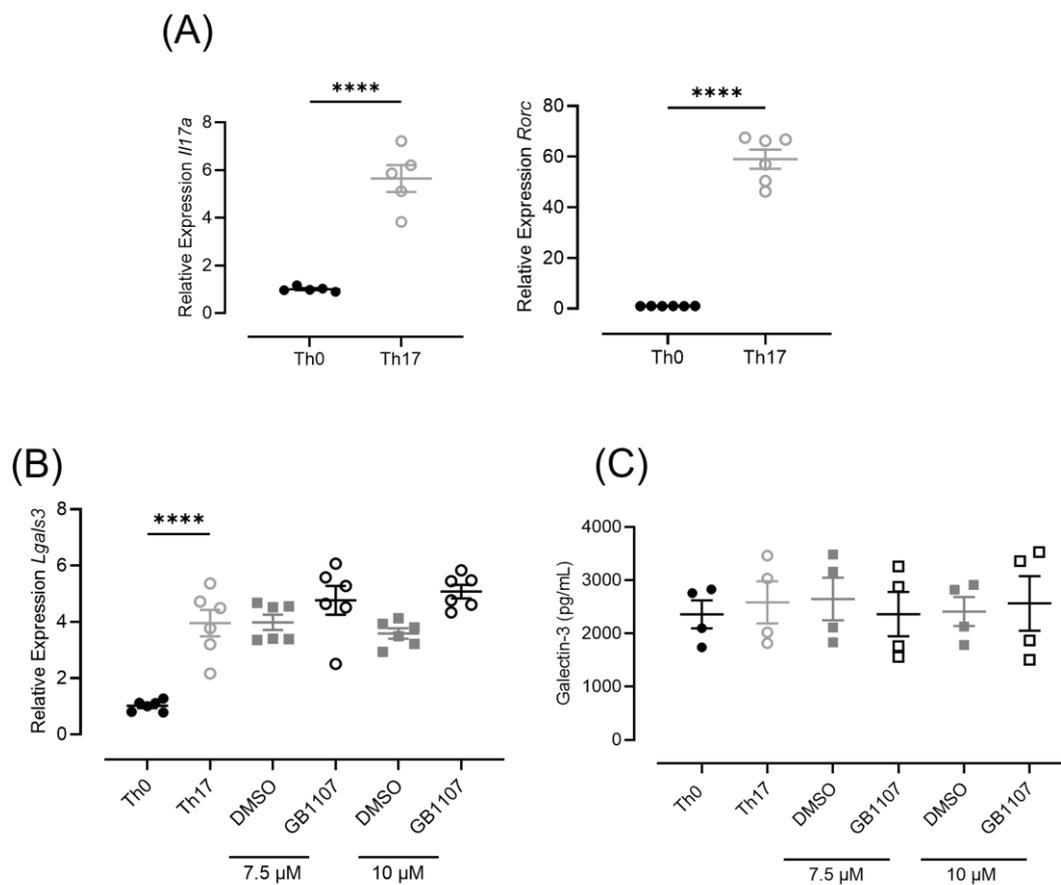


Figure 4.17 GB1107 does not directly alter galectin-3 expression

CD4 cells were cultured in Th17 differentiating conditions with and without GB1107 for 24 hours. Graphs show qPCR of (A) *Il17a* and *Rorc* expression in CD4 cells in Th17 differentiating conditions and (B) *Lgals3* in the presence of GB1107 or DMSO and (C) protein quantification by ELISA of galectin-3. Data are representative of 2 independent experiments and is shown as mean \pm SEM. Significance was calculated by one-way ANOVA with Tukey post-test where **** = $p < 0.0001$.

4.2.17 GB1107 inhibition of Th17 cell expansion requires galectin-3

Given that GB1107 does not alter *Lgals3* gene levels, it was proposed that GB1107 acts through an interaction involving the galectin-3 protein. Therefore, any inhibitory effects observed in response to GB1107 should be lost in galectin-3-deficient cells. To determine whether the inhibition in Th17 expansion mediated by GB1107 was galectin-3 dependent, I isolated CD4 T cells from *Lgals3*^{-/-} male mice and cultured the cells in Th17-polarising conditions with and without GB1107. Th17 expansion and inhibition was compared to that seen in CD4 cells from control wild-type male mice. As expected, there was approximately 40% Th17 expansion in wild-type (WT) cells, which was impaired in the presence of GB1107 (**Figure 4.18 A, C**). Consistent with an important role for endogenous galectin-3 in Th17 cell development identified in this Chapter, Th17 expansion in *Lgals3*^{-/-} CD4 T cells was significantly impaired (~50%) compared to wild-type cells (**Figure 4.18 B, D**). Furthermore, while significant inhibition of Th17 expansion was observed at 7.5 μ M and 10 μ M GB1107 in wild-type cells, this effect was absent in *Lgals3*^{-/-} cells.

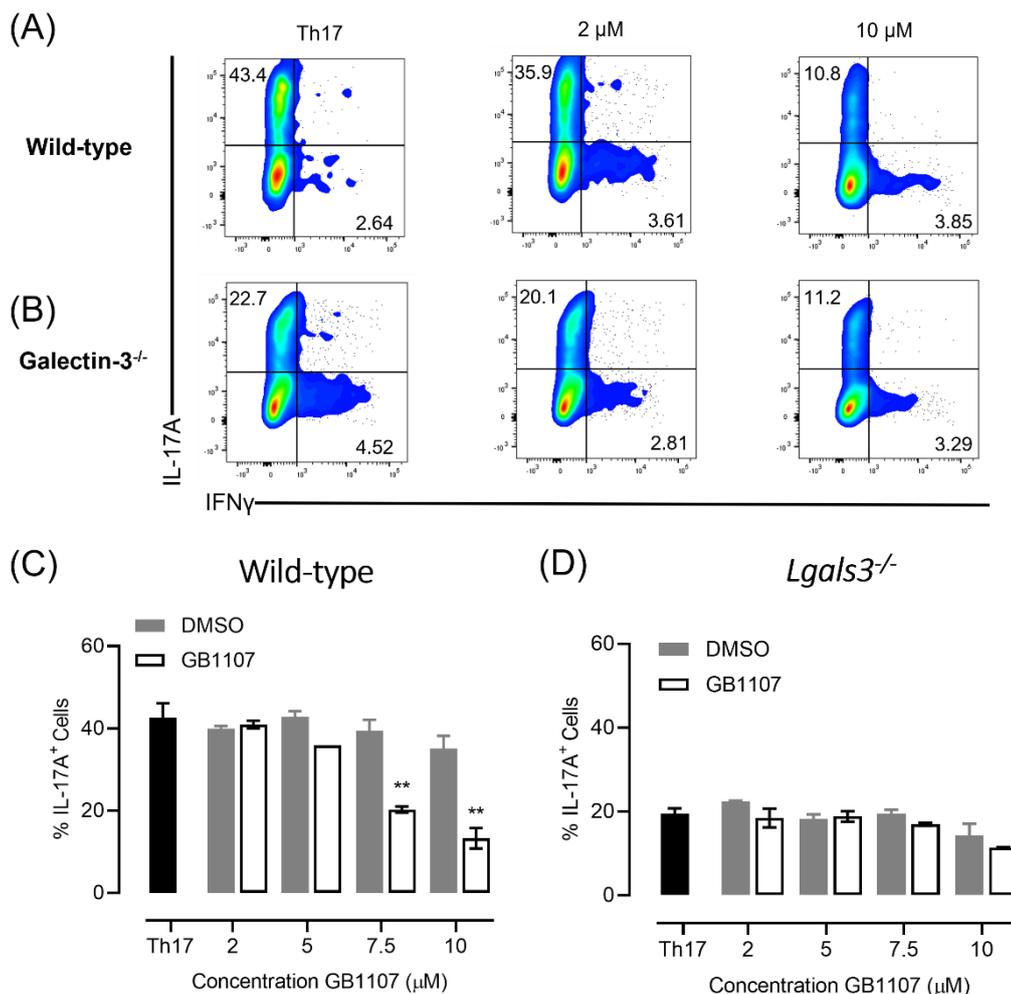


Figure 4.18 Reduced Th17 expansion in *Lgals3^{-/-}* cells.

CD4 cells were isolated from wild-type and *Lgals3^{-/-}* male mice and cultured in Th17 differentiating conditions with and without GB1107 for 96 hours. (A – B) Representative flow cytometry plots showing IL-17 and IFN γ -secreting CD4 T cells. (C) Graphs show bar chart of percentage of IL-17A expressing cells in (C) wild-type or (D) *Lgals3^{-/-}* cells in the presence of GB1107 or DMSO. Significance was calculated by 2-way ANOVA with Tukey post-test. Graphs are shown as mean \pm SEM and graphs are representative of 1 experiment.

4.3 Discussion

Lgals3 is increased at sites of inflammation, in activated peripheral CD4 T cells and in tissue infiltrating CD4 T cells

The link between galectin-3 and T cells is established in the literature and galectin-3 is required for IL-2 dependent cell growth in T cells (Joo et al. 2001). Here, the finding that *Lgals3* is significantly elevated in joint infiltrating CD4 T cells in inflammatory arthritis confirmed that galectin-3 is expressed in CD4 T cells and triggered the hypothesis that galectin-3 may play a role in modulating T cells during inflammation. Galectin-3 is increased in synovial fibroblasts in arthritic joints and correlates to CRP and IL-6 serum levels in patients with rheumatoid arthritis (Filer et al. 2009). Galectin-3 has been recognised in promoting inflammatory responses and knockdown of *Lgals3* have shown to alleviate pathology in EAE (Jiang et al. 2009) and inflammatory arthritis (Forsman et al. 2011). However, although there is a strong link between galectin-3 expression and pathology, the mechanisms by which galectin-3 determines disease outcome is still not fully understood. The aim of this Chapter was to test the hypothesis that galectin-3 modulates T cell phenotypes.

Studies initially set about establishing culturing conditions for the detection of galectin-3 in CD4 T cells. Investigations revealed that galectin-3 was detected intracellularly and extracellularly in CD4 T-helper cells. Initial studies used a single panel antibody (PE) to detect galectin-3 intracellularly and extracellularly. Half of the cells were permeabilised to detect extracellular and intracellular galectin-3 (equating to total galectin-3) and half were stained for surface galectin-3 only. The proportion of intracellular galectin-3 staining was determined by subtracting the surface galectin-3 staining from the total galectin-3 staining. However upon further consideration, an improved method of distinguishing between intracellular vs extracellular expression would be to use galectin-3 antibodies on different fluorochromes. Nonetheless, the experiment was a pilot experiment and did result in an initial observation of high galectin-3 expression in CD4 T cells. This led to establishing galectin-3 expression in resting CD4 T cells by flow cytometry and qPCR. The data presented in this Chapter agreed with observations by Joo *et al* who also noted a predominance of galectin-3 expression in the intracellular compartment of CD4 T cells (Joo et al. 2001). However, Joo's observations showed that approximately 16% of activated CD4 cells expressed intracellular galectin-3, whereas the data presented in this Chapter showed approximately 50% of CD4 cells stained positively for galectin-3. The high expression of

galectin-3 both intracellularly and extracellularly - regardless as to whether the cells were resting or activated - raised questions about the reliability of the flow cytometry methodology used for the intracellular detection of galectin-3 in CD4 T-helper cells. As optimised intracellular staining protocols for the detection of T cell effector cytokines (e.g., IL-17) was an important component of the work herein, qPCR was chosen as the preferred method to track galectin-3 (*Lgals3*) expression.

***Lgals3* is uniquely increased in Th17 cells, which offers a potential way to selectively target these cells**

Expression of *Lgals3* was higher in Th17 cells versus Th1 or Th2 cells. As a result of this striking elevation, the role of galectin-3 in this effector T cell subset became the research focus for the Chapter. A study by the Kuchroo group identified *Lgals3* in a transcriptional signature for pathogenic Th17 cells (Lee et al. 2012). This was the first and only published study that identifies a relationship between galectin-3 and pathogenic Th17 cells. While there have been studies that show the indirect effect of galectin-3 on controlling Th17 responses via dendritic cell cytokine induction of IL-23 (Fermin Lee et al. 2013), and studies that investigate galectin-3 deficiency in Th17 related models such as inflammatory arthritis (Forsman et al. 2011) and EAE (Jiang et al. 2009), little was known regarding how galectin-3 activity in CD4 T cells determines Th17 cell polarisation, or T cell intrinsic mechanisms involved in the relationship between galectin-3 and Th17-type responses. Furthermore, the development and testing of novel inhibitors that target galectin-3 have the potential to improve the treatment of diseases where CD4 T cells are pathogenic. Pharmacological targeting of galectin-3 using ligand for galectin-3, N-acetyllactosamine, has been tested in animal models of cardiovascular diseases and resulted in reduced myocardial inflammation and cardiac remodelling (Yu et al. 2013) but, to date, no studies have investigated the effect of targeting galectin-3 on Th17 mediated diseases.

The secretion of galectin-3 is unique to Th17 cells when compared to Th1 and Th2 cells. This is a significant finding as Th17 cells are linked with driving inflammation and may account for the selective inhibition of Th17 cells when targeting galectin-3 in my studies. IL-17 is linked to pathogenic effector T cell responses in inflammatory arthritis, and our group and others have shown elevated circulating IL-17 in experimental models of RA (Jones et al.

2015). Notably, both galectin-3 and Th17 cells have independently been linked with pathogenic outcomes in CIA (Geboes et al. 2009; Wang et al. 2010), RA (Maddur et al. 2012; Li et al. 2013) and vascular disease (Wang et al. 2011; Ding et al. 2012). The expression and secretion of galectin-3 from Th17 cells, and the pathogenic contribution Th17 cells makes towards inflammation, strengthen the rationale for using Th17-driven models as a system to test the therapeutic potential of targeting galectin-3.

GB1107 inhibits Th17 cell differentiation; and its mechanism of action is dependent on galectin-3

GB1107 was added to CD4 cell cultures and showed a concentration range of minimal toxicity between 1 μ M to 10 μ M. GB1107 was toxic to CD4 cells at concentrations above 50 μ M. While significant cell death was observed at 15 μ M and 20 μ M, 50% of cells remained viable and the death observed may have been selective cell death of effector CD4 cells and so these concentrations were included in initial studies. Interestingly, while cell death was observed in CD4 cells in Th17 differentiating conditions, there was no significant cell death observed in CD4 cells in Th1, Th2 or Treg differentiating conditions. The decrease in live cells observed in Th1 cells at 15 μ M and 20 μ M were due to issues with culturing conditions in the specific wells of that experiment and existed in both the DMSO and GB1107 treated cells, so it is likely that this was not due to a direct effect of GB1107 itself. It should be noted that the other wells (at lower concentrations) in the experiment were unaffected. There was increased cell death in cells treated with GB1107 at 15 μ M and 20 μ M in Th2 cell cultures suggesting toxicity, but this was not statistically significant when compared to DMSO. However, there was a higher percentage of live cells in Th17 differentiating conditions than in Th1 or Th2, with 70%, 40% and 50% cell viability respectively. CD4 cells in Treg conditions had 80% cell viability. This can be explained in part by Th17 conditions requiring survival signal IL-6 and TGF β and Treg conditions require TGF β , which aid cell survival and proliferation (Zhang et al. 2017).

GB1107 inhibited expansion of Th17 cells but did not alter Th1 expansion from mouse CD4 T cells. Th1 cells are raised in inflammation and are present in the CNS of mice with EAE (Ben-Nun and Cohen 1981), and the addition of Th1 differentiating cytokine IL-12 to mice with CIA exacerbates disease (Germann et al. 1996). Th1 cells are known to drive

autoimmune disease, both in partnership and independent of Th17 cells (Dardalhon et al. 2008; Luger et al. 2008). These are key results as it shows GB1107 elicits inhibitory effects in Th17 cells selectively and indicates that galectin-3 dependent Th17 expansion occurs independently of Th1 cells.

Inhibition of galectin-3 by GB1107 increased Th2 expansion. This is in contrast to studies reported in the literature that showed *Lgals3*^{-/-} mice with reduced Th2 responses in a model of asthma (Zuberi et al. 2004) and suppressed allergic airway inflammation in rats receiving a galectin-3-expressing plasmid suggesting that galectin-3 promotes a Th2 response (del Pozo et al.). However, the findings in this Chapter are consistent with that seen by Lukic *et al* whereby galectin-3 deficiency attenuated EAE by decreasing IL-17 production and promoted Th2 polarisation (Jiang et al. 2009). The same study showed elevated Treg cells in *Lgals3*^{-/-} mice undergoing EAE when compared to wild-type mice. Similar studies have shown that *Lgals3*^{-/-} mice have increased Treg cells in the spleen and the CNS (Fermino et al. 2013). Again, this is consistent with the data presented in this Chapter which showed inhibition of galectin-3 by GB1107 significantly increased Treg expansion. This also shows that while GB1107 inhibits the expansion of pathogenic cell phenotypes such as Th17 cells, it can also promote the 'anti-inflammatory' phenotypes such as Th2 and Treg cells. However, later assays showed that cells were apoptotic at 15 and 20 μ M GB1107. Therefore, further work is required to establish whether the effects observed in the reciprocal relationship and Th2 and Treg expansion are due to the direct action of GB1107, or if they are due to toxicity. One such approach in this would be repeat the experiments presented in this Chapter in cells from *Lgals3*^{-/-} mice to determine the true galectin-3 dependent effects on the reciprocal relationship between Th17, Th1 and Treg cells.

Galectin-3 supports Th17 cell differentiation

While studies report decreased IL-17 levels in *Lgals3*^{-/-} conditions (Forsman et al. 2011), there is no literature investigating the mechanism behind this. Given the striking decrease in Th17 expansion in the presence of GB1107 observed in this Chapter, it was important to ensure that the effects observed were due to specific galectin-3 dependent mechanisms in Th17 cells. While GB1107 did not have a direct effect on galectin-3 mRNA or protein levels, it is likely that the inhibitor binds to galectin-3 and inhibits its activity and role in downstream signalling that results in perpetuation in Th17 effector functions. GB1107 has a strong binding affinity for mouse galectin-3 with a dissociation constant (K_D) of 1710 nM

(information supplied by Galecto Biotech), making it possible that GB1107 binds and “neutralises” galectin-3 activity. As measuring GB1107’s binding to galectin-3 and its effect on galectin-3 activity is challenging and beyond the limits of this Thesis, CD4 cells from *Lgals3*^{-/-} mice were used to determine that the effects observed on Th17 expansion are specific to galectin-3. *Lgals3*^{-/-} cells had reduced Th17 expansion by approximately 50% when compared to wild-type cells highlighting that galectin-3 is important for Th17 differentiation. Furthermore, no additional reduction in Th17 expansion was observed in *Lgals3*^{-/-} cells treated with GB1107 compared to DMSO treated cells, until high concentrations of GB1107 were used. Due to the limited availability of *Lgals3*^{-/-} mice, studies involving CD4 T cells from these mice were only completed once and need to be repeated to ensure the results are reproducible. Nonetheless, the reduced Th17 expansion of GB1107 not only informs us that while galectin-3 may not have a role in the initial differentiation of CD4 cells to Th17 phenotype, it does have a role in the maintenance of these cells and that galectin-3 may contribute towards the pathogenic phenotype of these cells, as proposed by the Kuchroo group (Lee et al. 2012).

GB1107 inhibits Th17 cells via suppression of proliferation

Inhibition of galectin-3 elicited a clear effect on the suppression of proliferation of T cells in Th17 differentiating conditions. A clear reduction in rounds of cell proliferation was observed by reduction in CFSE staining and similarly, a significant reduction in Ki67 staining was also observed. However, it should be noted that while the data is shown based on lymphocytes, when CD3⁺CD4⁺IL-17⁺Ki67⁺ populations are quantified, there is a similar trend toward reduction upon inhibition of galectin-3. Ki67 expression has been quantified in CD4 T cells and has found to be reproducible and specific (Soares et al. 2010). There was two distinct Ki67-positive populations observed in the FACS plots. This may be in part due to variation in Ki67 expression levels depending on the stage of cell cycle that the cell is in. In a study by Miller *et al*, Ki67 expression was heterogenous depending on how long it spent in G0 and G1 phases (Miller et al. 2018). Inhibition of galectin-3 appeared to have a biased effect toward reducing the higher Ki67⁺ population observed on the FACS plots, suggesting that galectin-3’s role in proliferation may be more influential in one stage of the cell cycle compared to another. This has been shown in a study by Lin *et al* where galectin-3 has been identified in playing a role in promoting cell cycle arrest at the G2/M phase in an epithelial cell line without inducing apoptosis in the presence of dietary flavonoid genistein (Lin et al. 2000). However, no specific role of galectin-3 at specific stages of the cell cycle in

T cells has been identified. Nonetheless, as discussed in the Introduction of this Chapter, a role of galectin-3 in the proliferation of T cells has been identified in previous studies whereby addition of galectin-3 anti-sense oligonucleotides to activated murine T lymphocytes led to decreased proliferation, as determined by [³H]-thymidine incorporation to cells (Joo et al. 2001). However, this study is the first time that inhibition of proliferation in specific Th17 differentiating conditions have been reported.

High concentrations of GB1107 results in cell killing

Inhibition of galectin-3 caused a significant increase in IFN γ expressing cells in Th17 cultures cultured with GB1107. Interestingly, cells cultured in Th17 conditions in the presence of GB1107 also had a trend toward a Treg phenotype, as shown by increased FoxP3 expression but this was not significant and again, this increase was subtle. The trend toward enhanced IFN γ expressing cells and FoxP3 expressing cells may be in part explained by the effect of galectin-3 on T cell transcription factors. Addition of galectin-3 to leukocyte cell cultures has shown to downregulate T-bet and FoxP3 transcription factors (Vasil'eva et al. 2013). However, it could be argued that the effect observed was resultant of suppression of Th17 expansion by GB1107, and therefore cells remain in a "Th0" like state leading to spontaneous expression of IFN γ . This has been reported in the literature, where Th0 cells produce IFN γ (Aarvak et al. 1999). It may also be due to the apoptotic effect that GB1107 had on the cells. GB1107 induced AnnexinV staining in cells at 15 μ M and 20 μ M, indicating that the inhibitor may be causing non-specific killing at these concentrations. To confirm that the inhibitor is causing non-specific killing at these concentrations, a measure of apoptosis that could be used is a caspase activation assay that detects caspases 3 and 7. Caspases are activated in response to death receptors and apoptosis-inducing stimuli. Their activation results in the cleavage of nuclear proteins, mitochondrial proteins and plasma membrane proteins, which leads to cell death (Salvesen 2002). Detection of caspase 3 and 7 would be beneficial as they are activated regardless of the death-inducing stimuli. Caspase activation assays are much more specific in detecting true apoptosis than Annexin V and so would be useful in applying here to determine if GB1107 was causing non-specific killing of these cells. Nonetheless, the increase in Annexin V observed may in part explain for the significant increase in IFN γ expressing cells in Th17 cells cultures at these concentrations, as IFN γ can induce apoptotic pathways (Kotredes and Gamero 2013). Therefore, as higher concentrations of GB1107 caused apoptosis of CD4 cell cultures, studies thereafter used a smaller concentration range of 2 – 10 μ M GB1107.

This Chapter identifies and characterises galectin-3 expression in T helper cell subsets. Furthermore, studies presented in this Chapter have setup and established T cell bioassay that reproducibly shows an effect on T cell effector responses following GB1107 treatment. Combined with *Lgals3*^{-/-} mice, this provides a system to investigate the specificity of newly developed inhibitors that aim to target galectin-3. By using GB1107 as a tool understand galectin-3 dependent mechanisms in Th17 cells, these findings are the first step in understanding galectin-3's role in inflammatory states. This study shows a role of galectin-3 in the proliferation of Th17 cells and has established galectin-3 as a therapeutic target in Th17 mediated diseases and pathologies. **Chapter 5** of this Thesis will investigate if inhibition of galectin-3 by GB1107 in the Th17-driven model of antigen induced arthritis can modulate inflammation in an *in vivo* setting.

Chapter 5

Evaluating galectin-3 inhibition in antigen induced arthritis

5.1 Introduction

Antigen-induced arthritis is a murine model of inflammatory arthritis that is immune-complex mediated and drives a T cell-dependent pathology caused by administration of methylated BSA (mBSA) antigen to the knee joint of an immunized mouse (Jones et al. 2018). Contrary to the CIA model, the arthritis in AIA is confined to antigen-challenged knee joints only and therefore gives rise to an immediate and synchronous inflammatory response that follows a well-defined time course (van den Berg et al. 2007). The model has features of an acute inflammatory phase predominated by an innate leukocyte infiltrate, followed by a self-limiting chronic-like phase predominated by lymphocytes, followed by subsequent resolution of synovitis. This model involves priming of mice by injecting an emulsion containing mBSA in CFA under the skin and a solution of pertussis toxin into the peritoneal cavity 21 days before arthritis induction (Day -21). The mice receive a second boost to the immune response by administration of a second injection of mBSA in CFA a week later (Day -14). This subcutaneous boost to the immune system causes sufficient generation of IgG which is required for the robust arthritogenic reaction on Day 0 when the antigen (mBSA) is directly injected into the joint space of the knee (**Section 2.3.3**). Systemic priming is a key step in the induction protocol for both CIA and AIA. AIA is monoarticular, in contrast to CIA that is polyarticular. This means that arthritis is confined to the knee joint that is injected with the mBSA antigen. Mice develop an acute inflammatory response, and in the model run in our lab at Cardiff University, the acute response in wild-type mice peaks during the first 3 days following intra-articular injection. The acute phase of inflammation at Day 3 is characterised by innate immune cell infiltration dominated by neutrophils and macrophages. Day 10 is often used to model chronic-like synovitis (Jones et al. 2018). The chronic phase of AIA is characterised by a distinct T cell-mediated response in the joint, with some evidence of B cell infiltration (Meehan et al. 2021). Although joint swelling is resolving by Day 10, the chronic-like synovitis and breakdown of joint architecture is progressive beyond this point (van den Berg et al. 2007). AIA is typically performed on C57Bl/6 mice. It is used to study the molecular mechanisms underpinning adaptive immune-mediated joint pathology (Jones et al. 2018). In regard to the progression of the model, AIA is less aggressive than the CIA model in that the transition to the chronic T-cell dependent destructive phase of arthritis is slower. It happens over a period of weeks rather than days that we observed in the CIA model. Based upon experience of the CIA model documented in **Chapter 3** and the established *in house* expertise with the AIA model, I reasoned that the AIA model provided an excellent alternative model to study arthritis-

associated vascular constriction responses. To my knowledge, this is the first time that a study of this kind has been conducted in the AIA model. Furthermore, the predictable, reproducible, robust nature of the inflammatory pathology noted with AIA presented an opportunity to evaluate the role of galectin-3 in regulating joint swelling and arthritis-associated vascular constriction.

CD4 T cells play a central role in the pathogenesis of rheumatoid arthritis (RA) by activation of macrophages and synovial fibroblasts which, in turn, causes joint destruction. The number of IFN γ and IL-17-producing T-cells is higher in the synovium of patients with RA (Chemin et al. 2019) versus non-arthritic tissue. Biologics that target CD4 positive cells had limited success in treating RA. This indicated that other T-cell-mediated mechanisms were active in the joint and that these factors required investigation. Investigations have shown that CD4 T cells support antigen specific T cell reactivation in the joint space via engagement with articular dendritic cells during inflammatory arthritis (Prendergast et al. 2018). In studies using the AIA model, loss of CD25⁺ Treg cells in immunized mice prior to the induction of arthritis resulted in worse pathology. Furthermore, transfer of CD25⁺ Treg cells after administration of mBSA does not ameliorate disease, indicating that CD4 Treg cells play a regulatory role in the early stages of the model controlling inflammation (Frey et al. 2005). The pro-inflammatory and destructive role of Th17 cells was demonstrated in studies where neutralisation of IL-17 by using an anti-IL-17 antibody reduced the severity of CIA and prevented cartilage and bone destruction (Lubberts et al. 2004) and *Il17*^{-/-} mice had reduced knee joint swelling during AIA when compared to wild type mice (Baschant et al. 2011).

Few studies have investigated the molecular role of galectin-3 in the pathogenesis of AIA. A key study by Forsman *et al.* revealed that *Lgals3*^{-/-} mice with AIA showed reduced joint inflammation and erosive pathology (Forsman et al. 2011). Interestingly, *Lgals3*^{-/-} mice had significantly decreased IL-17 producing cells in the spleen compared to wild-type mice highlighting the potential of targeting galectin-3 for the control of inflammatory arthritis and Th17-mediated pathologies. Vascular dysfunction has not previously been reported in the AIA model, and furthermore, no studies on the effect on galectin-3 inhibition on vascular function in AIA have been published.

Hypothesis and Aims

My hypothesis is that AIA is associated with vascular changes and that targeting galectin-3 suppresses CD4 T cell responses to limit the vascular constriction deficit. To test this hypothesis, the following aims were set:

Aim 1: To determine 5HT induced vascular constriction responses in mice with AIA.

Aim 2: To characterise the systemic features of AIA.

Aim 3: To determine the effect of galectin-3 inhibition on joint and vascular inflammation.

5.2 Results

5.2.1 Antigen-induced arthritis features an early vascular constriction defect and a concurrent increase in serum galectin-3 levels.

Firstly, to determine if antigen-induced-arthritis (AIA) was an appropriate model to study vascular dysfunction associated with inflammatory arthritis, mice with AIA were culled at 3 timepoints in the model and vascular constriction responses were measured. Mice were culled on Day 0, where mice are immunologically primed but do not receive mBSA antigen to the synovium; Day 3 where mice are in the “acute” phase of inflammation; and Day 10 where swelling has resolved, but mice display ongoing chronic-like synovitis. Constriction responses were measured in mice from each group and compared to age and sex- matched naïve control mice.

Constriction responses from mice terminated at Day 0 show no constriction deficit (**Figure 5.1 A**) when compared to unchallenged control mice. Furthermore, circulating galectin-3 levels in primed mice did not significantly differ from the control mice (**Figure 5.1 B**). Mice terminated on Day 3 show a significant decrease in constriction responses, evidenced by a smaller R_{max} , when compared to control mice. Unchallenged mice and those systemically primed for AIA but without arthritis (Day 0) showed constriction responses of R_{max} of 8.4 mN, whereas mice with AIA terminated on Day 3 reached an R_{max} of 5.3 mN (**Figure 5.1 C**). The local administration of mBSA antigen to the joint is required to trigger vascular dysfunction in this model. The “acute” phase of AIA was associated with decreased constriction responses. The galectin-3 levels in the circulation at Day 3 post IA injection (**Figure 5.1 D**) was significantly elevated ($p < 0.001$) from 34 ng/mL in naïve mice to 71 ng/mL in mice with AIA.

By Day 10, AIA features a more chronic-like inflammatory response. Swelling typically peaks at Day 3 and resolves to near-normal values by Day 10. Interestingly at Day 10, constriction responses were restored (R_{max} of 8.9 mN) to the functional level noted in mice without AIA (**Figure 5.1 E**). Furthermore, the circulating galectin-3 levels were comparable to that in control mice (**Figure 5.1 F**). Therefore, AIA induction results in vascular changes and an increase in serum galectin-3 levels that follow a comparable time course to that of joint swelling.

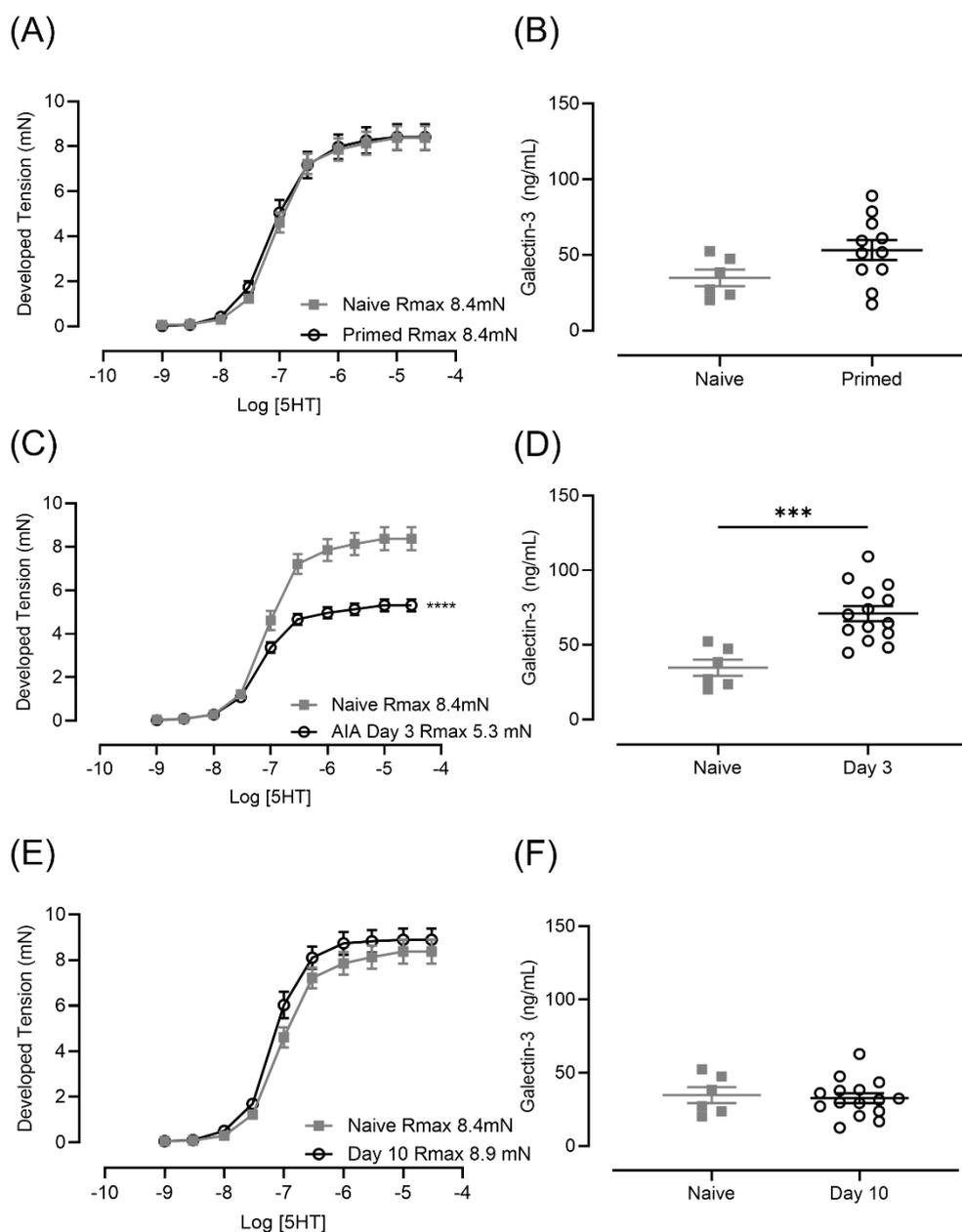


Figure 5.1: AIA induction causes decreased vascular constriction that coincides with elevated circulating galectin-3.

Antigen-induced arthritis was induced in wild-type mice and 5HT-induced constriction responses were measured at various time-points. (A) Mice primed with mBSA in CFA and pertussis toxin (n=2) were culled on Day 0 and constriction responses measured against naïve (n=5) mice. (B) Plasma was harvested from primed mice (n=11) and a galectin-3 ELISA performed. Data shown is not significant. (C) Constriction responses from mice culled on Day 3 (n=8) of the AIA protocol were compared to naïve mice (n=5). **** p<0.0001 as determined by 2-way ANOVA with Sidak post-test. (D) Plasma was harvested from AIA mice (n=14) Day 3 post IA injection and galectin-3 levels were quantified and compared to naïve mice (n=6). ***=p<0.001 as determined by t-test. (E) Constriction responses from mice culled on Day 10 of the AIA protocol and compared to naïve mice. n=5/group. (F) Plasma was harvested from AIA mice (n=15) Day 10 post IA injection and galectin-3 levels were quantified and compared to naïve mice (n=6). Graphs are shown as mean ± SEM.

5.2.2 Elevated T cell-associated cytokines at Day 3 of AIA

Vascular dysfunction and elevated systemic levels of galectin-3 are features of AIA at Day 3. T cells are required for the model, as evidenced by the use of nu/nu BALB/c mice - mice lacking a thymus and therefore unable to produce T cells – who were resistant to mBSA induced arthritis (Brackertz et al. 1977), but did develop arthritis following transfer of lymph nodes and spleens from syngeneic donors. To identify systemic T cell-associated cytokines that coincided with a reduction in vascular constriction, a multiplex assay was performed on plasma from naïve or AIA mice. T cell markers IFN γ and IL-17A were significantly upregulated in mice with AIA (**Figure 5.2 A, B**). Inflammatory markers TNF α and IL-6 were also significantly increased in mice with AIA compared to naïve mice (**Figure 5.2 C, D**). IL-5 was increased when compared to naïve controls, although this was not significant (**Figure 5.2 E**). Interestingly, the inflammatory response as a result of the induction of AIA did not affect circulating IL-4 levels, suggesting that the T cell cytokine response that results in vascular dysfunction is independent of Th2 signalling (**Figure 5.2 F**). Circulating IL-1 α levels decreased during AIA (**Figure 5.2 G**). Furthermore, IL-21 and IL-22 levels did not differ upon AIA induction and remained comparable to baseline levels observed in naïve controls (**Figure 5.2 H, I**).

The quantification of serum cytokine levels at Day 3 of AIA indicate that systemic inflammatory cytokines are induced following local antigen challenge. Here, peripheral T cell responses and their associated effector cytokines may contribute to vascular changes that underpin vascular dysfunction.

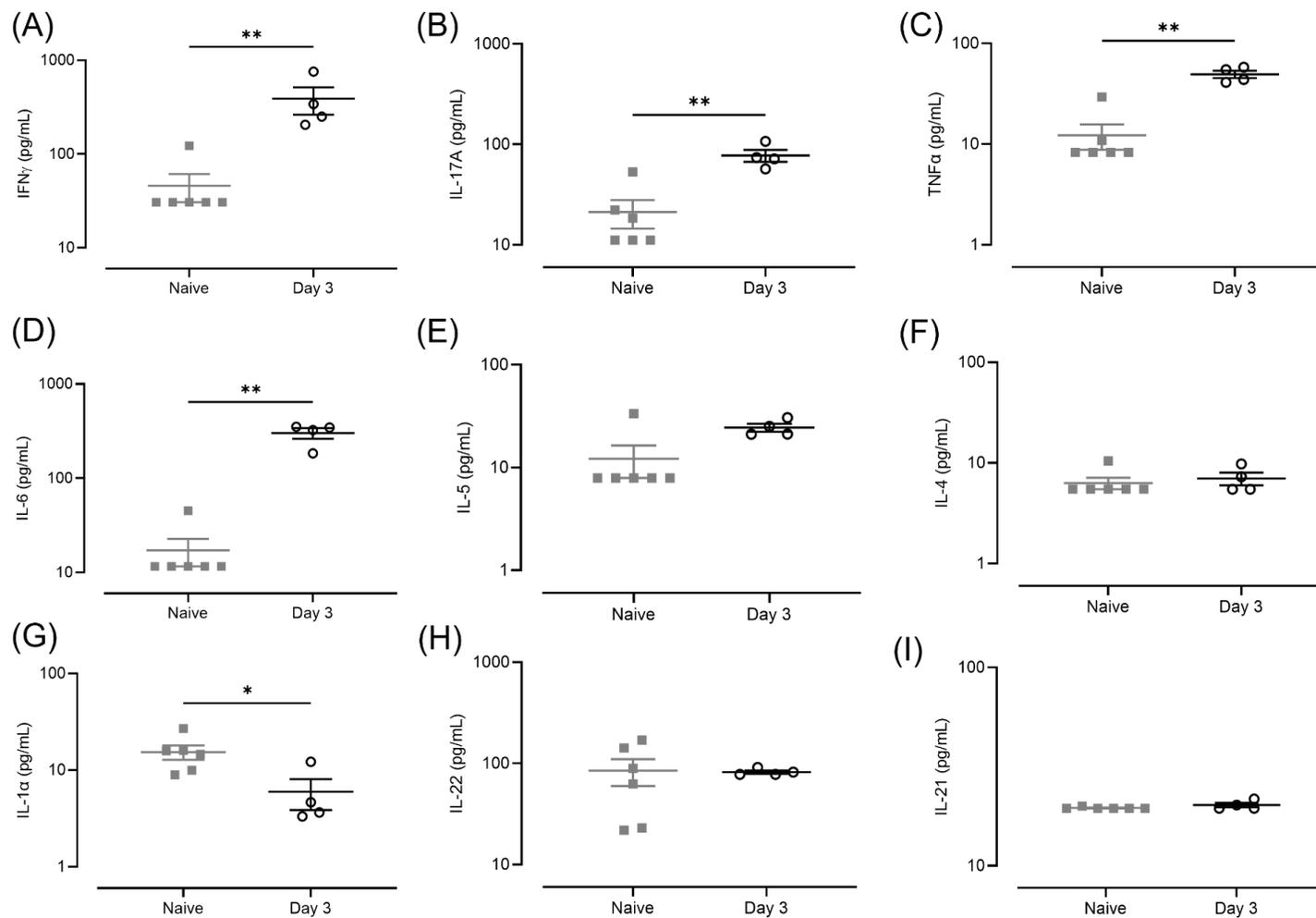


Figure 5.2: Systemic inflammation is a feature of AIA

To quantify circulating T cell related cytokines in mice with AIA, a LEGENDplex assay was performed on plasma from mice terminated on Day 3 (n=4) and compared to naïve mice (n=6). (A) IFN γ . (B) IL-17A. (C) TNF α . (D) IL-6. (E) IL-5. (F) IL-4. (G) IL-1 α . (H) IL-22. (I) IL-21. Graphs are shown as mean \pm SEM and significance is calculated by t-test. * = p < 0.05; ** = p < 0.01.

5.2.3 AIA results in aortic T cell infiltration at Day 3

The circulating cytokines in **Figure 5.2** are in direct contact with the blood vessel wall, which may promote endothelial activation that contributes to vascular inflammation, stiffness and defective constriction. However, the local environment such as the perivascular adipose tissue (PVAT) and the aorta is also infiltrated by immune cells during inflammatory arthritis, as evidenced by increased CD3 T cell and galectin-3 expression in the PVAT in the CIA model in **Chapter 3** of this Thesis. This change in vascular pathology influences vascular tone and contractility by promoting vascular remodelling and the proliferation of vascular smooth muscle cells (Savoia et al. 2011). To understand the T cell related signals occurring in the blood vessel and PVAT that could influence pathological mechanisms in the tissue, the expression of T cell inflammatory markers *Il17a* and *Ifny* was quantified in the aorta associated PVAT on Day 3 and Day 10 of AIA by qPCR. Both *Il17a* and *Ifny* expression in the aorta was increased at Day 3 and reached statistical significance at Day 10. Similarly, *Il17a* expression increased in the PVAT at Day 3 and Day 10 (**Figure 5.3 A**). However, *Ifny* expression in the PVAT remained comparable to that in healthy control mice (**Figure 5.3 B**). Heightened *Il17a* and *Ifny* expression in the aorta, but not the PVAT, combined with elevated IL-17A and IFN γ in circulating blood suggests that the local expression of these inflammatory cytokines may be important in their role in the vascular inflammation.

While the expression of *Il17a* and *Ifny* was significantly increased at Day 10, constriction responses at Day 10 were restored to baseline levels. Therefore, Day 10 was not an appropriate timepoint to test the hypothesis of this Chapter which was that targeting galectin-3 suppresses CD4 cell responses to limit the vascular constriction deficit. Albeit while genetic expression of these markers were not significantly increased at Day 3, there is a strong trend toward increased *Il17a* and *Ifny* expression in the aorta, suggesting that T cells are still involved in the vascular inflammation at Day 3.

Taking this into account, and that defective vascular constriction responses were observed at Day 3, Day 3 was chosen as the timepoint to study vascular inflammation in future studies. In addition to this, the regulated effect of galectin-3 levels in association with constriction responses, and CD4 T cell related expression in the vasculature resulted in the AIA model being chosen to investigate galectin-3 inhibition, via the use of GB1107, to test the hypothesis of this Chapter.

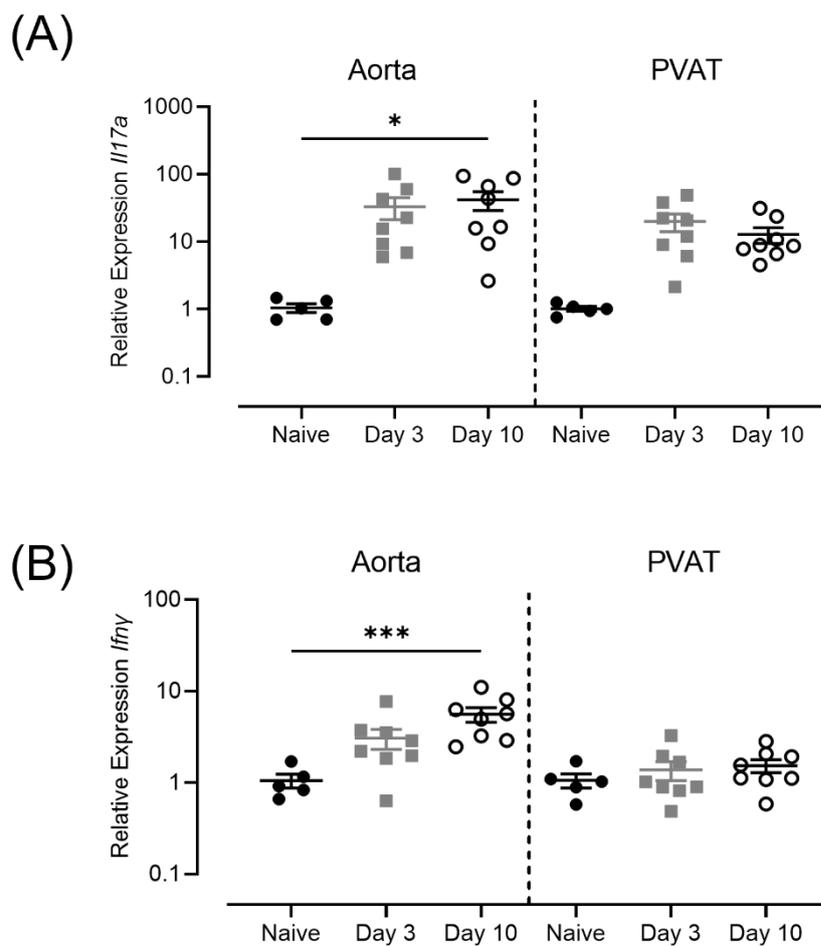


Figure 5.3: Increased expression of T helper cell signature cytokines in the aorta during AIA

Thoracic aortas and PVAT was harvested from mice at Day 3 and Day 10 of AIA. RNA was extracted and a qPCR performed. (A) *Il17a* expression was quantified in aorta and PVAT and is shown as relative expression to naïve sample and normalised to *Actb* housekeeping gene. (B) Graph showing *Ifny* expression in aorta and PVAT at Day 3 and Day 10 of AIA and compared to naïve mice. Graphs are shown as mean \pm SEM. Significance was calculated by one-way ANOVA with Tukey post-test. *= $p < 0.05$; ***= $p < 0.001$.

5.2.4 Inhibition of galectin-3 did not alter the incidence of AIA

To determine whether targeting galectin-3 using GB1107 influenced the progression of AIA, a pilot experiment with a small number of mice was carried out to form initial observations. This approach also enabled me to work within the constraints of the myograph, in that only a small number of tissue samples could be processed at any one time. Mice were dosed with 10 mg/kg GB1107 in vehicle propylene glycol in HPMC from Day -2 to termination on Day 3 via oral gavage. Mice were weighed daily from arthritis induction on Day 0 to Day 3. Weight loss was significantly decreased in mice with AIA treated with GB1107 when compared to naïve controls (**Figure 5.4 A**). The administration of mBSA to the knee joint normally triggers a robust inflammatory response that is quantified by measuring knee swelling. In untreated AIA, joint swelling in excess of 1.0 mm is expected on Day 1 if the systemic antibody titre is sufficient and the mBSA is delivered into the joint space after intra-articular injection on Day 0 (Nowell et al. 2009). Moderate swelling was noted in the GB1107 treated group (1.3 mm above baseline). However, only mild swelling (maximum of 0.6 mm) was observed in the group that received vehicle (**Figure 5.4 B**). mBSA-specific IgG antibody levels were quantified in the plasma of mice with and without AIA. The results were compared against plasma samples from previous AIA experiments where the swelling profile was higher and in the normal range for a typical experiment. Plasma mBSA-specific IgG titres were identical in the AIA pilot study and in the historical samples (**Figure 5.4 C**). This indicates that while swelling was decreased in this experiment, the immune response to mBSA was comparable to previous experiments.

To achieve the next aim of this Chapter, the effect of GB1107 on cellular infiltrate to the inflamed synovium and the lymph node was characterised by flow cytometry.

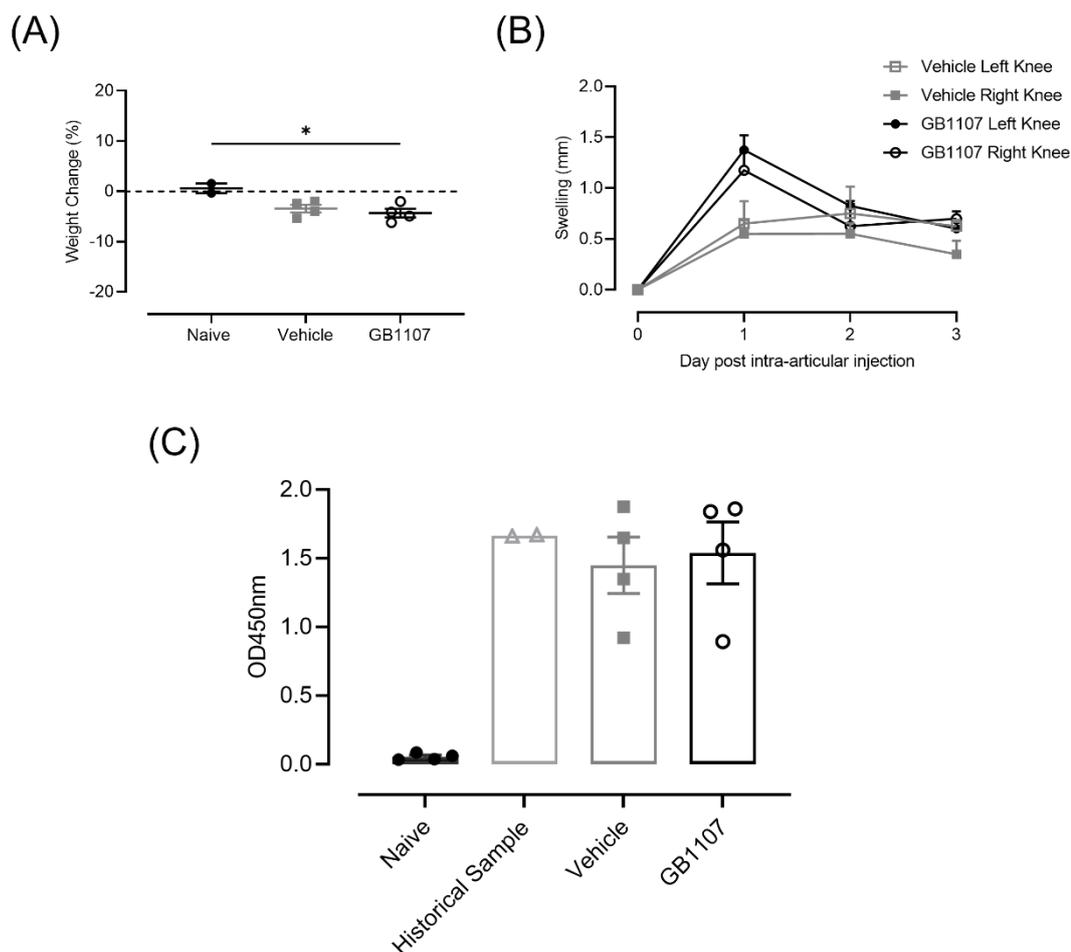


Figure 5.4: Establishing the antigen induced arthritis model

Antigen induced arthritis was established in wild-type male mice and were treated with 10 mg/kg GB1107 or vehicle (10% propylene glycol in 0.5% HPMC) once daily via oral gavage. (A) Weight loss across each group. Significance was calculated by one-way ANOVA with Tukey post-test. (B) Swelling was assessed by measurement of knee diameter on Day 0 prior to mBSA administration and everyday thereafter until termination. (C) mBSA specific antibodies were measured in the plasma of mice treated with vehicle or GB1107 and compared to antibodies from naïve mice and from plasma samples from historical experiments conducted in the lab. Graphs are shown as mean \pm SEM where $*=p<0.05$.

5.2.5 GB1107 reduces CD4 cell infiltrate to the synovium during AIA

During inflammatory arthritis, CD4 cells are recruited to the joint space and engage with dendritic cells to result in re-activation of T cells and perpetuation of inflammatory signal (Prendergast et al. 2018). The effect of GB1107 on CD4 infiltrate, and subsequent effector CD4 cells to the inflamed synovium were measured. Treatment with GB1107 reduced the proportion of CD3⁺CD4⁺ cells in the synovium (**Figure 5.5 A**). There was a trend toward reduced CD3⁺CD4⁺ cell number upon treatment with GB1107, but this was not significant (**Figure 5.5 B**). When CD4 effector cells were measured, while there was no effect observed in the proportion of IL-17-producing cells, treatment with GB1107 exhibited a trend toward decreased number of IL-17-producing cells in the synovium (**Figure 5.5 C, D**). Inhibition of galectin-3 by GB1107 significantly increased the proportion of IFN γ producing cells and a trend toward increased IFN γ expressing cell number (**Figure 5.5 C, E**). When IL-17⁺IFN γ ⁺ populations were studied, treatment with GB1107 increased the proportion of these cells, with the increase reaching statistical significance in the proportion of cells staining positively for IL-17 and IFN γ (**Figure 5.5 C, F**).

While GB1107 decreased CD4 cellular infiltrate to the synovium, further studies were carried out to evaluate the effect of GB1107 on the gene expression of T cell markers and galectin-3 in the inflamed synovium.

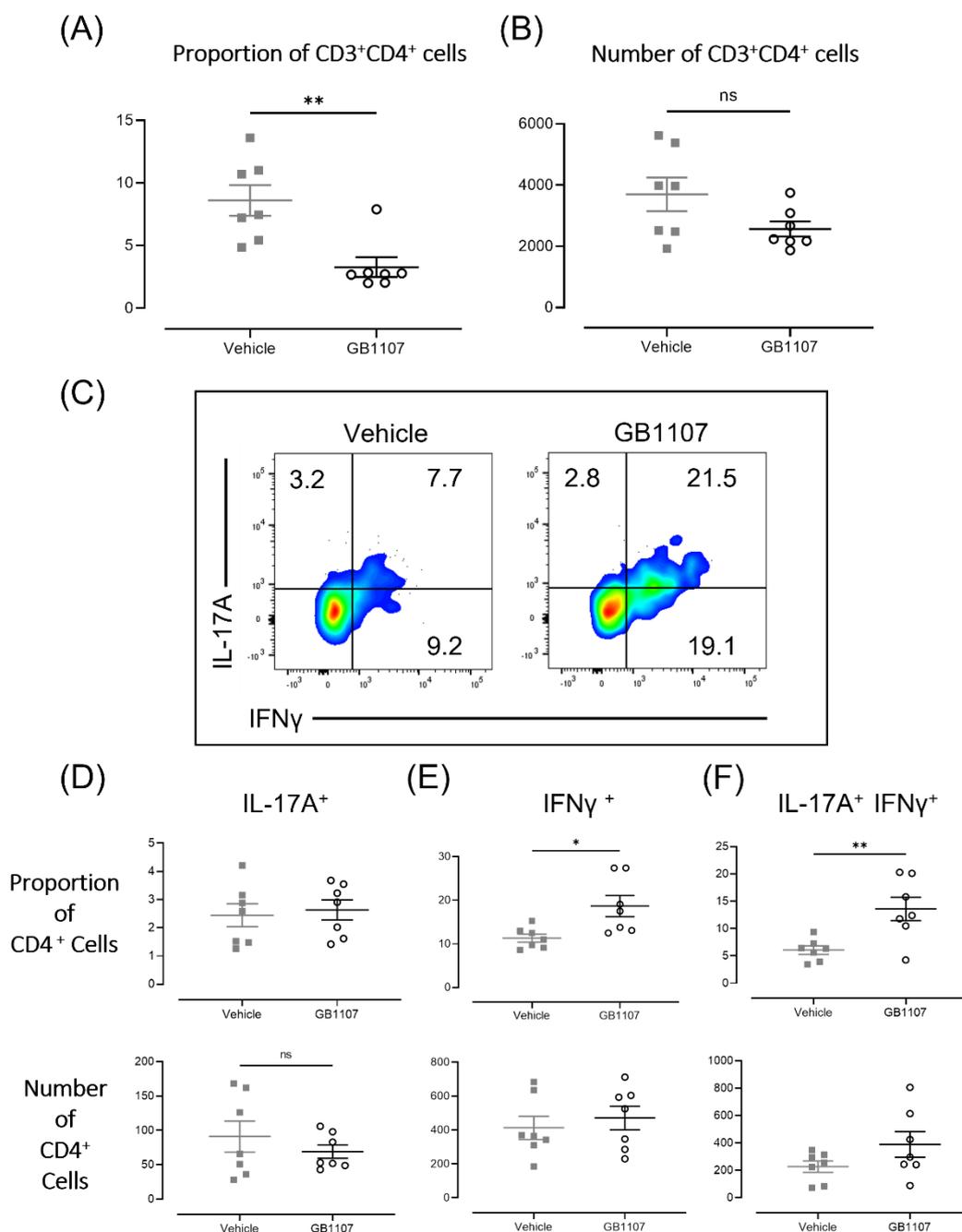


Figure 5.5: Treatment with GB1107 reduces CD3⁺CD4⁺ cell infiltrate to the synovium

Wild-type male mice undergoing AIA were treated with 10 mg/kg GB1107 or vehicle once daily via oral gavage. Synovium was collected and digested on Day 3 following termination and cells stained for live, CD3, CD4, IL-17A and IFN γ and run on a flow cytometer. (A) Graphs showing proportion and (B) number of CD3⁺CD4⁺ cells in the synovium after treatment with vehicle or GB1107. Cells and cell numbers are gated and based on live cells, as determined by negative ZombieAqua staining (BioLegend). Significance was calculated by t-test where **= $p < 0.01$. (C) Representative FACS plots of IL-17A vs IFN γ expressing cells collected from the synovium. Cells based on proportion of CD3⁺CD4⁺ cells. Large numbers are percentage of cells in each gate. (D) Proportion and number of IL-17A expressing cells, (E) IFN γ expressing cells and (F) IL-17A⁺IFN γ ⁺ expressing cells in the synovium. Data are shown as mean \pm SEM with 4 animals/group and samples run in duplicate. Significance was calculated by t-test where *= $p < 0.05$; **= $p < 0.01$.

5.2.6 Inhibition of galectin-3 does not alter T cell effector cytokine expression in the inflamed synovium

Gene expression analysis of the inflamed synovium was carried out in mice treated with GB1107 or vehicle. As the effect of GB1107 on galectin-3 levels in the synovium could not be reliably detected in the synovium by flow cytometry (**Section 4.2.3**), synovium was collected and processed for qPCR and *Lgals3* gene levels quantified. There was a trend toward decreased *Lgals3* in mice treated with GB1107, but this trend was very subtle with a relative change in expression from 1.0 to approximately 0.64 and wasn't statistically significant (**Figure 5.6 A**). *Il17a* and *Ifn γ* expression was unchanged following treatment with GB1107 (**Figure 5.6 B, C**). Together, this data shows that galectin-3 inhibition by GB1107 does not act by regulating the genetic expression of CD4 effector cells in the inflamed synovium.

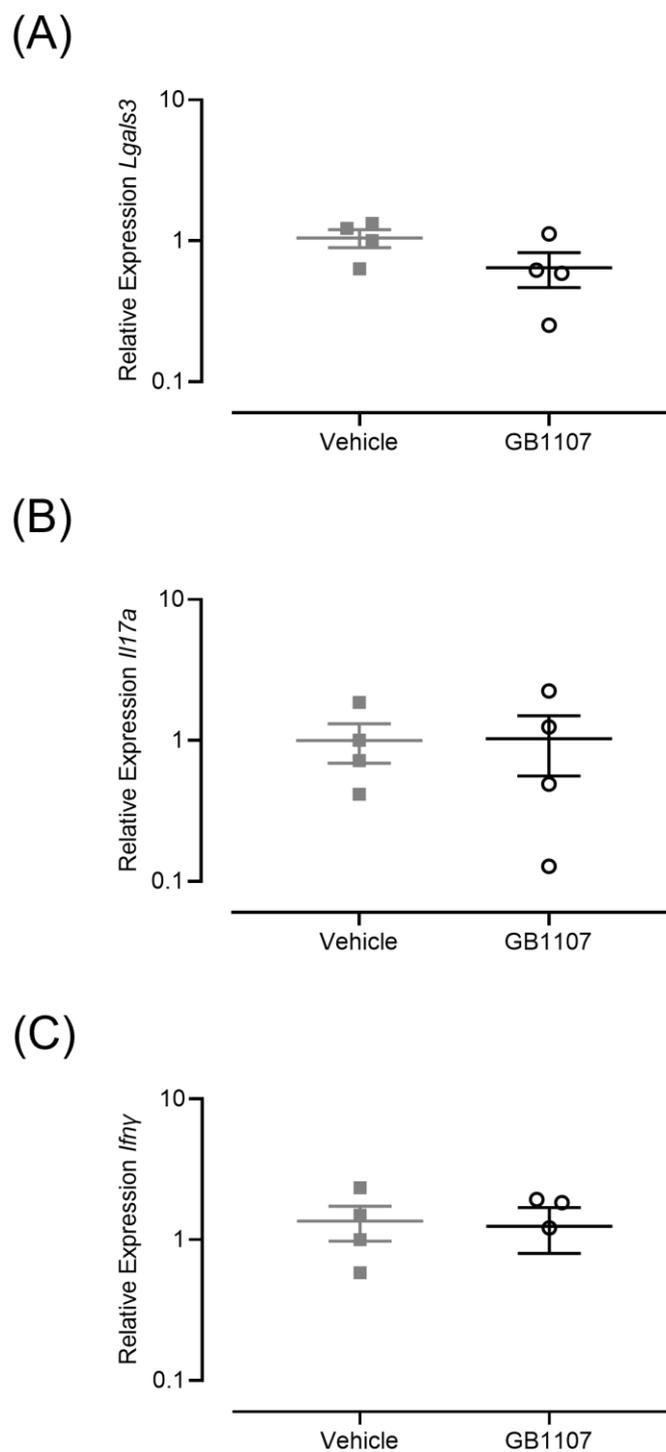


Figure 5.6: No differences in *Lgals3* or T cell markers following treatment with GB1107

Synovium was collected from mice with AIA at Day 3 of the protocol and homogenised in QiaZOL lysis reagent. RNA was extracted using chloroform separation technique and miRNeasy Micro kit (Qiagen) and qPCR performed. (A) Graph showing *Lgals3* (B) *Il17a* and (C) *Ifny* expression in synovium following treatment with GB1107. Data are shown as mean \pm SEM. n=4/group.

5.2.7 GB1107 reduces CD4 cells in the lymph node in AIA

Lymph nodes are reservoirs of lymphocytes and play a role in regulation of immune responses by facilitating the release of leukocytes, inflammatory mediators and drainage of extravasated fluid (Schwager and Detmar 2019). The effect of galectin-3 inhibition on draining lymph node lymphocyte populations was determined. Inguinal lymph nodes were collected from naïve mice with AIA treated with vehicle and mice with AIA treated with GB1107. CD4 cells were isolated from lymph nodes, stained for CD4 effector cells and flow cytometry performed. Mice with AIA, regardless if treated with vehicle or GB1107, had significantly decreased proportion of CD3⁺CD4⁺ cells in the lymph node when compared to naïve. When the number of cells in the lymph nodes were counted, the same trend was observed whereby mice with AIA had decreased CD3⁺CD4⁺ cell number compared to naïve mice (**Figure 5.7 A, B**). When CD4 effector populations were measured, there were no IL-17A producing cells in the lymph nodes (**Figure 5.7 C, D**). Treatment with GB1107 significantly increased both the proportion and number of IFN γ expressing cells in the lymph node (**Figure 5.7 C, E**) with approximately twice the number of IFN γ expressing cells in GB1107 treated lymph nodes compared to naïve lymph nodes. The overall decrease in CD3⁺CD4⁺ cells in the lymph nodes in mice with AIA may be in part due to the migration of these cells to distant sites of inflammation such as the synovium during this acute phase of the AIA model. One of these distant sites may include the vasculature, which was the next focus of the study.

5.2.8 No change in *Lgals3*, *Il17a* or *Ifny* expression in the vasculature in mice treated with GB1107

As shown in **Figure 5.3**, there is inflammatory cell infiltrate to the vasculature at Day 3 during AIA. To determine if this could be mediated by treatment with GB1107, RNA was extracted from aorta and PVAT and a qPCR carried out. *Il17a* expression was increased in the aorta and PVAT following AIA induction, but this was unchanged by GB1107 (**Figure 5.8 A**). Similarly, there was a trend toward increased *Ifny* expression in the aorta upon AIA induction but this was not significant and unchanged by treatment with GB1107, and there was no difference in *Ifny* infiltrate to the PVAT during AIA (**Figure 5.8 B**).

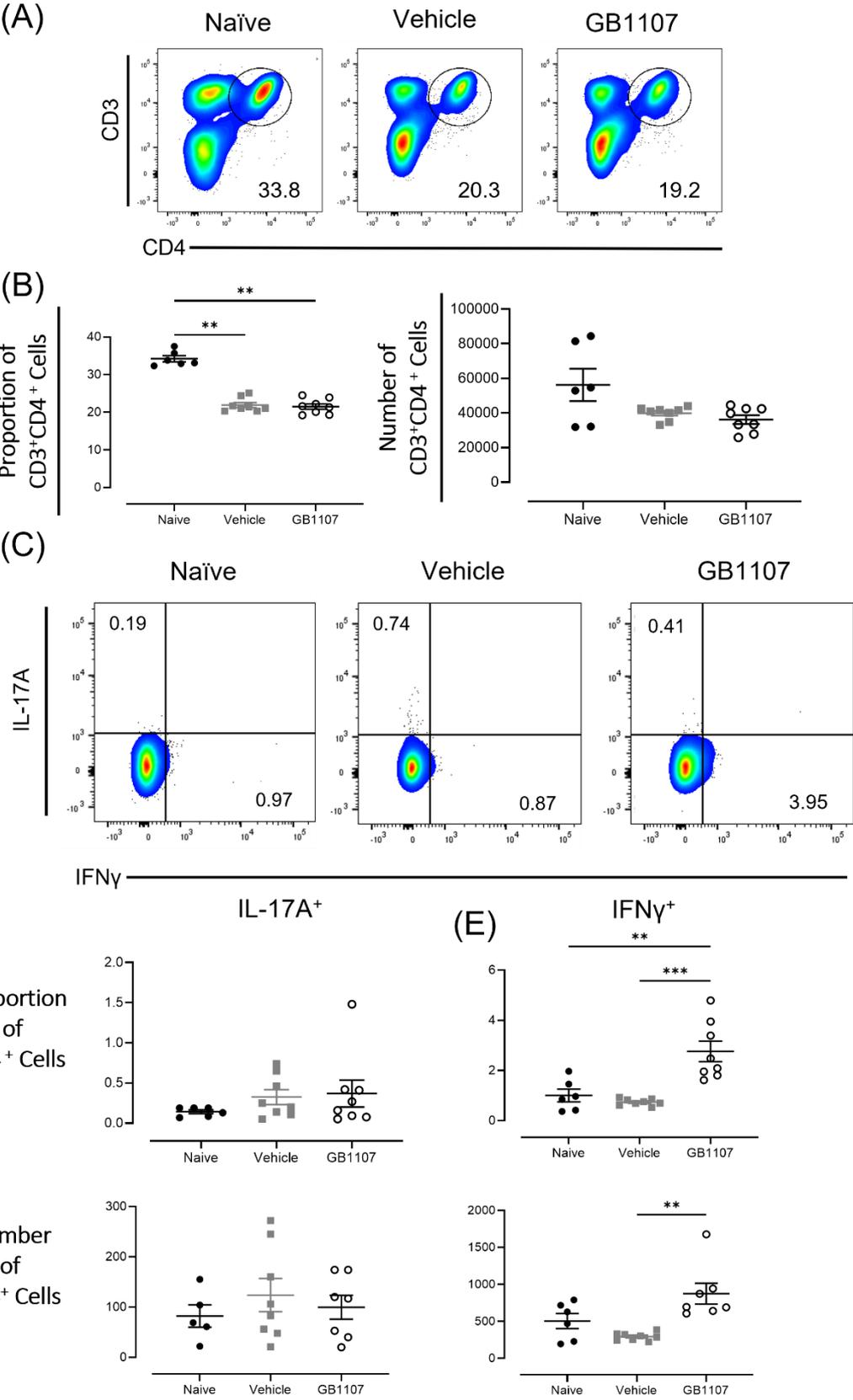


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Figure 5.7: Reduced T cells in the lymph node upon AIA induction

Lymph nodes were collected from mice with AIA and were homogenised to form single cell suspensions. Cells were stained for live cells, CD3, CD4, IL-17 and IFN γ before running on a flow cytometer. (A) Representative flow plots of CD3⁺CD4⁺ populations. Cells and cell numbers are gated and based on live cells, as determined by negative ZombieAqua staining (BioLegend). Large numbers are percentage of cells in each gate. (B) Graphs showing proportion and number of CD3⁺CD4⁺ cells in the lymph node in mice treated with vehicle or GB1107 and compared to naïve mice. Significance was calculated by one-way ANOVA with Dunn's post-test where **= $p < 0.01$. (C) Representative FACS plots of IL-17A⁺ and IFN γ ⁺ populations in the lymph node. Large numbers are percentage of cells in each gate. (D, E) Proportion and number of IL-17A⁺ and IFN γ ⁺ cells in the lymph nodes of mice treated with vehicle or GB1107 and compared to naïve controls. Data are shown as mean \pm SEM with 4 animals/group and samples run in duplicate. Significance is calculated by one-way ANOVA with Tukey post-test where **= $p < 0.01$; ***= $p < 0.001$. $n=4$ /group run in duplicate.

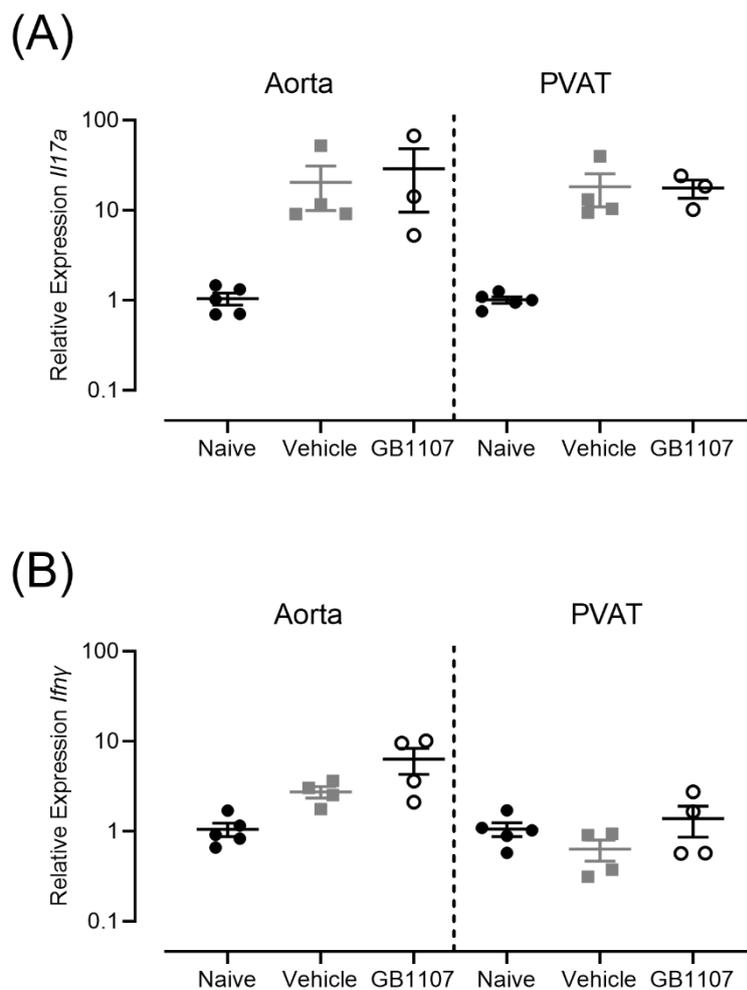


Figure 5.8: Gene expression analysis of the vasculature following treatment with GB1107

Thoracic aortas and surrounding PVAT was harvested from mice with AIA at Day 3 and RNA extracted using chloroform extraction method and miRNeasy extraction kit (Qiagen). Graphs showing (A) *Il17a* and (B) *Ifnγ* expression in the aorta and PVAT following treatment with vehicle (n=4) or GB1107 (n=4). n=5 in naïve group. Data is normalised to *Actβ* housekeeping gene. Data are shown as mean \pm SEM.

5.2.9 No significant difference in constriction responses in mice with AIA compared to naïve mice

In order to test if targeting galectin-3 suppresses CD4 T cell responses to limit vascular constriction defects, 5HT induced constriction responses in mice treated with galectin-3 inhibitor were determined by myography. Thoracic aortas from naïve mice achieved a R_{max} of 9.0 mN which is in keeping with previous studies described in **Section 5.2.1**. However, contrary to what was observed in previous studies, there was no significant differences between the constriction responses in naïve mice and mice undergoing AIA (**Figure 5.9 A**). The higher-than-expected R_{max} in vehicle treated mice may be a reflection of lower swelling in those mice. Nonetheless mice with AIA treated with vehicle exhibited the lowest R_{max} at 7.1 mN and mice treated with GB1107 had an R_{max} of 8.0 mN. This indicated that while the significant differences in constriction responses between mice with AIA and naïve mice was lost, there was still a trend toward decreased R_{max} in vehicle treated mice and a partially restored R_{max} in the GB1107 group.

As a final readout of the experiment, and to determine if treatment with GB1107 altered circulating galectin-3 levels, a galectin-3 ELISA was performed on plasma from mice with AIA. Mice treated with GB1107 had significantly higher circulating galectin-3 levels compared to naïve mice (**Figure 5.9 B**). This trend was identical to that observed in experiments run in the CIA model (**Figure 3.4 B**) which further strengthens the point that GB1107 does not directly target galectin-3 expression levels, as also evidenced in **Figure 4.17**, and is likely to indirectly target the activity of galectin-3, perhaps via its expression in cells.

It must be noted that this pilot experiment was statistically underpowered and had lower swelling than what is expected for wild-type mice in this experiment, but yielded interesting results nonetheless. Inhibition of galectin-3 reduced CD4 cell infiltrate during synovitis, and although no significant differences in constriction responses were observed between naïve and treatment groups in this experiment, there was a trend toward partially restored constriction responses in the GB1107 treated group. Therefore, to determine if the trend in constriction responses was a true effect of galectin-3 inhibition, a power calculation was performed which showed that the group sizes needed to be increased to allow for statistical tests to be performed and allow for conclusions to be drawn. Given this, the experiment was repeated.

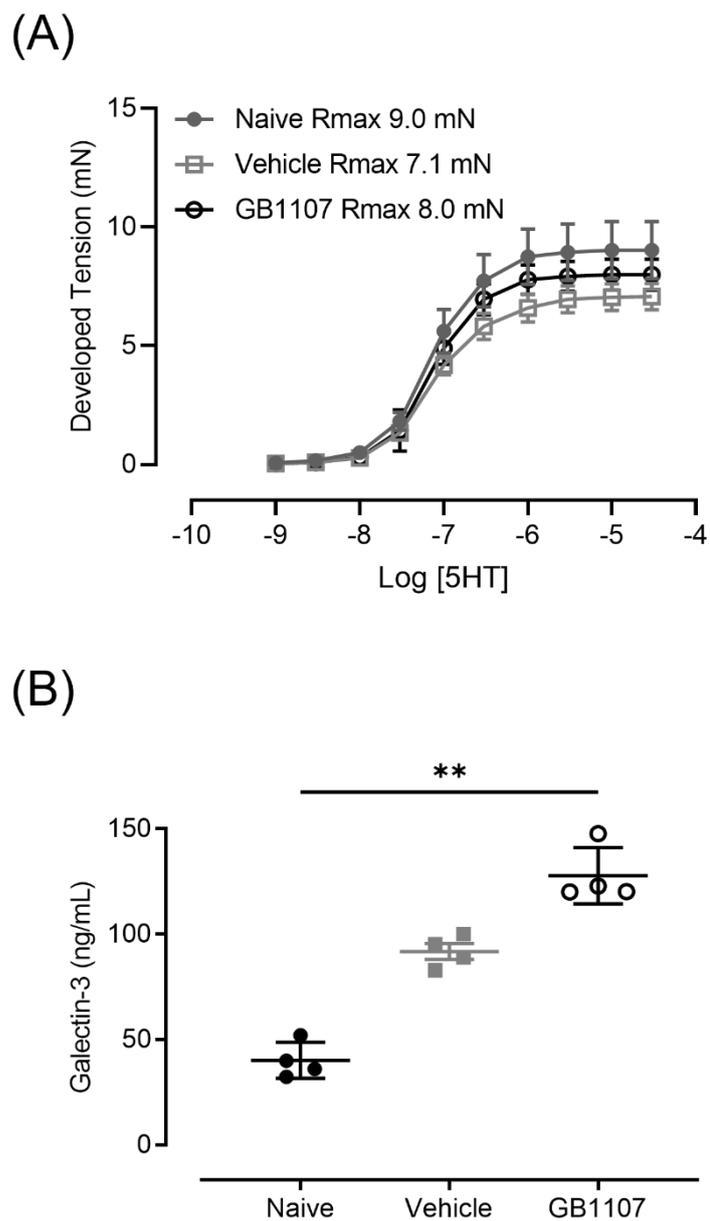


Figure 5.9: No significant change in vascular constriction responses upon AIA induction

Mice with AIA were culled on Day 3. (A) Thoracic aortas were harvested from mice treated with vehicle or GB1107 and 5HT induced constriction responses measured and compared to age-matched naïve mice. (B) Plasma was collected upon termination and a galectin-3 ELISA performed. Significance was calculated by one-way ANOVA with Dunn's post-test. Graphs are shown as mean \pm SEM. **= $p < 0.01$. $n = 4$ /group.

5.2.10 Treatment with GB1107 resulted in significantly reduced knee swelling in mice with AIA

Mirroring the previous experiment, mice were dosed with 10 mg/kg GB1107 daily from Day -2 prior to administration of mBSA intra-articularly until termination on Day 3. All mice in the AIA protocol lost weight, with mice in the vehicle group having significant weight loss when compared to naïve mice (**Figure 5.10 A**). A robust inflammatory response to the mBSA administration to the knee joint was observed. Knee swelling of 1.2 – 1.5 mm was measured in all mice following IA injection of mBSA. Mice treated with GB1107 had significantly less swelling than the vehicle group at Day 2 post IA injection (**Figure 5.10 B**). The swelling parameters reached the anticipated level for wild-type mice undergoing the AIA protocol (Nowell et al. 2009).

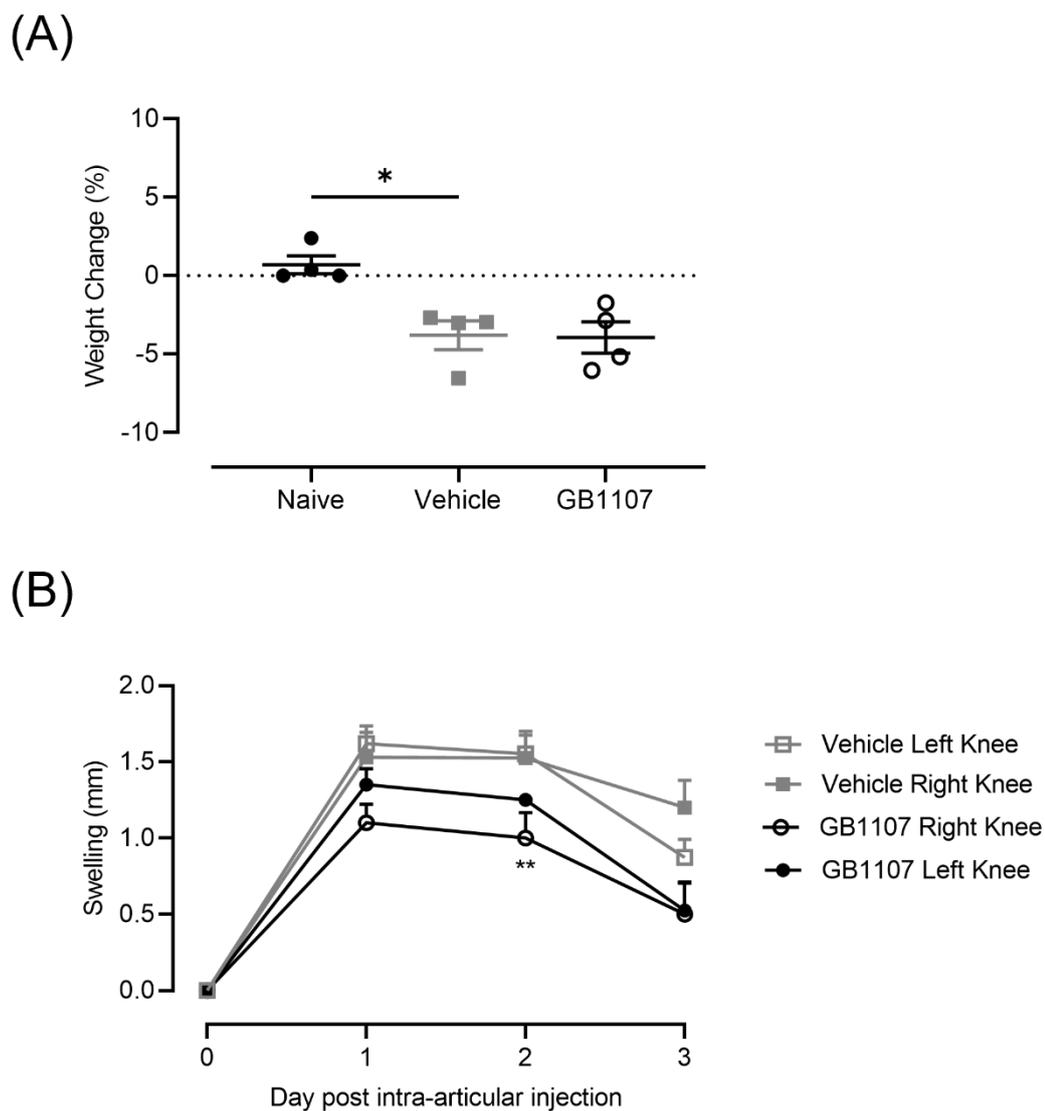


Figure 5.10: Significant decrease in knee swelling in mice treated with GB1107

Antigen induced arthritis was induced in male mice and mice were dosed with 10 mg/kg GB1107 or vehicle from Day -2 to Day 3 via oral gavage. (A) Graph showing weight change in mice from Day -2 to termination on Day 3. Significance was calculated by one-way ANOVA with Dunn's post-test. (B) Swelling was assessed in both knees daily using a calliper from Day 0 to termination. Significance was calculated by two-way ANOVA with Tukey post-test. *= $p < 0.05$; **= $p < 0.01$. Data are shown as mean \pm SEM.

5.2.11 Inhibition of galectin-3 with GB1107 promotes restoration of constriction responses

Thoracic aortas were used to measure vascular constriction by myography. Responses in naïve mice and AIA mice treated with vehicle or GB1107 were evaluated. Constriction responses in naïve mice reached an R_{max} of 7.9 mN. Values were consistent with those observed in initial studies, **Figure 5.1**. Constriction responses were consistently lower in mice with AIA that received vehicle ($R_{max}=4.6$ mN, $p=0.005$). Treatment with GB1107 fully restored vascular constriction responses (**Figure 5.11 A**). The average R_{max} for GB1107 treated mice was also 7.9 mN. For the first-time, in CIA or AIA, full restoration of constriction responses was achieved by treating arthritic mice with GB1107. A concomitant attenuation in knee swelling was also observed with GB1107. Next, the impact of GB1107 treatment upon circulating levels of galectin-3 was quantified by ELISA. Galectin-3 concentration was significantly higher in AIA than naïve mice, regardless of treatment. The galectin-3 level in mice treated with GB1107 was significantly higher than in mice given vehicle (**Figure 5.11 B**). This suggests that GB1107 may restore vascular function indirectly via its action on cells (e.g., Th17 cells) or the cytokines rather than directly by inhibiting galectin-3. Given the role of galectin-3 in Th17 proliferation discovered in **Chapter 4** of this Thesis, therefore highlighting galectin-3 as a key player in perpetuating pro-inflammatory processes, the next step was to determine if the effect of galectin-3 inhibition in disease inhibited Th17 cell specific responses in the model.

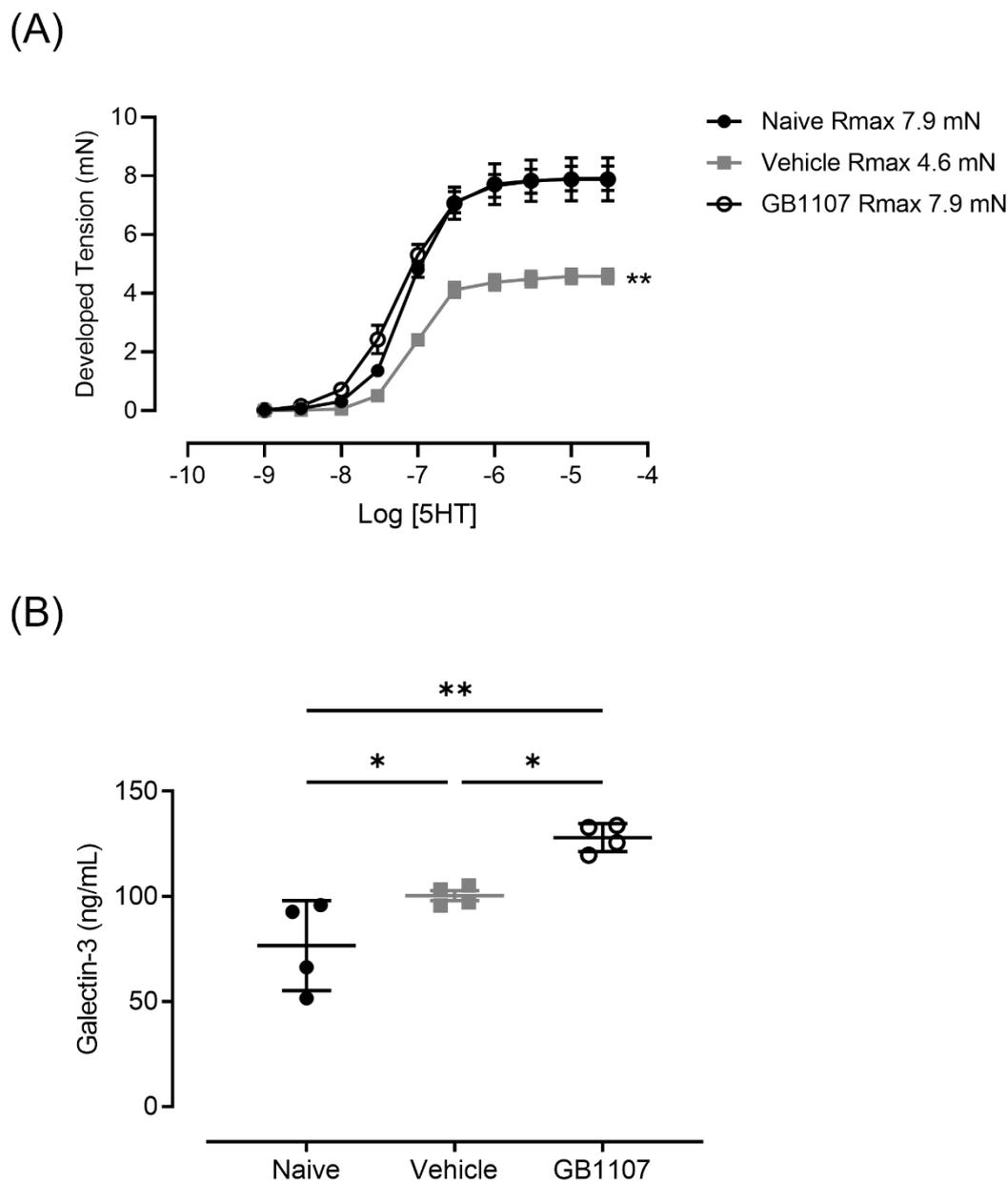


Figure 5.11: Treatment with GB1107 fully restored vascular constriction responses

Mice with AIA were terminated on Day 3. (A) Thoracic aortas were harvested and 5-HT induced constriction responses were measured. Significance was calculated by 2-way ANOVA with Sidak post-test where $**=p<0.01$. (B) Plasma was harvested on termination and a galectin-3 ELISA performed. $n=4/\text{group}$. Significance was calculated by one-way ANOVA with Sidak post-test where $*=p<0.05$; $**=p<0.01$. Graphs are shown as mean \pm SEM.

5.2.12 GB1107 does not affect the Th17 mBSA specific response

To assess whether GB1107 inhibited Th17 specific responses, inguinal lymph nodes from mice with AIA were harvested, mashed to single cell suspension and re-stimulated with mBSA. Cells were cultured for 24, 48 and 72 hours. The supernatants were collected at each timepoint and IL-17A secretion from cells to the supernatants was measured in each sample by ELISA. The baseline for Th17 secretion for healthy mice was established using lymph nodes from naïve mice. These were used as the experimental controls. Lymph nodes from mice with AIA were also included for completeness. They were not re-stimulated with mBSA and were designated unstimulated samples.

IL-17A secretion increased with time from lymph nodes from mice with AIA. No differences in IL-17A output was observed in any group at 24 hours post stimulation. IL-17A secretion was significantly higher in stimulated lymph nodes from vehicle and GB1107 treated mice when compared to naïve mice at 48 hours (**Figure 5.12**). This effect was further increased at 72 hours post stimulation where again, lymph nodes from both vehicle and GB1107 treated mice had significantly higher IL-17A secretion when compared to naïve mice. While there was IL-17A secretion from unstimulated lymph nodes from mice with AIA, this effect was not significant when compared to lymph nodes from naïve mice. Notably, treatment with GB1107 did not affect IL-17A secretion from lymph nodes after stimulation with mBSA, indicating that GB1107 doesn't affect the Th17 mBSA specific response. Therefore, it is likely that the restoration of vascular constriction responses in mice treated with GB1107 occur independently of the effect of GB1107 on IL-17 responses described in **Chapter 4** of this Thesis. It is possible that GB1107 may be acting via other mechanisms that could result in other cells or cytokine activation to result in IL-17 secretion. To determine what effect GB1107 had on the cytokine responses during AIA, a multiplex assay was carried out.

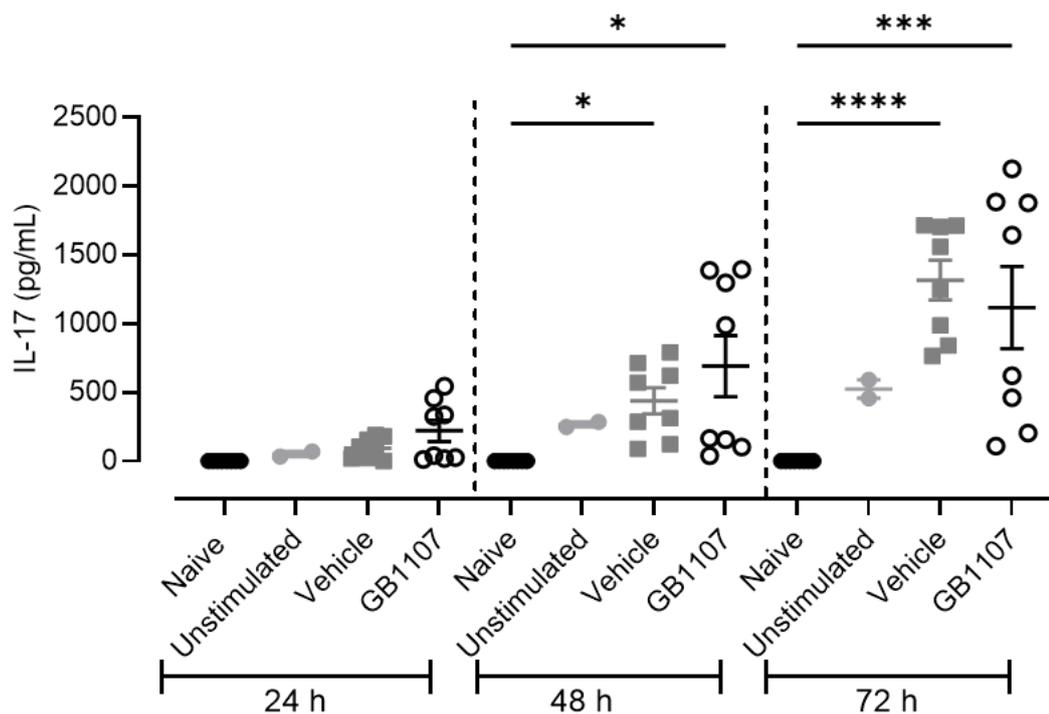


Figure 5.12: GB1107 does not affect mBSA specific Th17 responses

Inguinal lymph nodes were recovered from mice with AIA treated with vehicle or GB1107 and were re-stimulated with 20 $\mu\text{g}/\text{mL}$ mBSA. Cells were cultured at 37°C and supernatants were collected at 24, 48 and 72 hours post seeding and an IL-17 ELISA performed. Unstimulated refers to lymph nodes from mice with AIA but did not receive re-stimulation with mBSA. Significance was calculated by 2-way ANOVA with Tukey post-test where $*=p<0.05$; $***=p<0.001$; $****=p<0.0001$. Data are shown as mean \pm SEM.

5.2.13 GB1107 treatment does not modulate the systemic T cell-associated cytokine response

As outlined in **Figure 5.2**, T cell related cytokines are elevated during AIA. To determine if treatment with GB1107 had an effect on these cytokines, a multiplex assay was carried out on plasma from mice from both experiments presented in this Chapter. Concurrent with that seen in **Figure 5.2**, IFN γ , IL-17A, TNF α and IL-6 levels were significantly increased in AIA (**Figure 5.13 A, B, C, D**). However, treatment with GB1107 did not alter the levels of these cytokines. Th2-related cytokines IL-5 and IL-4 were significantly increased during AIA but again, this was not changed by GB1107 (**Figure 5.13 E, F**). Circulating IL-10 levels were unchanged upon induction of AIA (**Figure 5.13 G**). Conversely to that observed in **Figure 5.2**, circulating levels of Th17 cytokine IL-22 were significantly increased in mice with AIA, but this was unchanged by treatment with GB1107. (**Figure 5.13 H**).

These findings suggest that circulating cytokines - although in direct contact with the blood vessel wall - bear little impact on the contractility of the blood vessel, and that GB1107 does not directly impact circulating cytokine levels *in vivo*.

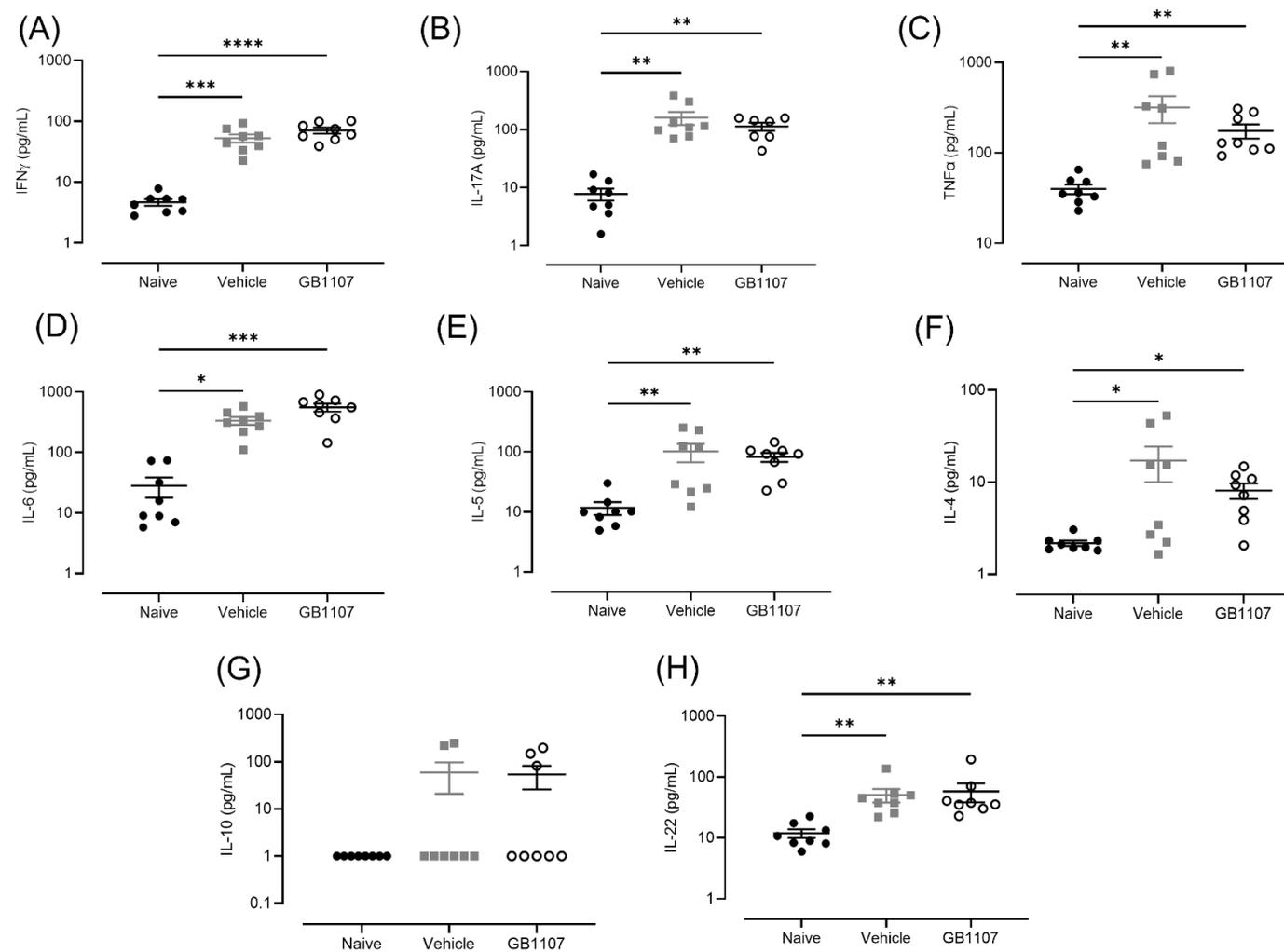


Figure 5.13: GB1107 did not alter levels of circulating cytokines in AIA

Multiplex analysis on plasma from mice with AIA treated with vehicle or GB1107 compared to age-matched naïve mice. (A) IFN γ ; (B) IL-17A; (C) TNF α ; (D) IL-6; (E) IL-5; (F) IL-4; (G) IL-10; (H) IL-22. n=8 animals per group. Significance is calculated by one-way ANOVA with Dunn's post-test where *= $p < 0.05$; **= $p < 0.01$; ***= $p < 0.001$; ****= $p < 0.0001$. Data are shown as mean \pm SEM.

5.3 Discussion

AIA is a localised model with systemic effects, and features a constriction response deficit

The hypothesis of this Chapter was that AIA is associated with a vascular change and that targeting galectin-3 suppressed the CD4 T cell response to limit vascular constriction defects. **Chapter 4** of this Thesis identified a role of galectin-3 in Th17 expansion and proliferation, and so the AIA model was employed to test the effect of inhibiting galectin-3 in an *in vivo* setting as the AIA model has strong CD4 cell involvement, including Th17 cells (Ebbinghaus et al. 2012; Jones et al. 2015; Jones et al. 2018). However any systemic features and the effect of localised inflammation in the synovium on vascular function was not reported in the literature prior to initiating this study. The constriction deficit at Day 3 and subsequent restoration to baseline at Day 10 of the model suggested that the processes underlying the acute phase of the model drives vascular dysfunction. Circulating galectin-3 levels are elevated at Day 3 and fall to baseline at Day 10, suggesting that galectin-3 may also have a role in the acute phase of disease. Galectin-3 is involved in inflammatory responses and is raised during acute inflammation (Numano et al. 2015). A study by Gittens *et al* identified a role for galectin-3 in facilitating leukocyte recruitment during acute inflammation. *Lgals3*^{-/-} mice have defective leukocyte rolling and emigration, due to decreased binding to E-selectin (Gittens et al. 2017). While the exact mechanism underlying defective constriction responses are not fully understood, the elevated galectin-3 levels observed at Day 3 that coincide with defective constriction responses suggest that they may be related to galectin-3-mediated leukocyte infiltrate to the blood vessel and surrounding vascular structures (e.g., PVAT), that result in blood vessel wall thickening and arterial stiffening. This has been observed in other models of diabetes where mice with elevated expression of TNF α and CRP in the PVAT display a constriction deficit to vasoconstrictors phenylephrine and endothelin-1 (Azul et al. 2020).

While a relationship between acute inflammation, circulating galectin-3 levels and defective constriction responses was identified, this study also met the second aim of the Chapter which was to characterise the systemic features of AIA. The presence of vascular inflammation and elevated cytokines in the blood proved that local inflammatory insult to the synovium results in a systemic response and systemic disease. IL-17A was significantly increased in the model, as previously reported (Jones et al. 2015), further highlighting that AIA is a T cell mediated disease, and that Th17 cells are involved in the model. Interestingly,

Th17 cytokine IL-22 levels were unchanged upon induction of AIA. This finding is contrary to previous studies that showed elevated IL-22 serum levels during inflammatory arthritis in pristane induced arthritis in rats, and that IL-22 correlated with clinical score in the model (Wang et al. 2017). These findings indicated that IL-22 may have played a role in driving inflammation during inflammatory arthritis, but this was not observed in the present study. However, T cell mediated pathology was further evidenced by the expression of *Il17a* and *Ifn γ* in the aorta during the model. It can be hypothesised that it is this T cell infiltrate to the aorta that contributes towards defective vascular constriction responses. However, further studies would need to be performed to confirm this. These could include determining if the relationship between galectin-3 and Th17 cells, as described in **Chapter 4**, and the relationship between galectin-3 and E-selectin, as discovered by Gittens *et al* (Gittens et al. 2017), exists in AIA by performing immunofluorescence co-staining of the aorta. Functional studies could be carried out by performing myography on *Il17a*^{-/-} mice undergoing the AIA protocol or by measuring Th17 cell infiltrate to the vasculature by flow cytometry or qPCR.

Inhibition of galectin-3 by GB1107 reduces CD4 cell trafficking

The final aim of the Chapter was to test the inhibition of galectin-3 in AIA on joint and vascular inflammation. Mice were dosed with 10 mg/kg GB1107 from Day -2 to Day 3. For these series of experiments, GB1107 was prepared in vehicle propylene glycol in HPMC. The dose and vehicle preparation for GB1107 was recommended by Galecto Biotech. Inhibition of galectin-3 significantly reduced CD3⁺CD4⁺ cells in the synovium. Galectin-3 is expressed in CD4 cells, indicating that inhibiting galectin-3 alleviates joint disease. There was a trend toward reduction in the number of IL-17A expressing cells in mice treated with GB1107 but this was not significant. However, there was an increase in the proportion of IFN γ expressing cells in the synovium of mice treated with GB1107. IFN γ has a protective effect in the early stage of AIA. *Ifn γ* ^{-/-} mice with AIA had exacerbated joint disease with significantly higher number of CXCR2⁺ neutrophils than wild-type mice (Williams et al. 2007). In a similar study by Irmmler *et al*, *Ifn γ* ^{-/-} mice with AIA also had increased production of IL-17 from lymph nodes and splenocytes and increased histopathological scores of arthritis (Irmmler et al. 2007).

The same trend was observed in the draining lymph nodes whereby inhibition of galectin-3 resulted in significantly increased IFN γ expressing cells, again indicating that GB1107 was mediating anti-inflammatory effects at this early stage of disease. However, induction of

AIA resulted in reduced CD3⁺CD4⁺ cells in the draining lymph nodes. This may be due to the migration of CD3⁺CD4⁺ cells from the lymph nodes via efferent lymphatic vessels to distant sites of inflammation, such as the synovium and the vasculature. This has been observed in the literature whereby sheep were infused with various antigens such as LPS or influenza virus, directly to the afferent popliteal lymph nodes and lymph was continuously collected from the efferent popliteal lymph node and lymphocytes were then isolated and quantified. This study showed a significant increase in lymphocyte trafficking from the lymph node that peaked at Day 3 after exposure to the antigen (Cahill et al. 1976). This suggests that the decrease observed in CD3⁺CD4⁺ cell in the lymph node upon induction of AIA may be due to increased lymphocyte trafficking to the blood and the distant sites.

The series of *in vivo* experiments produced in this Chapter were presented as 2 separate experiments. The first experiment was run in small numbers as a pilot study to form initial observations. However, it must be noted that while the data showed some promising results on the effect of the inhibition of galectin-3 on joint and vascular inflammation, the first experiment did not have the robust inflammatory response that was expected for the AIA model. Swelling post intra-articular injection was lower than expected in the vehicle group. Furthermore, although the trend was as expected, the significant differences in constriction responses between naïve mice and mice with AIA was lost. This, combined with low swelling, suggested that the inflammatory response observed in the first experiment did not reach a threshold required to result in co-existing vascular defects. However, the reduction in lymphocyte infiltrate to the synovium, the promotion of anti-inflammatory IFN γ mediating effects by GB1107, followed by the trend of partially restored constriction responses in the GB1107 group when compared to vehicle treated mice, led to the experiment being repeated to determine if the observations in the constriction responses were a true result.

Resolution of constriction responses is independent of IL-17 mediated responses

Upon repeating the *in vivo* experiment, a more robust inflammatory response was observed, with larger knee swelling in both groups. Interestingly, treatment with GB1107 fully restored vascular constriction responses, but this was independent of IL-17 responses. Measurement of IL-17A secretion from inguinal lymph nodes after re-stimulation with mBSA showed that treatment with GB1107 did not affect mBSA specific Th17 responses. Furthermore, when circulating cytokines were quantified, treatment with GB1107 did not alter IL-17A levels, further highlighting that the effect on vascular constriction responses is

independent of the IL-17A response. Furthermore, treatment with GB1107 had minimal effect on most circulating cytokines, with the exception of a trend toward decreased TNF α , which is also observed in the literature. Circulating galectin-3 levels positively and significantly correlate with TNF α in patients with acute myocardial infarction (Alturfan et al. 2014). However, it is difficult to distinguish if the link is correlative or causative and would require more mechanistic studies to determine this. Such studies could include measuring TNF α plasma levels in *Lgals3*^{-/-} mice with AIA.

Given that treatment with GB1107 fully restored constriction responses but did not affect the IL-17A response means that there is another mechanism - or mechanisms - causing the constriction deficit. It is likely to be caused by a number of factors. While T cells are expressed in the vasculature at both Day 3 and Day 10 of the model, early stages of the AIA model is characterised by macrophage infiltration to the synovium. Interestingly, while macrophages are found in the articular cartilage 5 days post mBSA administration, T-helper cells are also found in clusters surrounding the synovial vasculature (Dijkstra et al. 1987). This suggests that not only is both innate and adaptive immune responses involved in early timepoints in the model and therefore T cells could be involved in defective constriction responses, but also that T cells may be migrating from the blood to the tissue and perpetuating inflammatory responses. It is likely to be a combination of these complex series of inflammatory responses that haven't yet been fully elucidated that result in a constriction deficit. However, inhibition of galectin-3 has shown to fully restore the restoration of constriction responses, giving insight that galectin-3 has a definitive role in perpetuating, or aiding, the inflammatory response to result in a constriction deficit. Future experiments could use immunohistochemistry of the vasculature and identify cells of the innate and adaptive immune system, along with co-staining for galectin-3, following treatment with vehicle or GB1107 as it may be more informative and give better appreciation to the pathology in terms of immune cell infiltrate, endothelium thickness and vascular remodelling.

This Chapter identified a new model for investigating the mechanisms underlying vascular inflammation associated with inflammatory arthritis. Additionally, this study has shown that the AIA model is a model with systemic inflammatory features that has exhibited a regulated effect on constriction responses, with defective constriction responses at Day 3 that is restored at Day 10. Inhibition of galectin-3 in the AIA model reduced CD3⁺CD4⁺ infiltrate to the synovium, enhanced the anti-inflammatory properties of IFN γ and restored vascular constriction responses in mice with AIA.

Chapter 6

General Discussion

6.1 Summary of Work

The hypothesis for this Thesis states that “galectin-3 promotes the inflammatory response to result in vascular inflammation”. Galectin-3 is a key driver of inflammation in many diseases including cancer, fibrosis and cardiovascular diseases. Galectin-3 and its binding protein (G3BP) are increased in the sera and synovial fluid of RA patients (Ohshima et al. 2003). They are highly expressed in synovial fibroblasts recovered from the inflamed joints of RA patients (Filer et al. 2009). Notably, galectin-3 levels directly correlate with the number of atherosclerotic plaques found in patients with coronary artery disease (CAD) (Ozturk et al. 2015), and *Lgals3*^{-/-} mice have smaller atherosclerotic plaques in the aortic arch and thoracic aorta (MacKinnon et al. 2013). These studies presented the foundation for asking the question ‘does galectin-3 link inflammatory joint disease with arthritis-associated vasculopathy?’. The data presented in this Thesis describes the early steps in a research pathway that begin to answer this question. The literature also highlights the potential importance of galectin-3 as an immunomodulatory target for treatment of inflammation-associated conditions like arthritis, cardiovascular disease and arthritis-associated cardiovascular disease.

In summary, **Chapter 3** of this Thesis investigates the effect of galectin-3 inhibition in CIA, a mouse model of inflammatory arthritis. It mimics the systemic nature of rheumatoid arthritis. Mice with CIA have vascular inflammation and exhibit defective constriction responses to serotonin during established arthritis (Reynolds et al. 2012). Studies outlined in **Chapter 3** identified a constriction deficit in the preclinical phase of CIA and showed an increase in the tissue expression of CD3 T cells and levels of galectin-3 in the PVAT. This illustrated that not only did vascular damage precede the development of macroscopic arthritis but also identified galectin-3 as a potential target to regulate early inflammatory changes in the aorta. There is a need for therapeutic strategies that target and prevent this early pathology in the aorta. It could help reduce cardiovascular morbidity associated with RA. 40% of patients with RA die of cardiovascular disease (Dhawan and Quyyumi 2008). Indeed RA is a risk factor for CVD (Crowson et al. 2013). **Chapter 4** focussed on a mechanistic approach and investigated the role of galectin-3 in CD4 T cells. Galectin-3 expression was characterised in CD4 T cells, followed by the development of a bioassay that tested the effect of galectin-3 inhibition in CD4 effector cell responses. The use of GB1107 and cells derived from *Lgals3*^{-/-} mice *in vitro* in Th17 differentiating conditions showed a role of galectin-3 in Th17 proliferation. Furthermore, it established galectin-3 as a potential therapeutic target in Th17 mediated diseases such as rheumatoid arthritis and

multiple sclerosis. **Chapter 5** moved to using a different *in vivo* model of inflammatory arthritis, antigen induced arthritis, to investigate the role of galectin-3 in vascular inflammation in a CD4 T cell dependent pathology. The AIA model was chosen as it serves as an excellent model to study the molecular mechanisms underpinning arthritis progression. The C57Bl/6 strain of mice are highly susceptible to AIA which allows for investigations to be performed into the role of immune pathways during inflammatory arthritis (i.e., cytokines, transcriptional regulators and immune cells) by the use of gene deficient mice. The AIA model also gives rise to a robust inflammatory response that follows a well-defined time course. Therefore, using the AIA model to study inhibition of galectin-3 in vasculopathy associated to inflammatory arthritis was advantageous due to the model possessing a more controlled regulation of inflammation when compared to the CIA model. Inhibition of galectin-3 in AIA led to the reduction in CD4 T cells in the inflamed synovium. This further highlighted the galectin-3 dependent mechanisms in CD4 T cells. Additionally, inhibition of galectin-3 in AIA resulted in the full restoration of constriction responses, suggesting that the hypothesis of this Thesis that galectin-3 promotes the inflammatory response to result in vascular inflammation can be accepted. Together, this Thesis provides new insights into how targeting galectin-3 may have therapeutic applications for the control of CD4 T cell-mediated pathologies, including in inflammatory joint diseases, cardiovascular disease and arthritis-associated-cardiovascular disease.

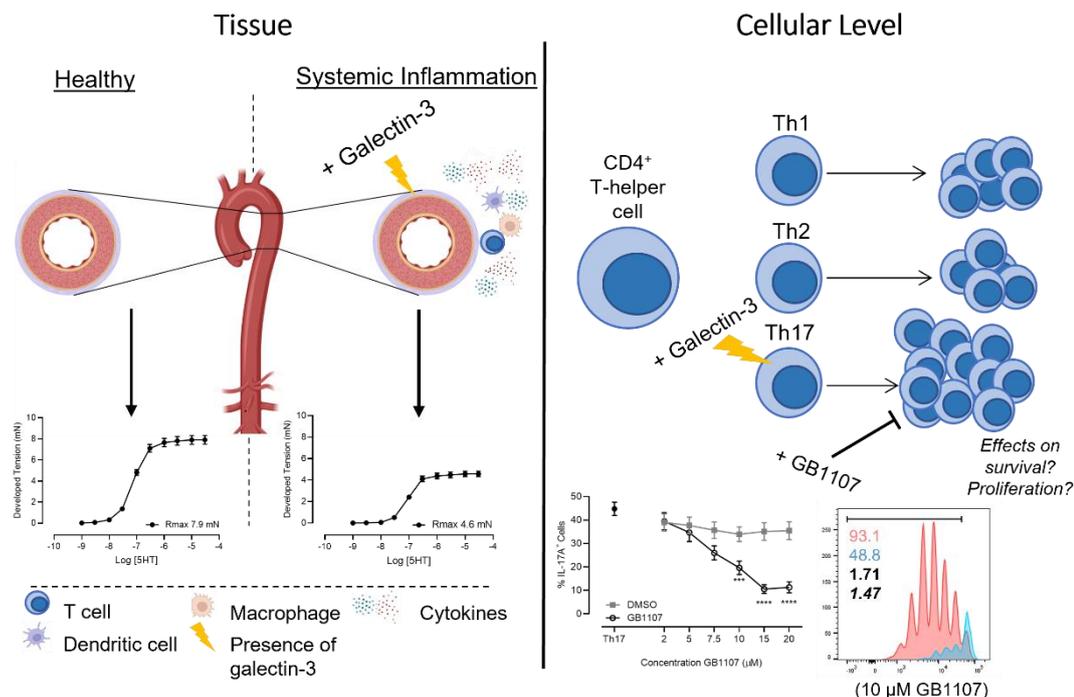


Figure 6.1: Overview of the main research findings in this Thesis

Findings of this Thesis can be loosely separated based on findings at tissue levels and at a cellular level. Systemic inflammation in the vasculature as a result of CIA or AIA leads to constriction defect, which led to delineating some of the potential mechanisms underlying the deficit to limit arthritis associated co-morbidities. Firstly, this Thesis showed that galectin-3 is highly expressed in a subset of CD4 T-helper cells, Th17 cells, which are considered pathogenic cells during autoimmunity. Inhibition of galectin-3 using GB1107 showed a reduction in Th17 expansion, and upon further investigation, a reduction in proliferation of Th17 cells using CFSE and Ki67 proliferating assays was observed. This indicated that galectin-3 may have a role in promoting the proliferation and cell survival of Th17 cells. Furthermore, when mice with inflammatory arthritis were treated with GB1107, complete restoration of vascular constriction responses were observed. Therefore, this Thesis gives new insights to potential galectin-3 dependent mechanisms during inflammation that can be applied to a wide range of diseases where Th17 cells are involved.

6.2 Arthritis-associated cardiovascular pathology

Approximately 40% of RA patients die from cardiovascular diseases like hypertension, atherosclerosis and myocardial infarction (Dhawan and Quyyumi 2008). The molecular mechanisms that could explain the reasons for deaths attributed to cardiovascular causes are not fully understood in RA patients. Certain risk factors such as presence of HLA-DRB1 alleles, rheumatoid factor and pre-existing cardiovascular diseases leading to inflammation in blood vessels such as hypertension and diabetes significantly increases the risk of cardiovascular diseases in RA patients. Vascular dysfunction can have detrimental effects - not only on major blood vessels by contributing towards plaque formation, and subsequent plaque rupture, but also directly on the joint and organ tissues. Similar to the processes observed in the microvasculature, damage to the capillary beds in the joint leads to inappropriate passage of leukocytes, chemokines, cytokines and metabolites to the tissue. Furthermore, damaged microvasculature due to vascular dysfunction compromises oxygen transport in and out of tissue leading to ischaemic damage (Bordy et al. 2018). While the primary site of inflammation is synovial tissue in RA, features of joint pathology are also shared in pathological changes seen in major blood vessels such as the aorta. For example, activated T cells are expressed in atherosclerotic plaques as well as the rheumatoid synovium (Pasceri and Yeh 1999) and, similar to the joint, cytokines play a key role in fuelling inflammation by stimulating immune cell proliferation and differentiation. In the blood vessel such as the aorta, this results in promotion of immune cells binding to endothelial cells to cause an increase in vascular permeability, thereby promoting atherogenic processes (Sprague and Khalil 2009). Enhanced endothelial permeability leads to endothelial activation, which in turn induces chemokine expression and adhesion molecules (i.e. ICAM-1, VCAM-1) furthering leukocyte migration to the joint space in the synovial tissue, or the intima in the blood vessel (Urman et al. 2018). Galectin-3 is expressed in the inflamed synovium in RA (Ohshima et al. 2003), and is expressed in atherosclerotic plaques (Papaspzyridonos et al. 2008) and inflamed PVAT (Sime et al. 2017). Galectin-3 is expressed in many of the immune cells that perpetuate the pro-inflammatory processes and may be the link between joint destruction and vascular dysfunction. Galectin-3 is expressed in T cells, and in this Thesis, T cells and galectin-3 were increased in the inflamed vasculature during CIA. Furthermore, inhibition of galectin-3 in AIA decreased T cells in the inflamed synovium. So, while the exact mechanisms underlying the link between arthritis and associated cardiovascular diseases aren't fully elucidated, a number of factors are likely to contribute. These factors may include increased circulating

cytokines, enhanced recruitment of leukocytes and adhesion molecules as a result of joint disease, and galectin-3's pro-inflammatory properties, which all work in concert to result in disease.

6.3 The local inflammatory environment and its influence on defective constriction responses

The functional readout of vascular inflammation in this Thesis was the measurement of 5HT induced constriction responses. The CIA model is the gold standard mouse model for mimicking the systemic nature of rheumatoid arthritis. Importantly for this Thesis, vasculopathy develops with CIA. The vasculopathy was not fully characterised but some early histological changes are reported in the aorta and associated PVAT (Sime et al. 2017). These histological changes were accompanied by defective constriction responses as described in the literature (Reynolds et al. 2012; Williams et al. 2016; Sime et al. 2017). Constriction deficits were noted in other models of vascular inflammation. A rat model of type 2 diabetes showed decreased constriction responses to endothelin-1 and phenylephrine, combined with increased expression of CRP, CCL2 and TNF α in the PVAT of diabetic rats when compared to controls (Azul et al. 2020). Interestingly Azul *et al* observed that the PVAT of thoracic aortas were modified under inflammatory conditions such as diabetes. The environment which surrounds the blood vessel is vital in influencing the behaviour and contractile nature of the vessel. Therefore, the environment of the PVAT is important when investigating vascular inflammation. The main function of the PVAT is providing mechanical support to the blood vessel, thermogenesis, lipid storage and maintaining vascular tone by the release of adipocyte-derived relaxing factor (ADRF). The anti-contractile properties of PVAT are established and extensively reported in the literature (Xia and Li 2017). The PVAT is involved in vascular inflammation during various diseases such as atherosclerosis, hypertension and vascular ageing (Brown et al. 2014; Chen et al. 2021). PVAT dysfunction is the imbalance between the production and release of protective factors such as ADRF and the presence of pro-inflammatory cells and cytokines such as T cells and TNF α (Kim et al. 2019). The PVAT expands during disease due to increased infiltration in immune cells and expansion of adipocytes (Lehman et al. 2010). Galectin-3 has been targeted in vascular inflammation and links inflammation with fibrosis in cardiovascular disease (Besler et al. 2017). This Thesis investigated the direct effect of galectin-3 on constriction responses. The addition of galectin-3 to thoracic aortas did not directly cause defective constriction responses, as evidenced in **Chapter 3**. However, only a single concentration of 1 ng/mL galectin-3 was used, and a single incubation timepoint of 2

hours was used before measuring constriction. This concentration and timepoint was chosen as it was reported in the literature as a sufficient concentration and length of time to induce an effect in constriction responses (Gillham et al. 2008), but further experiments would need to be carried out to determine if higher concentrations of galectin-3 are required to induce a change. Conversely, galectin-3 is expressed in the PVAT of mice with inflammatory arthritis and so may be indirectly influencing the release of vasoactive substances from the PVAT to result in a constriction deficit. For example, galectin-3 has been identified as promoting endothelial dysfunction by increasing LOX-1 expression, the receptor for oxidised LDL (oxLDL) uptake (Ou et al. 2019). Additionally, galectin-3 facilitates leukocyte rolling and emigration to sites of inflamed microcirculation (Gittens et al. 2017), again suggesting its role in promoting a local inflammatory environment to indirectly result in defective constriction responses. The infiltration of CD3⁺ T cells into PVAT during CIA may also promote defective constriction responses. Studies using mice with deletion of the T cell chemokine RANTES, also known as CCR5, were protected against T cell infiltration to the vasculature (Mikolajczyk et al. 2016) and did not develop vascular dysfunction in a model of hypertension. Adoptive transfer of T cells induced vascular disease which demonstrated the T-cell dependent nature in a model of hypertension. Furthermore, administration of angiotensin II (resulting in hypertension) caused an increase in CD3⁺CD4⁺CD8⁻ T cells in the PVAT in a model of hypertension and increased TNF α production from T cells - suggesting a role and potential therapeutic target of T cells in the management of high blood pressure (Guzik et al. 2007). Animal models of hypertension show T cell infiltration to the PVAT can precede and even exceed macrophage infiltration in an animal model of hypertension (Nosalski and Guzik 2017). CD4⁺ cells have been identified in the PVAT but studies have shown a large proportion of CD3⁺CD4⁺CD8⁻ T cells in the PVAT as $\gamma\delta$ T cells. However, these $\gamma\delta$ T cells are a source of IL-17 and are likely to promote pro-inflammatory signalling and further propagation of PVAT and vascular dysfunction. To illustrate this, in a study by Li *et al*, to measure the heart's response to hypertension, mice were infused with angiotensin II for 7 days and IL-17A expression measured. mRNA levels of IL-17A were significantly increased in mice with AngII, and co-localisation of IL-17A and CD3 cells were detected by immunofluorescence in the heart. Interestingly, deletion of $\gamma\delta$ T cells resulted in significantly decreased IL-17A cells in the heart, but not in the hearts of CD4^{-/-} mice, indicating that $\gamma\delta$ T cells were the major source of IL-17A cells in hypertension in cardiovascular diseases (Li et al. 2014). These studies illustrate that T cells are necessary for the propagation of vascular dysfunction, and that IL-17A is an important target in

cardiovascular diseases and can be targeted in diseases where Th17 cells are involved, but also where $\gamma\delta$ T cells are involved such as psoriasis and Crohn's disease. The secretion of these proinflammatory cytokines from infiltrating immune cells to the PVAT further stimulates immune cell proliferation and differentiation, vascular cell growth and migration and vascular permeability on endothelial cells (Sprague and Khalil 2009). These processes ultimately leads to and contributes towards PVAT dysfunction to result in the induction of reactive oxygen species, vascular oxidases and eNOS dysfunction in the endothelium, resulting in altered constriction and relaxation of the vessel wall (Nosalski and Guzik 2017). Additionally, the use of $CD4^{-/-}$ mice could be used in the CIA model to determine if the vascular dysfunction observed in the studies presented in this Thesis are T-cell dependent mechanisms. $CD4^{-/-}$ mice with CIA are less susceptible - but are not resistant - to developing arthritis (Ehinger et al. 2001). Furthermore, the use of conditional knockout mice in models of inflammatory arthritis where $CD3^{+}CD4^{+}$ T cells are knocked out of the aorta would determine if vasculature dysfunction during arthritis is T cell dependent. Following this, conditional knockouts could further be used and *Lgals3* could be knocked out from $CD4^{+}$ cells – or indeed other immune cells - during inflammatory arthritis to understand galectin-3's specific contribution to inflammation and vascular dysfunction. Unravelling the $CD4$ dependent mechanisms in contractile dysfunction would be beneficial in further understanding the role and involvement of $CD4$ cells in arthritis associated vasculopathy.

The CIA model was initially chosen as the model to study vasculopathy associated with inflammatory arthritis. Typically, a global morphological change in adipose tissue, including in the PVAT, and an increase in galectin-3 PVAT expression is observed in CIA. Galectin-3 was also identified as a potential biomarker for detection of early vascular pathology in CIA (Sime et al. 2017). However, phenotypic changes were noted in the CIA model for the duration of this research project, which led to the loss of constriction deficit and the CIA model was no longer an appropriate model for investigating vasculopathy associated with inflammatory arthritis.

6.4 Application of galectin-3 inhibition in other T cell mediated diseases

This Thesis focussed on galectin-3 inhibition in two models of inflammatory joint disease - the CIA model and the AIA model. Galectin-3 inhibition can be applied to a wide array of pathologies due to its wide expression profile in cells and tissues, and upregulation in

diseases such as cancers (Farhad et al. 2018; Wang et al. 2018), vascular diseases such as atherosclerosis (Papaspyridonos et al. 2008; Gao et al. 2020), and arthritis (Ohshima et al. 2003; Filer et al. 2009). More specifically, galectin-3 is also expressed in immune cells involved in these diseases such as macrophages (MacKinnon et al. 2008), dendritic cells (Breuilh et al. 2007) and T cells (Yang et al. 1996).

In this Thesis, inhibition of galectin-3 was investigated in relation to vasculopathy associated to inflammatory arthritis. While GB1107 failed to show any impact on vasculopathy or arthritis parameters in the CIA model, this was due to the acute onset and severe progression of the model. Furthermore, this was not due to an absence of galectin-3 in the pathogenesis of the disease - confirmed by elevated circulating galectin-3 in the plasma in mice with CIA; **Chapter 3** - nor an issue with the efficacy of the inhibitor, as a clear effect of GB1107 was observed in *in vitro* studies in **Chapter 4**. Intervention with GB1107 did not change galectin-3 levels in the systemic circulation nor did it impact on constriction responses in CIA. The probable reason for this was because of the rapid transition for the model to the chronic stage which was not controlled by galectin-3. This reasoning is in line with the findings in **Chapter 5** in using a milder, less systemic AIA model where GB1107 did restore vascular constriction responses. Interestingly, this was without affecting the systemic levels of galectin-3, suggesting that GB1107 does not directly affect circulating galectin-3 levels and is acting in an indirect way. Studies investigating the effect of inhibition of galectin-3 have given conflicting results in the literature. In a study where galectin-3 was inhibited by MCP-1 in a mouse model of abdominal aortic aneurysm (AAA), mice had decreased aortic dilatation and vascular smooth muscle cell loss and macrophage content when compared to control mice, which was associated with a decrease in galectin-3 mRNA and protein expression (Fernandez-García et al. 2017). While this study suggested that inhibition with galectin-3 by MCP-1 was beneficial and reduced the development of AAA, other studies involving patients with osteoarthritis have shown that treatment with MCP-1 for 12 weeks had no effect on arthritis severity (Andrews et al. 2020), indicating that the success of MCP-1 is highly dependent on the disease in which it is applied to. Furthermore, while MCP-1 may show some promising results in pre-clinical studies, it has not shown any benefit when treating patients in clinical trials and so its success is limited and short lived.

Other galectin-3 inhibitors have shown more promising results in controlling T-cell mediated pathology. Pharmacological inhibition of galectin-3 in diseases where T cells play a role have yielded some interesting results. In mouse models of mammary carcinomas and

prostate adenocarcinomas, administration of galectin-3 inhibitor GR-MD-02 intra-venously, in combination with anti-OX40 antibody (aOX40), resulted in significantly increased survival. This coincided with heightened CD8 T cell density in the tumour, therefore promoting anti-cancer properties, and reduced monocytic myeloid derived suppressor cells (MDSCs) in the tumour, relating to decreased pro-tumorigenic properties. The effect of galectin-3 inhibition in combination with aOX40 reduced immune suppression during cancer and increased CD8 T cell recruitment to result in tumour cell regression (Sturgill et al. 2021), making pharmacological targeting of galectin-3 towards immune cells in cancer treatment favourable. Furthermore, similar effects were observed using GB1107 in a mouse model of non-small cell lung cancer (NSCLC). Treatment with GB1107 increased CD8 T cell infiltration to the tumours and increased anti-tumour M1 macrophage polarization (Vuong et al. 2019). This not only shows the application of GB1107 in targeting specific T cells in other diseases, but these studies also demonstrate a potential galectin-3 dependent role in CD8 T cell suppression. GB1107 has shown to have benefit in targeting pathogenic effector CD4 T cells in this Thesis and in treating vasculopathies associated to inflammatory arthritis. Moreover, GB1107 can now be applied in targeting inflammatory diseases where T cells are pathogenic, and where inhibition of galectin-3 has been identified as therapeutic target such as colitis (Tsai et al. 2016), multiple sclerosis (MS) (de Jong et al. 2020), and in cancer immunotherapy where enhancing T cells are beneficial to promote the death of malignant cells, such as the studies above.

6.5 Is galectin-3 the main driver of vascular inflammation?

Upon switching to a different *in vivo* model of inflammatory arthritis, the AIA model exhibited a more controlled regulation of inflammation, and a contractile deficit at Day 3. This allowed antigen induced arthritis to be used as a tool to not only investigate vasculopathies associated to inflammatory arthritis, but to also determine the effect of the inhibition of galectin-3 on arthritis associated contractile dysfunction. Inhibition of galectin-3 alleviated swelling and decreased levels of CD4 T cells in the inflamed synovium. Notably, treatment with GB1107 fully restored vascular constriction responses back to baseline, stimulating the question of is galectin-3 the main driver of the vascular inflammation that leads to the contractile deficit? Is inhibition of galectin-3 enough to single-handedly restore the constriction deficit? The hypothesis for this Thesis is that galectin-3 promotes the inflammatory response to result in vascular inflammation. This Thesis has shown that

galectin-3 does promote the inflammatory response by promotion of Th17 proliferation, and knockdown of galectin-3 reduces systemic IL-17-producing splenocytes in AIA (Forsman et al. 2011), showing a clear link between galectin-3 and Th17 cells in the model. Furthermore, galectin-3 inhibition in other disease contexts such as cancer have shown to have beneficial effects in increasing CD8 T cell number to improve outcome (Vuong et al. 2019). However, stating that inhibition of a single protein such as galectin-3 is enough to restore vascular constriction responses directly and single-handedly would be a rather simplistic view of a complex process occurring in the blood vessel that has a number of factors at play during health and during inflammation. Therefore, I would suggest that the restoration of constriction responses upon inhibition of galectin-3 in AIA is likely due to the indirect effects of galectin-3 inhibition on CD4 T cells, and in other immune cells not investigated in this Thesis, during the inflammatory responses.

6.6 Future perspectives

This Thesis had a number of novel findings (**Section 6.1**). However, it does also have a number of weaknesses that could be explored in future investigations. This Thesis focussed on the CD4 T-helper cell as the key cell type of interest during vascular pathology. Galectin-3 is expressed in T cells and can modulate their activation (Gilson et al. 2019). More specifically, this Thesis focussed on the relationship between galectin-3 and Th17 cells, as galectin-3 expression was uniquely elevated in that effector subset. Galectin-3 is located both intracellularly and extracellularly and GB1107 targets both forms of galectin-3. Future studies could investigate if the function of galectin-3 required for Th17 cell proliferation is dependent on intracellular galectin-3 or extracellular galectin-3. This could be determined by the use of specific galectin-3 inhibitors such as GBO149 or GB1265 that targets extracellular galectin-3 only, available from Galecto Biotech, and would further enhance our understanding of galectin-3 biology in CD4 T cells and further delineate the galectin-3 dependent mechanisms in effector CD4 cell subsets. Additionally, the availability of *Lgals3*^{-/-} mice would confirm for the role of galectin-3 in vascular inflammation to be determined and vascular constriction responses to be measured in inflammatory arthritis. This could be determined by measuring vascular constriction responses in *Lgals3*^{-/-} mice with AIA, but also measuring inflammatory infiltrate to the vasculature in the absence of galectin-3, which would further elucidate the role of galectin-3 in recruitment of pro-inflammatory immune cells during vascular inflammation. Furthermore, vasculopathy is present in other

models of systemic inflammation, and so investigations into the effect of galectin-3 inhibition using GB1107 and other such galectin-3 inhibitors in other models of vascular disease such as hypertension and atherosclerosis could illustrate the potential of targeting galectin-3 in these diseases. Finally, the use of next generation sequencing methods such as RNA sequencing of the inflamed PVAT and aorta in mice with AIA would be beneficial and would provide insight into what genes and signalling pathways are involved in the vasculature during inflammation. Single cell sequencing of the inflamed PVAT and aorta during AIA at Day 3 and Day 10 would give an understanding of the potential mechanisms underlying the defect, and subsequent resolution in constriction responses at the single cell level and RNA sequencing would give insight as to what is occurring at the tissue level. In tandem to this, a proteomic approach could be taken that could allow gene changes to be related to alterations in protein expression in the tissue. This combined omics approach could further enhance the understanding in the relationship between arthritis-associated cardiovascular comorbidities.

Taking a broader view, the application of the findings in this Thesis have capacity for further development. Galectin-3 is required for the proliferation of Th17 cells – an important discovery for the consideration of therapeutic targets in diseases where CD4 effector cells are pathogenic. Taking this into account, the targeting of galectin-3 is desirable for application in the clinic. There are several clinical trials underway targeting galectin-3 in a wide range of diseases, as outlined in **Table 1.4**. However, there are multiple cellular sources of IL-17 and targeting Th17 cells in autoimmune diseases such as rheumatoid arthritis and Crohn's disease have had limited success in the clinic (Miossec and Kolls 2012). Recently, a monoclonal antibody against IL-17A, secukinumab, has shown promising results in clinical trial in patients with rheumatoid arthritis (Huang et al. 2019), but it is yet to be FDA approved for the disease. Targeting galectin-3 as an adjunct therapy to cytokine blockade may be hugely advantageous. By halting the promotion of inflammatory processes and regulating the proliferation of pathogenic effector CD4 T cells in patients with vascular inflammation, even before they are symptomatic, the potential for galectin-3 inhibition in the clinic to prevent deaths in diseases of the heart and circulation may be substantial.

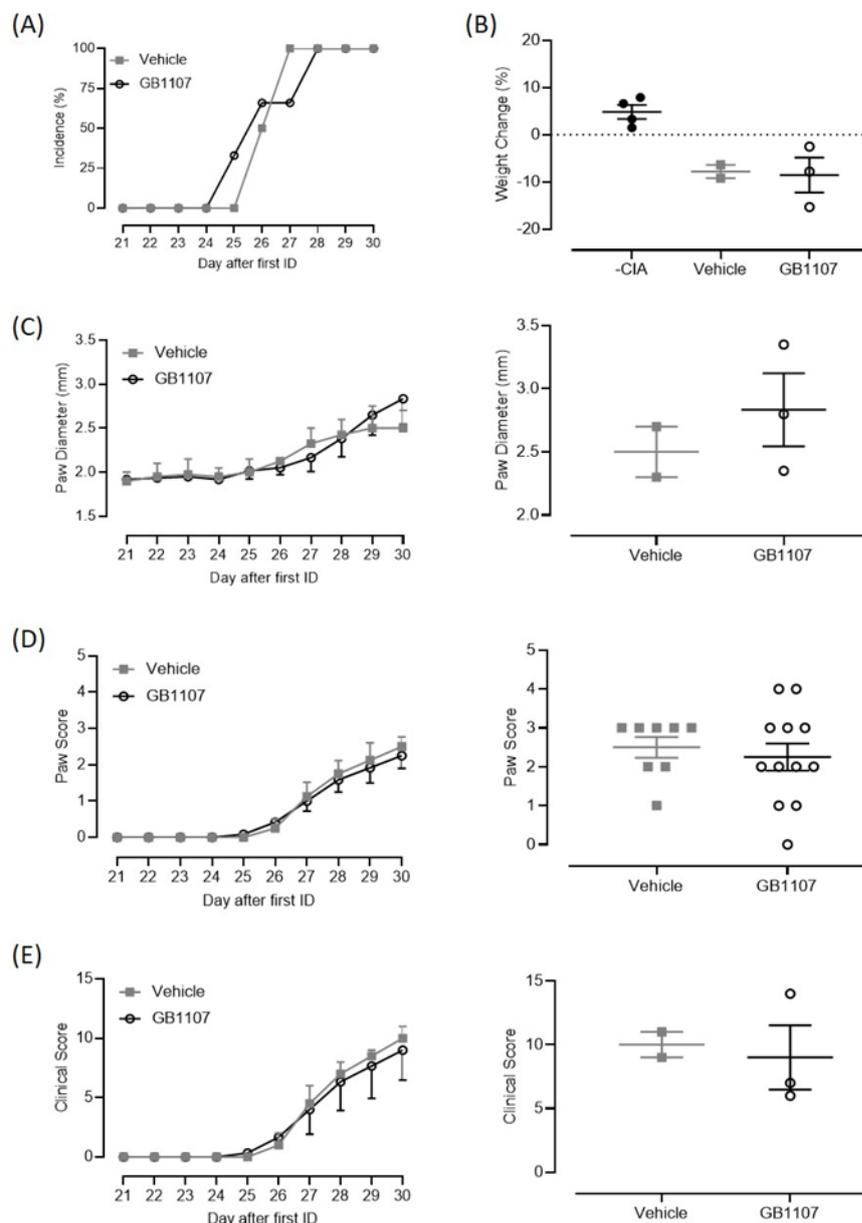
6.7 Concluding Remarks

In summary, this Thesis demonstrated that the CIA model can be used to study vasculopathy associated to established inflammatory arthritis. However, a phenotypic change in the model, potentially caused by repeated infections in the animal facility in Cardiff University hampered the studies in this Thesis to investigate the effect of galectin-3 inhibition in vascular inflammation in CIA. It did, however, characterise the preclinical phase of vascular inflammation and identified a contractile deficit prior to the development of arthritis. These findings identified galectin-3 as a potential therapeutic target in an early driver of vascular disease. Additionally, the research conducted in this Thesis established a bioassay for the testing of novel galectin-3 inhibitors and discovered a unique role for galectin-3 in Th17 cell proliferation. Finally, this Thesis showed that inhibition of galectin-3 fully restored defective constriction responses associated with inflammatory arthritis in the AIA model. Further work into the inhibition of galectin-3 in vascular inflammation and beyond has huge potential to benefit patients and prevent deaths associated with arthritis.

Appendix

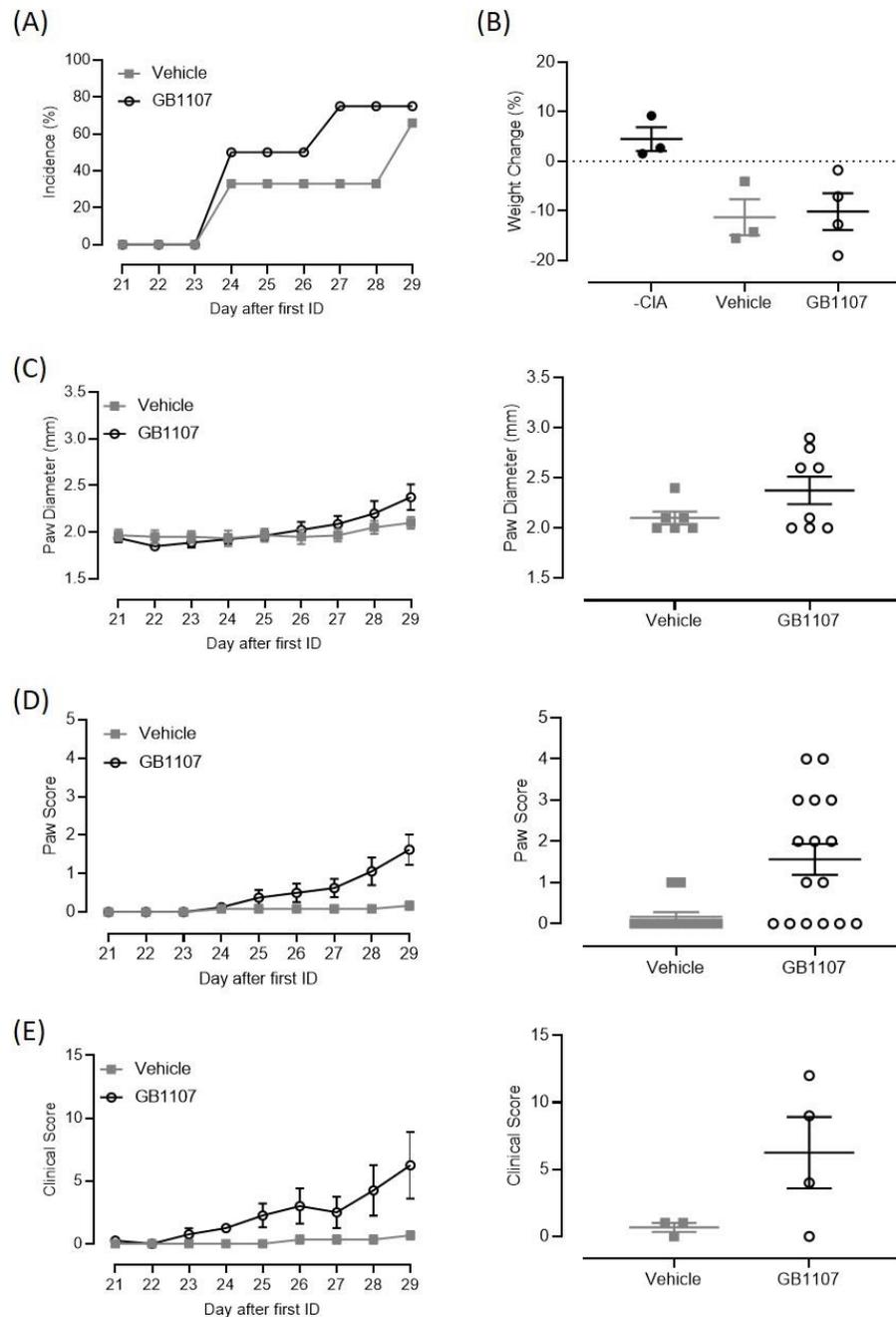
Supplemental Figures

CIA Experiment 1

**Supplementary Figure 1: No efficacy observed in GB1107 in HPMC vehicle**

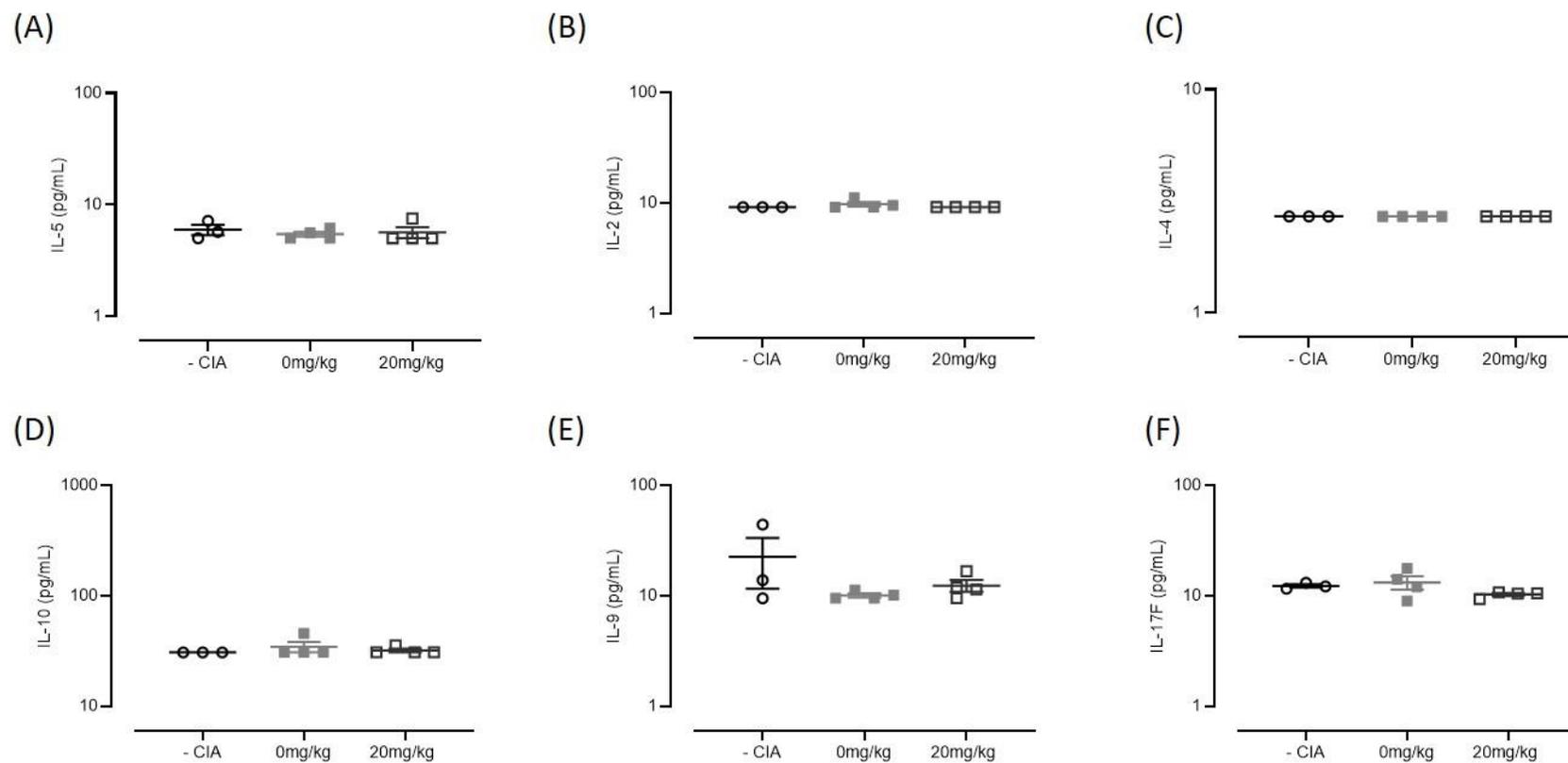
Inhibition of galectin-3 with GB1107 during CIA (n=3) showed accelerated progression of arthritis when compared to vehicle treated group (n=2). (B) Percentage of weight change in mice with no CIA (n=4), vehicle (n=2) and GB1107 (n=3). (C) Paw diameters of both hind paws were measured daily until termination. (D) Paw score from all four paws from Day 21 - 30 (left) and at termination (right). (E) Clinical score is the sum of the four paw scores from each mouse. This experiment was terminated at Day 30 of the CIA protocol. Graphs are shown as mean \pm SEM.

CIA Experiment 2



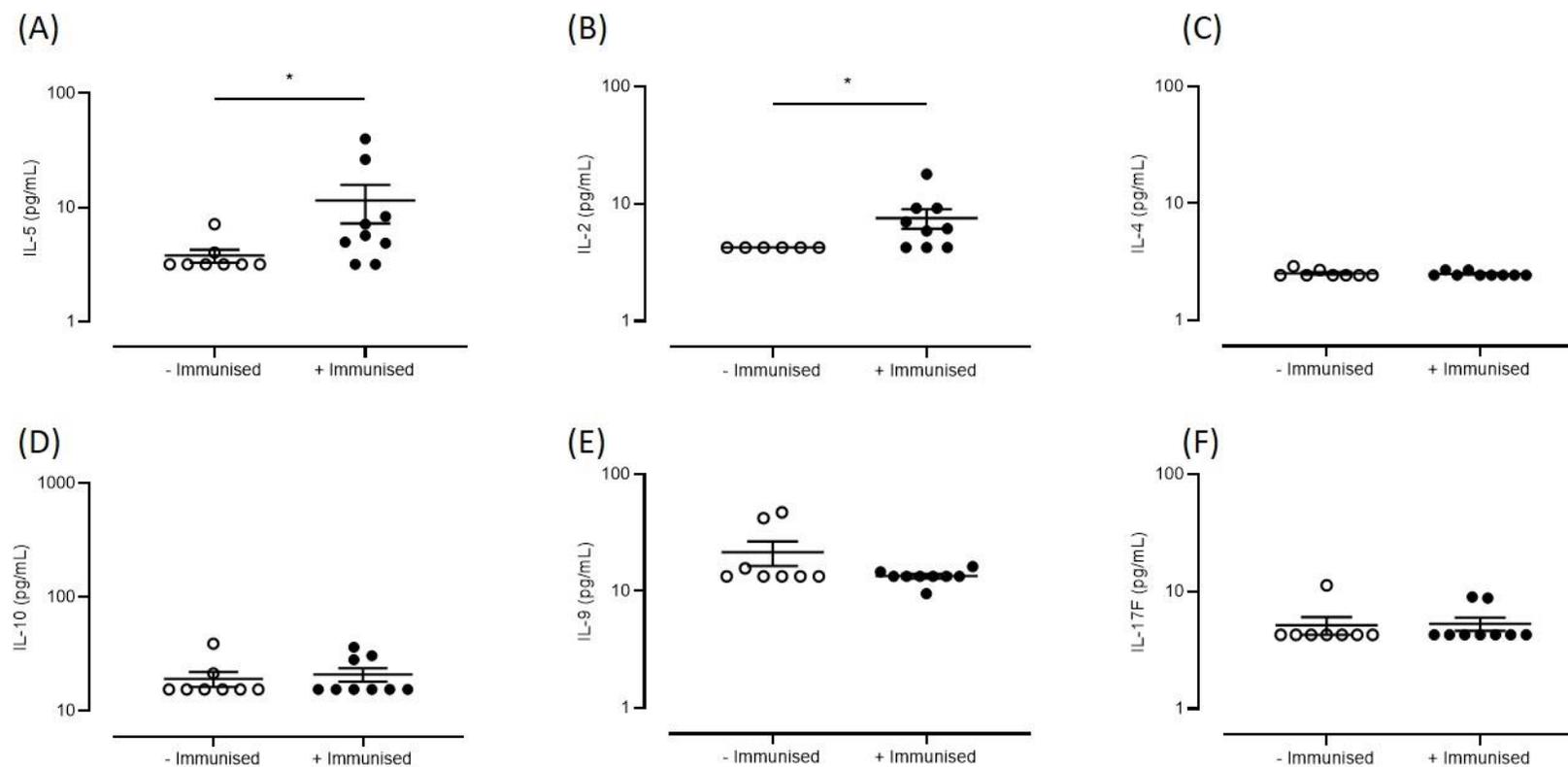
Supplemental Figure 2: No efficacy observed in GB1107 in HPMC vehicle when mice are treated with enrofloxacin

Inhibition of galectin-3 with GB1107 during CIA (n=4) showed accelerated progression of arthritis when compared to vehicle treated group (n=3). (B) Percentage of weight change in mice with no CIA (n=3), vehicle (n=3) and GB1107 (n=4). (C) Paw diameters of both hind paws were measured daily until termination. (D) Paw score from all four paws from Day 21 - 29 (left) and at termination (right). (E) Clinical score is the sum of the four paw scores from each mouse. Mice were treated with antibiotic enrofloxacin from Day 1 to 7. This experiment was terminated at Day 29 of the CIA protocol. Graphs are shown as mean \pm SEM.



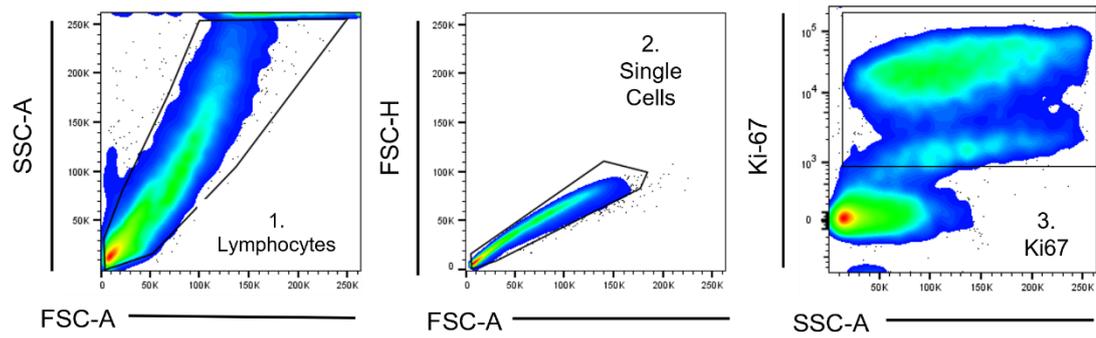
Supplemental Figure 3: Quantification of circulating cytokines at Day 27 of the CIA protocol

To determine if 20 mg/kg had an effect on various pro and anti-inflammatory cytokines, plasma was harvested and multiplex carried out. (A) IL-5. (B) IL-2. (C) IL-4. (D) IL-10. (E) IL-9. (F) IL-17F. Graphs are shown as mean \pm SEM.



Supplemental Figure 4: Quantification of circulating cytokines at Day 21 of the CIA protocol.

To determine if 20 mg/kg had an effect on various pro and anti-inflammatory cytokines, plasma was harvested and multiplex carried out. (A) IL-5. (B) IL-2. (C) IL-4. (D) IL-10. (E) IL-9. (F) IL-17F. Graphs are shown as mean \pm SEM and *= $p < 0.05$ as determined by unpaired t-test.



Supplemental Figure 5: Gating strategy for Ki67-positive populations

Lymphocytes were based on FSC-A vs SSC-A, followed by singlets based on FSC-A vs FSC-H. Ki67-positive populations was determined by SSC-A vs Ki67.

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