The Role of Modified Fc Fragments in Treating Autoimmune Diseases: A Potential Replacement for Intravenous Immunoglobulin

by

Bonnie J.B. Lewis

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy Laboratory Medicine and Pathobiology University of Toronto

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Abstract

High-dose (1-2 g/kg) intravenous immunoglobulin (IVIg), and more recently, subcutaneously delivered immunoglobulin (SCIg), are used to treat a variety of autoimmune diseases; however, there are challenges associated with product production and availability. These challenges have provided incentives to develop a human recombinant fragment crystallizable region (Fc) as a more potent alternative to IVIg and SCIg for the treatment of autoimmune diseases where its mechanism has been suggested to be Fc-dependent. Various Fc multimers have been produced that show enhanced efficacy compared to IVIg for amelioration of disease in animal models, such as primary immune thrombocytopenia (ITP) and rheumatoid arthritis (RA). Recently, a recombinant human immunoglobulin (Ig) G1 Fc hexamer entitled Fc-µTP-L309C was produced by CSL Behring by fusing the 18 amino acid (aa) IgM tailpiece to the C-terminus of a variant human IgG1 Fc with a point mutation at position 309. To better understand whether Fc-µTP-L309C is good replacement therapy for IVIg/SCIg, I first examined its efficacy in a mouse model of ITP. I demonstrated that Fc-µTP-L309C is more efficacious than SCIg at ameliorating ITP and at blocking Fc gamma receptor (FcyR)-mediated phagocytosis. With the limited amount of studies performed on the therapeutic efficacy of IVIg/SCIg in RA, I decided to investigate the efficacy of IVIg and SCIg in the K/BxN serum transfer model and in the endogenous K/BxN model of RA and compare it to that of Fc-µTP-L309C. Again, I showed that Fc-µTP-L309C is

more efficacious than SCIg at ameliorating RA in the K/BxN mouse models. Furthermore, I showed that Fc- μ TP-L309C affected both the innate and the adaptive immune system in the K/BxN endogenous model of RA. Fc- μ TP-L309C decreased auto-antibody (Ab) production by B cells and the subsequent deposition of auto-Abs on the articular cartilage. Fc- μ TP-L309C also increased transforming growth factor beta (TGF- β) and forkhead box P3 (FoxP3)⁺ T regulatory cells (Tregs) in the joints of K/BxN mice. Fc- μ TP-L309C also blocked Fc γ RIII on neutrophils and prevented interleukin-1 beta (IL-1 β) release from neutrophils. Thus, Fc- μ TP-L309C serves as a potential replacement for IVIg for Ab mediated autoimmune diseases and its mechanism is likely multifactorial and different depending on the disease.

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Contributions

Bonnie J.B. Lewis (author) solely prepared this thesis. All aspects of this body of work, including the planning, execution, analysis, and writing of all original research and publications was performed in whole or in part by the author. The following contributions by other individuals are formally acknowledged:

Dr. Donald Branch (supervisor)- mentorship; laboratory resources; guidance and assistance in planning, execution, and analysis of experiments as well as manuscript/thesis preparation.

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Heather Whetstone (SickKids research scientist) embedded and processed samples for histology.

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List of Abbreviations

aa: amino acid Aa: Aggregatibacter actinomycetemcomitans Ab: antibody AMR: Ashwell-Morell receptor ACPA: anti-citrullinated peptide antibody ACR: American College of Rheumatology APC: antigen presenting cell ASH: American Society for Hematology BLT1: leukotriene B4 receptor 1 B-lys: B-lymphocyte stimulator Breg: regulatory B cell BSA: bovine serum albumin CAIA: collagen antibody induced arthritis CCL: CC chemokine ligand CXCL: CXC chemokine ligand CCR: CC chemokine receptor CD: cluster of differentiation CDAI: Clinical Disease Activity Index CI: chemotactic index CIA: collagen induced arthritis CII: type II collagen CRP: C-reactive protein CTLA: cytotoxic T lymphocyte antigen C5aR: complement component 5a receptor DAB: diaminobenzidine

DAS: Disease Activity Score DC: dendritic cell DIPJ: distal interphalangeal joint DMARD: disease modifying anti-rheumatic drug EBV: Epstein-Barr virus ELISA: enzyme-linked immunosorbent assay ELISpot: enzyme-linked immune absorbent spot ESR: erythrocyte sedimentation rate EULAR: European League Against Rheumatism F(ab')2: antigen binding fragments FBS: fetal bovine serum Fc: fragment crystallizable region FcyR: Fc gamma receptor FcRn: neonatal Fc receptor FLS: synovial fibroblast FOXP3: forkhead box P3 GM-CSF: granulocyte-macrophage colony stimulating factor GP: glycoprotein GWAS: genome wide association study G6PI: glucose-6-phosphate isomerase H&E: hematoxylin and eosin H. pylori: Helicobacter pylori HIV: human immunodeficiency virus HCV: hepatitis C virus HLA: human leukocyte antigen HSA: human serum albumin IC: immune complex

IFN: interferon Ig: immunoglobulin IL: interleukin IIIC: immobilized immune complexes IPF: immature platelet fraction ITP: immune thrombocytopenia **ITP-BAT: ITP Bleeding Assessment Tool** i.p.: intraperitoneal i.v.: intravenous IVIg: intravenous immunoglobulin IWG: International ITP Working Group JAK: Janus kinase KO: knockout L: ligand LTB4: leukotriene B4 LPS: lipopolysaccharide MCPJ: metacarpophalangeal joint M-CSF: macrophage-colony stimulating factor MHC: major histocompatibility complex MIF: macrophage migration inhibitory factor mm: millimeter MMA: monocyte monolayer assay MRI: magnetic resonance imaging NET: neutrophil extracellular trap NFκB: nuclear factor kappa-light-chain-enhancer of activated B cells NK: natural killer NOD: non-obese diabetic mice

NSAID: non-steroidal anti-inflammatory drug PADI: peptidylarginine deiminase PBMC: peripheral blood mononuclear cell PBS: phosphate buffered saline PFA: paraformaldehyde PI: phagocytic index PIPJ: proximal interphalangeal joint P. gingivalis: Porphyromonas gingivalis PTPN22: protein tyrosine phosphatase, non-receptor type 22 RA: rheumatoid arthritis RAW: RAW246.7 RANKL: receptor activator of nuclear factor kappa-B ligand RBC: red blood cell RF: rheumatoid factor Rh: rhesus RLU: relative light units RNA: ribonucleic acid **ROS:** reactive oxygen species s.c.: subcutaneous SCID: severe combined immunodeficient SCIg: subcutaneous immunoglobulin SD: standard deviation SDAI: Simplified Disease Activity Index SE: shared epitope SIGNR1: DC-SIGN-related protein 1 SLE: systemic lupus erythematosus STAT: signal transducer and activator of transcription

Syk: spleen tyrosine kinase TCR: T cell receptor TGF: transforming growth factor Th: T helper TNF: tumour necrosis factor TPO: thrombopoietin Treg: regulatory T cell Tregitope: regulatory T cell epitope VZV: varicella-zoster virus WT: wildtype

Chapter 1 1 Introduction

1.1 Immune Thrombocytopenia (ITP)

Immune thrombocytopenia (ITP) is a condition of low platelets, which can occur from primary causes or secondary to an underlying disease, such as an autoimmune disease or an infection. Thus, the two forms of this disorder can be described as either primary or secondary ITP. Part of my thesis focuses on primary ITP and it will be discussed in detail below.

1.1.1 Etiology and Diagnosis of Primary ITP

Primary ITP is an autoimmune disease that is characterized by increased platelet destruction in the spleen and liver and/or decreased platelet production in the bone marrow (Rodeghiero et al., 2009). The International ITP Working Group (IWG) defines primary ITP as an immune mediated disorder characterized by an isolated thrombocytopenia (absolute platelet count <100 x 10⁹/L) in the absence of any secondary causes (Rodeghiero et al., 2009). The diagnosis of primary ITP is one of exclusion (Provan et al., 2010). An increased risk of bleeding poses the biggest clinical problem for primary ITP however, bleeding symptoms may not always be present (Lambert and Gernsheimer, 2017; Neunert et al., 2011; Neunert et al., 2019; Provan et al., 2010; Provan et al., 2015; Rodeghiero et al., 2009).

Secondary ITP is an immune-mediated thrombocytopenia that is secondary to other manifestations such as drugs, infections, or diseases (Cines et al., 2009).

Primary ITP is mainly due to IgG auto antibodies (Abs), which primarily target glycoprotein (GP) IIbIIIa and GPIb-IX-V on the surface of platelets and megakaryocytes (Boylan et al., 2004; He et al., 1994). The cause of platelet auto-Ab production in primary ITP is unknown. Genetic predispositions in primary ITP are uncommon however, there are genetic polymorphisms in

cytokines and Fc gamma receptors ($Fc\gamma Rs$) that may increase a person's risk of developing the disease (Breunis et al., 2008; Carcao et al., 2003; Foster et al., 2001; Pehlivan et al., 2011; Rischewski et al., 2006; Wu et al., 2005).

Molecular mimicry may play a role in the development of cross reactive platelet auto-Abs as certain viral and bacterial pathogens such as human immunodeficiency virus (HIV), hepatitis C virus (HCV), *Helicobacter pylori* (*H. pylori*), and varicella-zoster virus (VZV) may express antigens that are similar to platelet glycoprotein (GP) IIIa (Li et al., 2005; Rand and Wright, 1998; Takahashi et al., 2004; Zhang et al., 2009). Although the absence of these bacterial and viral infections leads to remission in most patients with primary ITP, there is still a high variation in response rates in patients infected with *H. pylori*. (Stasi et al., 2009).

The diagnosis of primary ITP can be further characterized into phases whereby newly diagnosed ITP is from diagnosis to 3 months, persistent ITP is 3-12 months after diagnosis and chronic ITP is more than 12 months after diagnosis (Rodeghiero et al., 2009). This characterization is important because patients with newly diagnosed and persistent ITP have significantly higher remission rates than patients with chronic ITP (Rodeghiero et al., 2009; Neunert et al., 2011; Provan et al., 2010; Provan et al., 2015; Lambert and Gernsheimer, 2017). Thus, physicians usually recommend a more rigorous treatment plan for patients with chronic ITP (Rodeghiero et al., 2009).

1.1.2 Epidemiology of Primary ITP

The incidence of primary ITP varies based on age, gender and location. For adults, the incidence rate is 3.3/100 000 adults per year with a prevalence of 9.5 per 100 000 adults (Rodeghiero et al., 2009). These rates vary slightly based on whether you live in North America, Europe, or Asia. In adults under the age of 65, the prevalence of primary ITP in women versus in men is generally higher (Bennett et al., 2011; Fogarty 2009; Moulis et al. 2014; Schoonen et al., 2009; Terrell et al., 2012). There is also a higher risk of thrombosis in older adults shortly after diagnosis (Doobaree et al., 2016).

For children, the incidence rate is between 1.9 and 6.4/100 000 children per year (Terrell et al., 2012). The prevalence of primary ITP in children is about 4.6 per 100 000 children in Europe and 7.2 per 100 000 in North America (Segal and Powe, 2006).

1.1.3 Symptoms of ITP

Symptoms of ITP are mainly hemorrhagic symptoms such as purpura, petechiae, ecchymosis, epistaxis and other forms of bruising in the skin, oral cavity bleeding, gastrointestinal bleeding and rarely, intracranial hemorrhage (Moulis et al., 2014; Neunert et al., 2015; Rodeghiero et al., 2013). Most studies have shown that patients are not at risk for intracranial hemorrhage unless they have had a significant hemorrhage in the past (Cortelazzo et al., 1991; Melboucy-Belkhir et al., 2016). Purpura and petechiae are typical symptoms of ITP and refer to small red or purple dots on the skin caused by bleeding underneath the skin (Neunert et al., 2011; Provan et al., 2010). Ecchymoses (bruises) are another type of bleeding symptom in the skin, which are usually larger than petechiae and occur on the back of the thigh (Neunert et al., 2011; Provan et al., 2010). These bleeding symptoms and others are further described in **Table 1.1**.

Table 1.1 Definition of Bleeding Symptoms in ITP.

Modified from Rodeghiero, F., Michel, M., Gernsheimer, T., Ruggeri, M., Blanchette, V., Bussel, J.B., Cines, D.B., Cooper, N., Godeau, B., Greinacher, A., et al. (2013). Standardization of bleeding assessment in immune thrombocytopenia: report from the International Working Group. Blood *121*, 2596-2606. (Rodeghiero et al., 2013).

Site of Bleeding	Manifestation	Definition
Skin		
	Petechiae	Red or purplish discoloration
		in the skin with a diameter of
		0.5-3 mm that does not
		blanche with pressure and is
		not palpable

	Ecchymosis	Flat, rounded, or irregular
		red, blue, purplish, or
		yellowish green patch, larger
		than a petechia. Elevation
		indicated spreading of an
		underlying hematoma into the
		superficial layers of the skin
	Hematoma	Bulging localized
		accumulation of blood, often
		with discoloration of
		overlying skin
Visible mucous membranes		
	Petechiae, purpuric macules,	Same as for skin
	and ecchymosis	
	Bulla, vesicle, and blister	Visible raised, thin-walled,
		circumscribed lesion
		containing blood. Each bulla
		(.5 mm) is larger than a
		vesicle. Bullae, vesicles, and
		blisters should be counted
		together as bulla
	Epistaxis	Any bleeding from the nose
		may be anterior or posterior
		and unilateral or bilateral
	Gingival bleeding	Any bleeding from the
		gingival margins
	Subconjunctival hemorrhage	Bright red discoloration
		underneath the conjunctiva at
		onset; may assume the

		appearance of an ecchymosis
		over time
Muscles and soft tissues		
	Hematoma	Any localized collection of
		blood visible, palpable, or
		revealed by imaging. May
		dissect through fascial planes

In general, the severity of bleeding does not necessarily correlate with platelet counts, except in patients with absolute platelet counts less than 20×10^9 /L (Rodeghiero et al., 2013). Instead, it has been recently suggested that the severity of bleeding is related to the time from diagnosis with newly diagnosed patients having the highest risk (Altomare et al., 2016; Middelburg et al., 2016; Panzer et al., 2007). However, these studies need to be further investigated and it is still controversial as to whether the management of ITP should be based on absolute platelet count, time from diagnosis, or bleeding symptoms.

There were many attempts in the 1990s to early 2000s to create a bleeding assessment tool with adequate specificity (Bolton-Maggs and Moon, 1997; Buchanan and Adix, 2002; Page et al., 2007). After many failed attempts, the ITP specific Bleeding Assessment Tool (ITP-BAT) was developed in 2013 by the IWG (Rodeghiero et al., 2013). The ITP-BAT is a systemic evaluation of bleeding symptoms in the skin, visible mucosae, and organs, with gradation of severity and with specific definitions of the manifestations and descriptions of grading standards. Under each of these categories, the bleeding severity is graded from 0 to 4 where the highest grade means that there is more than one symptom in each category (Rodeghiero et al., 2013). The ITP-BAT is further described in Rodeghiero et al. (Rodeghiero et al., 2013).

The ITP-BAT allows physicians to differentiate bleeding symptoms at more than one site to facilitate different disease management strategies. However, further assessment needs to be done as to whether there is any correlation between bleeding severity and treatment strategies (Rodeghiero et al., 2013). Despite this, research has shown that adult patients with ITP that have a history of bleeding are at risk of experiencing bleeding symptoms again in comparison to

patients who do not have a history of bleeding (Rodeghiero et al., 2013; Psaila et al., 2009). However, most children with ITP undergo spontaneous remission without any treatment, although most of these patients still experience skin bruising and bleeding (Kuhne et al., 2001; Zeller et al., 2005).

1.1.4 Prognostic Markers of Primary ITP

There are currently no laboratory tests to diagnose primary ITP or to identify bleeding risk in patients. There are blood tests for absolute platelet counts and for anti-platelet Abs however, these are not necessarily indicative of primary ITP (George, 1990; Kiefel et al., 1987). As briefly mentioned before, significant research has been done to identify potential biomarkers that may be predicative of bleeding risk in primary ITP. The immature platelet fraction (IPF) measures young platelets that have recently been released into the circulation and are considered indicators of higher platelet production in the bone marrow (Briggs et al., 2004). They also contain a higher concentration of ribonucleic acid (RNA) than mature platelets (Schmoeller et al. 2017). The IPF was first characterized by Briggs et al., who found that the IPF had a larger flow cytometric forward light scatter, which showed that these platelets have a larger diameter (Briggs et al., 2004). Increases in the IPF in primary ITP has been associated with less severe bleeding events (Barsam et al., 2011).

In addition to IPF, platelet function has been correlated with bleeding severities in primary ITP. Platelets have been stimulated with a variety of agents and the expression levels of common platelet activation markers such as GPIIb/IIIa and cluster of differentiation (CD) 42b, have been correlated with the severity of bleeding in primary ITP (Frelinger et al., 2015; van Bladel et al., 2014). Additionally, a recent study by Middelburg et al., corrected for platelet function, defined as platelet reactivity to various stimulants, in cohorts of patients with different platelet counts, using $<32 \times 10^{9}$ /L as the lowest cohort and $>132 \times 10^{9}$ /L as the highest cohort (Middelburg et al., 2016). They demonstrated that increased platelet reactivity was associated with decreased risk of bleeding, especially for patients with lower platelet counts (Middelburg et al., 2016).

This research may allow for clinically available tests to assess bleeding risk in patients with primary ITP. For now, the history, physical examination, absolute platelet count, and blood

smear, to look for platelet abnormalities, need to be considered for diagnosis of primary ITP. There are a few additional tests that have been recommended such as, *H. pylori* testing, HIV and hepatitis C status, as well as a direct antiglobulin test, quantitative immunoglobulin (Ig) level testing, blood type and bone marrow examination (Provan et al., 2019; Provan et al., 2010). However, there are conflicting results regarding whether some of these tests are necessary (Purohit et al., 2016).

1.2 Pathogenesis of Primary ITP

Primary ITP is an autoimmune disease where a person's platelets are destroyed by their own immune system. This autoreactivity results in decreased platelet production from megakaryocytes in the bone marrow and increased peripheral platelet destruction in the spleen and liver (Cines et al., 2014; Cooper et al. 2006; McMillan, 2007). The pathogenesis of primary ITP is complex and will be described in detail in the upcoming sections.

Impaired megakaryopoiesis is a cause for low platelet counts in primary ITP (Khodadi et al., 2016). Studies using light microscopy have shown that primary ITP patients have an increased proportion of premature megakaryocytes and a decreased proportion of platelet producing megakaryocytes (Dameshek and Miller, 1946) Additionally, the characteristics of megakaryopoeisis such as, granularity, cytoplasmic vacuolization and nuclear condensation, are decreased in megakaryocytes, which prevents platelet production (Malara et al., 2015; Nugent et al., 2009). Studies with radiolabeled platelets have shown that during states of blood loss, the megakaryocytes in healthy individuals were able to produce 10x as many platelets as the megakaryocytes from patients with primary ITP (Cines et al., 2014; Nugent et al., 2009). Further studies using electron microscopy showed that megakaryocytes from these patients more frequently undergo apoptosis (Houwerzijl et al., 2004; Houwerzijl et al., 2006).

Although decreased platelet production by megakaryocytes is an important aspect of primary ITP, platelet destruction in the periphery is the main cause for low platelet counts in primary ITP. Using Chromium-51 labeled platelets, Harker and Finch showed that the life span of platelets was about 10 days in healthy humans, and only a few hours to days in patients with ITP (Harker,

1970; Harker and Finch, 1969). The shorter life span of platelets in ITP was also confirmed by other groups using Chromium-51 labeled platelets or Indium-111 labelled platelets (Branehog et al., 1975; Branehog et al., 1974; Kernoff et al., 1980). The theory of increased platelet destruction in the spleen was also supported by the effectiveness of splenectomy in raising platelet counts in patients with primary ITP and this theory will be discussed further below, in addition to the destruction of platelets in the liver (Blanchette and Freedman, 1998; Branehog et al., 1975; Louwes et al., 2001; Nugent et al., 2009).

1.2.1 Platelet Involvement in Primary ITP

There are unique differences between healthy platelets and platelets in patients with primary ITP, which may contribute to the mechanism of this disease. CD47 is a common protein expressed on all cells and it serves as a ligand for signal regulatory protein α on neutrophils, monocytes and DCs to downregulate phagocytosis (Seiffert et al., 1999). It has been shown that CD47 expression on senescent platelets is significantly lower in patients with primary ITP in comparison to healthy controls, which could contribute to enhanced platelet destruction (Catani et al., 2011; Olsson et al., 2005).

Senescent platelets are cleared from the circulation primarily by macrophage-mediated phagocytosis in the spleen however, these same platelets can be cleared. through the Ashwell-Morell receptor (AMR) on liver hepatocytes and Kupffer cells (Cines et al., 2014; Grovosky et al., 2010; Grovosky et al., 2015; Hoffmeister, 2011; Hoffmeister and Falet, 2016; Rumjantseva and Hoffmeister, 2010). The AMR recognizes desialylated platelets, which has been identified as a determinant for the removal of senescent circulating platelets (Rumjantseva and Hoffmeister, 2011). This process may be enhanced during primary ITP (Li et al., 2015). Shao et al. reported that a patient with refractory ITP had significantly increased platelet desialylation and with tamiflu treatment, a sialidase inhibitor, the desialylation of the platelets gradually decreased and the platelet count of the patient recovered (Shao et al., 2015). This case report was corroborated by two other independent studies (Alioglu et al., 2010; Jansen et al., 2015).

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Recently a study has attributed CD8⁺ cytotoxic T cells as an inducer of platelet clearance in the liver via platelet desialylation. CD8⁺ T cells induced the lysosomal sialidase, neuraminidase 1, expression on the surface of platelets, which increased platelet desialylation and platelet destruction in the liver (Qiu et al., 2016). Inhibiting platelet desialylation or blocking the clearance of desialylated platelets in the liver may be a novel therapeutic strategy for primary ITP patients.

1.2.2 Antigen Presenting Cells (APCs) in Primary ITP

Monocytes and macrophages have been shown to be involved in both the innate and the adaptive anti-platelet immune responses in primary ITP. Activated splenic macrophages are highly phagocytic and can engulf antiplatelet Ab opsonized platelets in primary ITP through $Fc\gamma RI$ and $Fc\gamma RIIIA$ (Kuwana and Ikeda, 2005; Norris et al., 2018). Monocytes that express $Fc\gamma RIIIA$ were increased in primary ITP patients and could promote the expansion of T helper (Th) 1 cells, while inhibiting the expansion of T regulatory cells (Tregs) (Zhong et al., 2012). A shift in the balance of inhibitory and activating $Fc\gamma Rs$ on monocytes in primary ITP patients has been shown to occur after corticosteroid treatment and *H. pylori* eradication (Asahi et al., 2008; Liu et al., 2011). These findings suggested that a decreased threshold for $Fc\gamma R$ activation may play a role in primary ITP.

In addition to monocytes and macrophages, dendritic cells (DCs) have been shown to activate T cells to respond to auto-antigens on platelets in primary ITP. DCs were cultured from the blood and spleens of primary ITP patients and it was found that CD86, a co-stimulatory ligand for T and B cells, was highly expressed on these cells (Catani et al., 2006). When these DCs were pulsed with platelets, they were able to increase T cell proliferation. There may be an association between increased antigen presenting capability of DCs and an expansion of autoreactive T and B cells in primary ITP (Catani et al., 2006).

CD205, an important ligand for immune tolerance induction, was decreased on splenic DCs from primary ITP patients (Zhang et al., 2015). This may help to explain the Treg deficiency seen in primary ITP patients. Moreover, Sehgal et al. found that primary ITP was associated with an increase in plasmacytoid DCs, which had an increased capacity to release interferon (IFN) α and to activate T cells (Sehgal et al., 2013).

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1.2.3 CD4⁺ T Cells in Primary ITP

In primary ITP, CD4⁺ T cells are commonly Th1 polarized such that there is an imbalance of Th1 and Th2 cells, which is common in many other autoimmune diseases as well (Carlton et al., 1995; Ma et al., 2012; Ogawara et al., 2003; Panitsas and Mouzaki, 2004; Wang et al., 2005). Previous work has shown that there is increased serum interleukin (IL)-2, IFN- γ and IL-10, but not IL-4 or IL-6 in patients with primary ITP suggesting that this Th1 polarization happens early on in the disease (Semple and Freedman, 1991; Semple et al., 1996). Additionally, these Th1 cells have been shown to have an increased survival and proliferation capacity (Ma et al., 2012; Semple et al., 1996; Yamashita et al. 2008).

Stasi et al. suggested that CD4⁺ T cell activation and Th1 responses may be responsible for the activation of CD8⁺ T cell- and Ab-mediated platelet destruction in primary ITP (Stasi et al., 2007). Both rituximab and splenectomy restored the balance between Th1 and Th2 cells in primary ITP patients, indicating the importance of Th2 subsets in resolving inflammation (Panitsas and Mouzaki, 2004; Stasi et al., 2007).

1.2.4 T Helper 17 (Th17) Cells in Primary ITP

Th17 cells play a controversial role in several autoimmune diseases, including primary ITP (Ji et al., 2012; Maddur et al., 2012; McKenzie et al. 2013; Ye et al., 2015). Zhang et al. first reported increased Th17 cells in the peripheral blood of patients with primary ITP (Zhang et al., 2009). Rocha et al. showed a significant increase of IL-17 in the plasma of patients with primary ITP and increased expression of other cytokines secreted by Th17 cells, such as IL-22 and IL-23 (Cao et al., 2011; Rocha et al., 2011). Furthermore, the levels of IL-17, IL-22 and IL-23 were found decreased in patients who responded to corticosteroid treatment (Cao et al., 2012; Li et al., 2015; Ye et al., 2015). The increased levels of Th17 cells and Th17 cytokines in primary ITP have been confirmed by several other studies (Guo et al., 2009; Hu et al., 2011; Hu et al., 2012; Ji et al., 2012; Ma et al., 2008; Ye et al., 2015 Zhang et al., 2009; Zhu et al., 2010).

However, the quantification of certain Th17 cytokines such as IL-17, TGF- β and IL-6 in the plasma from primary ITP patients was comparable to healthy controls, which contradicted the

previously mentioned results (Cao et al., 2011). Also, the same group reported that Th17 cells in the peripheral blood mononuclear cells (PBMCs) of primary ITP patients did not vary from healthy controls (Cao et al., 2011). These results were corroborated by another group (Sollazzo et al., 2011). In general, there are conflicting results with regards to the role of Th17 cells in primary ITP and this needs to be further investigated.

1.2.5 T Regulatory (Treg) Cells in Primary ITP

Treg deficiency is associated with many autoimmune diseases (Dejaco et al., 2006; Einstein and Williams, 2009; Noack and Miossec, 2014; Sakaguchi et al., 2006; Wing and Sakaguchi, 2010). Many studies have shown that in patients with primary ITP, there is a significant deficiency or dysfunction of Tregs (Fahim and Monir, 2006; Liu et al., 2007; McKenzie et al., 2013; Sakakura et al., 2007; Yu et al., 2008; Zhang et al., 2009). This may explain the loss of tolerance, abnormal cytokine production, and skewed Th1/Th2 ratios (Beaten and Kuchroo, 2013; Rocha et al., 2011; Ma et al., 2008; Ware and Howard, 1993). The peripheral deficiency of Tregs in primary ITP could be restored after treatment with corticosteroids, rituximab, or intravenous immunoglobulin (IVIg) (Audi et al., 2011; Li et al., 2011; Ling et al., 2007; Stasi et al., 2008; Yu et al., 2008). However, it is still unclear whether restoring Treg function in primary ITP patients ameliorates the disease or is just a marker of restored immune tolerance.

The decreased number of Tregs has also been shown to be associated with impaired cross-talk between Tregs and DCs such that immune tolerance is impaired (Catani et al., 2013; Ling et al., 2007). The importance of tolerance induction by DCs in primary ITP was further suggested by Sigaram et al., who showed that IVIg primed DC regulatory activity (Sigaram et al., 2006).

Treg deficiency in the periphery in autoimmune diseases may occur through a loss of cell surface markers that are involved in contact-dependent suppression such as cytotoxic T lymphocyte antigen (CTLA)-4. Plasma CTLA-4 levels have been found to be decreased in newly diagnosed ITP patients (Zhu et al., 2015). In addition, it was found that CTLA-Ig stimulated DCs from patients with primary ITP promotes the formation of Tregs *in vitro* (Xu et al., 2012).

Dysfunctional Tregs may occur through a deficiency in the transcription of forkhead box P3 (FOXP3) or in the production of immunosuppressive cytokines, such as TGF- β or IL-10 (Catani et al., 2013; Chen et al., 2014; Li et al., 2015). In addition, Tregs can be influenced by cytokines in their microenvironment such as IL-2, IL-12, and IL-21, which can lead to their dysfunction and polarization towards an inflammatory phenotype. (Andersson et al., 2002; Clough et al., 2008; King and Segal, 2005; Tesse et al., 2012; Thornton et al., 2004).

A shift in theTh17/Treg balance towards a Th17 phenotype is common in autoimmunity (Noack et al., 2014). The Th17/Treg ratio may be a good predictor of bleeding symptoms in primary ITP. Ji et al. showed that patients with ITP who had platelet counts below $30 \ge 10^9$ /L with active bleeding symptoms had a skewed Th17/Treg ratio in comparison to patients with primary ITP who had platelet counts above $30 \ge 10^9$ /L without active bleeding symptoms (Ji et al., 2012).

1.2.6 CD8⁺ T Cells in Primary ITP

Olsson et al. was the first group to show that CD8⁺ T cells mediate platelet lysis in primary ITP and their work was confirmed by Zhang et al. and others (Olsson et al., 2003; Qiu et al., 2016; Zhang et al., 2006; Zhao et al., 2008). Additionally, Li et al showed that CD8⁺ T cells could also mediate the destruction of megakaryocytes in primary ITP (Li et al., 2007) and Olsson et al., showed that CD8⁺ T cells could inhibit thrombopoiesis by megakaryocytes (Olsson et al., 2008). Direct killing by CD8⁺ T cells was proposed to be mediated through the activation of a variety of death receptor and T cell homing pathways and through increased granzyme production (Sood et al., 2008; Olsson et al., 2012; Olsson et al., 2008). Although the total number of CD8⁺ T cells seemed to be comparable between patients with primary ITP and healthy individuals, cytotoxic CD8⁺ T cells were increased in primary ITP. These cytotoxic T cells decreased after treatment, indicating an imbalance of subpopulations of CD8⁺ T cells in the pathogenesis of this disease (Stasi et al., 2007).

Increased numbers of CD8⁺ T cells were found in primary ITP patients without auto-Abs, suggesting that T cell autoimmunity can be elicited separately from the humoral response (Zhao et al., 2008). Evidence of a T cell response separate from Ab-mediated autoimmunity was further shown in primary ITP patients who did not respond to rituximab therapy in whom increased levels of splenic CD8⁺ T cells were detected (Audia et al., 2013). In addition, the authors found increased expression of human leukocyte antigen (HLA)-DR on CD8⁺ T cells and increased production of granzyme B in CD8⁺ T cells, indicating increased CD8⁺ T cell activation in primary ITP (Audia et al., 2013). It is possible that the altered cytokine environment as a result of B cell depletion affects T cell subsets, as the interplay between these cells is essential in a systemic autoimmune response (Shlomchik et al., 2001).

1.2.7 B Cells in Primary ITP

Ab opsonization of platelets leading to their destruction was the earliest mechanism discovered in the pathogenesis of primary ITP. The failure of B cell tolerance gives rise to autoreactive B cells which produce anti-platelet Abs. Approximately 60% of primary ITP patients have platelet specific IgG Abs directed against GPIIb/IIIa (~70%) or GPIbIXV (~25%), which are proteins expressed on the platelet surface that aid in platelet activation and aggregation for clot formation (Cines et al., 2014; He et al., 1994; McMillan et al., 1987; Van Leeuwen et al., 1982).

The production of anti-platelet Abs by B cells may involve multiple factors such as CD4⁺ T cell help, enhanced Th1 and Th17 responses, Treg dysfunction/deficiency as well as B cell defects and the cytokines in the microenvironment. B-lymphocyte stimulator (B-lys) was found to be significantly increased in the peripheral blood of primary ITP patients, which is important for promoting B cell survival, proliferation and differentiation (Yu et al., 2011).

Autoreactive IgG anti-platelet Abs could induce the development of primary ITP in several different ways, including increasing platelet destruction in the spleen, decreasing platelet production by suppressing megakaryocytopoiesis, or interfering with platelet function via apoptosis induction (Chang et al., 2003; Goette et al., 2016; Kuwana et al., 2009; McMillan et al., 2004; Najaoui et al., 2012). Of these mechanisms, Ab-mediated platelet destruction and enhanced clearance *in vivo* through FcγR-mediated phagocytosis is considered the most important mechanism of thrombocytopenia in primary ITP and will be discussed in further detail below (Provan et al., 2010; Cines et al., 2009).

1.2.8 Fc Gamma Receptor (FcγR) Mediated Platelet Destruction in Primary ITP

Fc γ R-mediated phagocytosis is the major way that platelets are destroyed in primary ITP. Fc γ Rs are part of a family of Fc receptors that recognize the fragment crystallizable region (Fc) of IgG (Ravetch et al., 2001). Monocytes, macrophages, DCs and neutrophils express all four types of Fc γ Rs, including the activating receptors Fc γ RIA, Fc γ RIIA and Fc γ RIIC Fc γ RIIIA and Fc γ RIIIB in humans and Fc γ RI, Fc γ RIII and Fc γ RIV in mice that promote phagocytosis, and the inhibitory receptor Fc γ RIIB that suppresses phagocytosis in both humans and mice (Nimmerjahn et al., 2008). As the Fc of IgG Abs could be recognized by multiple Fc γ Rs with different affinities, the overall response is dependent on the activation/inhibition ratio (Nimmerjahn et al., 2008).

The importance of $Fc\gamma R$ -mediated phagocytosis in primary ITP has been extensively researched. Early evidence came indirectly from the observation that splenectomy could significantly increase platelet counts in patients with primary ITP, suggesting that remission could be achieved when platelets are not being destroyed by phagocytosis in the spleen (Blanchette and Freedman, 1998; Hirsch and Dameshek, 1951; Luiken et al., 1977; McMillan, 1981). In 1974, Handin et al. showed that Ab-coated platelets could be phagocytosed by autologous peripheral leukocytes *in vitro*, by neutrophils, suggesting the phagocytosis of Ab-platelet complexes *in vivo* in primary ITP (Handin and Stossel, 1974). Later, the effectiveness of IVIg in primary ITP management and related studies further improved our understanding of the $Fc\gamma R$ -mediated phagocytosis of platelets in the pathogenesis of the disease (Nagelkerke et al., 2015).

In 1981, Imbach et al. reported that IVIg was effective in pediatric chronic ITP, and in the following two years Fehr et al. and Newland et al. showed similar findings in adult chronic ITP (Fehr et al., 1982; Imbach et al., 1981; Newland et al., 1983). It was hypothesized that high dose IVIg given at 400 mg/kg for 5 consecutive days raised platelet counts in primary ITP through the blockade of Fc γ Rs leading to decreased Fc mediated phagocytosis of anti-platelet IgG-coated platelets. This was corroborated by Clarkson et al. who showed that infusion of monoclonal Abs against both Fc γ RII and Fc γ RIIIA into patients could induce an immediate increase of platelet counts in patients with primary ITP (Clarkson et al., 1986). In an analogous set of observations, intravenous (i.v.) anti-D Ig infusions were also found to increase platelet counts in rhesus (Rh)⁺

ITP patients, suggesting anti-D opsonized red blood cells (RBCs) may competitively block FcγR-mediated platelet phagocytosis in primary ITP (Lazarus and Crow, 2003; Salama et al., 1984; Salama et al., 1986).

It is now well accepted that $Fc\gamma R$ -mediated phagocytosis of Ab-bound platelets is a major mechanism responsible for the increased platelet destruction in the spleen. Monocytes and macrophages have been shown to bind and phagocytose Ig-opsonized platelets via their $Fc\gamma Rs$, explicitly contributing to platelet clearance (Kuwana et al., 2009). Although the spleen is the major site for platelet destruction, more than one third of patients fail splenectomy. This suggests that there are other sites of platelet destruction, such as the liver. In addition to activating receptors, the inhibitory receptor $Fc\gamma RIIB$ has also been shown to have decreased patterns of expression on both monocytes and macrophages after *H. Pylori* infection in primary ITP patients (Asahi et al., 2008; Wu et al., 2012). Later, Satoh et al. found a genetic polymorphism of the $Fc\gamma RIIB$ gene in patients with primary ITP (Satoh et al., 2013). Overall, the increased platelet destruction in primary ITP may be a consequence of the increased activation/inhibition ratio of the differing activating and inhibitory $Fc\gamma Rs$. Further studies are still needed to confirm the role of $Fc\gamma RIIB$ receptors in primary ITP.

1.3 Treatment of Primary ITP

The IWG and the American Society for Hematology (ASH) published guidelines for the diagnosis and management of primary ITP (Neunert et al., 2011; Neunert et al., 2019; Provan et al., 2019; Provan et al., 2010). Patients with platelet counts above $50 \ge 10^9$ /L should be under observation without any treatment unless there is active bleeding (Neunert et al., 2011; Neunert et al., 2019; Provan et al., 2019; Provan et al., 2010). Patients with platelet counts below $50 \ge 10^9$ /L should be under /L but above $20 \ge 10^9$ /L are again not necessarily treated without active bleeding. Patients with platelet counts below $20 \ge 10^9$ /L, however, are at a significant risk of bleeding and thus are treated whether these symptoms occur (Provan et al., 2010).

1.3.1 First Line Therapies for Adult Primary ITP

First-line treatments for primary ITP include corticosteroids, IVIg, and anti-D for Rh⁺ patients (Provan et al., 2010). Corticosteroids are the conventional first line therapy for primary ITP patients with prednisone, given at 1 mg/kg/day for 2 to 4 weeks, being the most common treatment strategy (Cuker et al., 2016; Matschke et al., 2016). High dose dexamethasone, given at 40 mg/day for 4 days, is another corticosteroid that is commonly prescribed for primary ITP and is now being considered as a better steroid than prednisone because it is thought to increase remission rates (Din et al., 2015; Matschke et al., 2016; Wei et al., 2016). Although steroids are inexpensive, they are also associated with systemic and potentially severe side effects that greatly reduce the quality of life of patients (Provan et al., 2010).

IVIg and anti-D are more expensive, but they are better tolerated (Provan et al., 2010). IVIg is recommended at 0.4 g/kg for 5 days/month or 1 g/kg for 1-2 days/month (Provan et al., 2010). Anti-D is recommended for RhD⁺ non-splenectomised patients with primary ITP and without autoimmune hemolytic anemia at a dose between 50-75 μ g/kg for 4-5 days (Provan et al., 2010)

1.3.2 Second Line Therapies for Adult Primary ITP

Second-line treatments are recommended for patients who fail one or more of the first-line therapies with the goal of achieving platelet counts above 50×10^9 /L (Provan et al., 2010). Some of the common second-line treatment options include azathioprine, cyclosporin A, cyclophosphamide, rituximab, splenectomy, thrombopoietin (TPO)- receptor antagonists (Provan et al., 2010). Splenectomy is not recommended to newly diagnosed or persistent ITP patients due to the invasiveness of the surgery. Although most patients experience long term remission, 20% of patients fail to respond and another 10% of patients relapse after an initial response (Provan et al., 2019). Rituximab and TPO-receptor antagonists have clear advantages including low toxicity and improved quality of life (Provan et al., 2010).

1.3.3. Treatment of Pediatric Primary ITP

The main difference between adult and pediatric primary ITP is that most children undergo spontaneous remission and rarely experience active bleeding. For newly diagnosed children with primary ITP, children whose platelet counts are above 20×10^9 /L without any bleeding symptoms are to be observed but not treated (Provan et al., 2010). For children with platelet counts below 20×10^9 /L or with active bleeding, IVIg, anti-D, and prednisone are recommended as first-line treatments (Provan et al., 2010). However, anti-D therapy has a black box warning highlighting the risks of hemolysis and renal failure, prompting concern from physicians regarding its use in children (Despotovic et al., 2012).

1.4 Animal Models of ITP

Animal models of human diseases allow us to investigate disease pathogenesis and to evaluate potential therapeutics. Several animal models of ITP are important to our understanding of the disease pathogenesis and are used widely today to evaluate potential therapeutics (Neschadim and Branch, 2015; Neschadim and Branch, 2016; Semple, 2010). Animal models of ITP can be divided into two major categories: passive ITP (anti-platelet serum/Ab induced) and active ITP (T cell induced).

1.4.1 Passive ITP

Several mouse models of ITP have been developed over the years. These models rely on the passive transfer of Abs that are either from the serum of affected animals, monoclonal Abs, or Abs from other species that are cross reactive with mouse platelet antigens. Early mouse models relied on the transfer of anti-platelet Abs from serum (Corash and Levin, 1990; Cox et al., 1991). However, this serum was replaced with monoclonal Abs such as the mouse 6A6 Ab (mouse IgG2A), derived from male (NZW x BXSB)F1 (W/BF1) mice that develop SLE (Mizutani et al., 1993), or the rat anti-mouse CD41 (anti-GPIIb, clone MWReg30, rat IgG1: λ Ab) (Crow et al., 2001; Katsman et al., 2010; Song et al., 2003).
These passive Ab models are characterized by the rapid depletion of platelets through FcγRmediated phagocytosis by macrophages. The platelet antigens that are recognized in these mouse models are CD61 (GPIIIa) and CD41 (GPIIb), which are the same proteins that are usually targeted in the human disease (Cines et al., 2014). The repeated administration of monoclonal Abs to CD61 or CD41 for 6 consecutive days at escalating doses prolongs and maintains a platelet nadir such that it mimics chronic ITP (Katsman et al., 2010; Neschadim and Branch, 2016). However, only Abs to CD41 affects bone marrow megakaryocyte counts in addition to affecting the rate of platelet destruction through phagocytosis (Guo et al., 2018).

Passive ITP models have been extensively utilized to rapidly evaluate the efficacy of various therapeutics. Many of the therapeutics that have been tested in these models are small molecule-based therapeutics or recombinant Fcs that serve as potential replacements for IVIg (Purohit et al., 2014; Purohit et al., 2013; Zuercher et al., 2016). It is worth noting that the results obtained from these models must be accompanied by other studies as these models do not have a breach in immune tolerance.

1.4.2 Active ITP

Active ITP involves the transfer of alloreactive splenocytes into mice. One of the first models used lethally irradiated BALB/c mice engrafted with bone marrow from severe combined immunodeficient (SCID) mice, which were then given splenocytes from patients with chronic ITP (Dekel et al., 1998). These mice produced anti-human platelet Abs that were cross-reactive with mouse platelets.

The most commonly used active ITP mouse model involves the transfer of splenocytes from CD61-knockout (KO) mice, previously immunized against CD61-wildtype (WT) platelets, into SCID mice (Aslam et al., 2012; Chow et al., 2010). These mice develop severe ITP within 2 weeks following the transfer and produce anti-CD61 Abs (Chow et al., 2010). This model highlighted the importance of T cells in the immunopathogenesis of ITP. Chow et al. showed that the depletion of CD4⁺ T cells abrogates ITP and that CD8⁺ cytotoxic T cells lyse platelets in ITP (Chow et al., 2010).

This model has also been critical in establishing a role for Tregs in the immunopathogenesis of ITP, whereby Aslam et al. showed that Tregs are sequestered in the thymus, which may explain the lack of peripheral Tregs and the perturbed immune tolerance in ITP patients (Aslam et al., 2012). This study was corroborated by human findings, which showed that Tregs are decreased in primary ITP patients and that their suppressive functions are reduced (Arandi et al., 2007; Arandi et al., 2014; Ji et al., 2012).

Recently, a novel mouse model of ITP was demonstrated using splenocytes from CD41-KO mice, previously immunized against CD41-WT platelets, into SCID mice (Li et al., 2019). They showed that this model may represent a superior alternative to the chronic ITP model developed by Chow et al. because CD61 is expressed on vascular endothelial cells in addition to platelets and megakaryocytes (Byzova et al., 1998). Additionally, CD61 KO mice experience placental defects and post-natal hemorrhage (Hodivala-Dilke et al., 1999; Reynolds et al., 2002). In contrast, CD41 KO females are fertile and do not have placental defects and CD41 KO pups are viable (Li et al., 2019). In this mouse model, transferred SCID mice experience reduced platelet counts, increased anti-platelet antibody levels in the serum and reduced mature megakaryocytes in the bone marrow (Li et al., 2019).

1.5 Intravenous Immunoglobulin (IVIg) in Primary ITP

IVIg is the pooled plasma from thousands of donors and it is mainly composed of polyvalent IgG Abs, with trace amounts of IgA and IgM (Gelfand, et al., 2006; Lazarus and Crow, 2003; Sewell and Jolles, 2002). IVIg was initially used to treat primary immunodeficiency (Etzioni and Pollack, 1989). In addition to primary immunodeficiency, IVIg is also used as a therapy for several autoimmune diseases and inflammatory conditions (Ballow 2011; Brandt and Gershwin, 2006; Etzioni and Pollack 1989; Jolles et al., 2017; Kaveri et al., 2008; Oates et al., 1991; Pyne et al., 2002; Stangel et al., 1998). Additionally, high dose IVIg has become a first line therapy for autoimmune diseases such as primary ITP and Kawasaki disease (Bierling and Godeau, 2004; Bussel, 2006; Etzioni and Pollack, 1989; Fehr et al., 1982; Imbach et al., 1981; Lazarus and Crow, 2003; Tarantino, 2006).

In 1981, Imbach et al. first reported that high dose IVIg given at 400 mg/kg over 5 consecutive days raised platelet counts in pediatric patients with primary ITP (Imbach et al., 1981). The same group subsequently showed that more than 80% of patients with primary ITP responded to IVIg shortly after treatment (Imbach et al., 1985). IVIg was also found to be effective in treating adult primary ITP, with comparable response rates (Bussel et al., 1983; 14; Newland et al., 1983; Provan et al., 2010). However, it seemed as though patients needed continuous IVIg treatment in order to treat their disease (Bussel et al., 1983; Fehr et al., 1982; Newland et al., 1983; Provan et al., 2010).

There are several mechanisms of action that have been proposed for the immunomodulatory effects of IVIg however, this topic is still heavily debated. One of the earliest proposed mechanisms of IVIg includes the blockade of activating $Fc\gamma Rs$ on macrophages by IVIg (Bussel, 2000). Fehr et al. showed that the clearance of radiolabeled erythrocytes was delayed by IVIg administration, suggesting that IVIg worked to treat primary ITP by inhibiting platelet clearance by blocking $Fc\gamma Rs$ on macrophages (Clarkson et al., 1986; Fehr et al in 1982). However, there is also evidence that contradicts this theory because multiple independent studies have shown that the antigen binding fragments (F(ab')2) of IVIg could ameliorate primary ITP in human patients (Burdach et al., 1986; Crow et al., 2001; Tovo et al., 1984).

Another theory that has been proposed for the mechanism of action of IVIg involves the upregulation of inhibitory $Fc\gamma RIIB$ on effector cells, which increases their threshold for activation (Samuelsson et al., 2001; Schwab et al., 2014). Using an Ab-induced passive mouse model of ITP in $Fc\gamma RI$, $Fc\gamma RIIB$ and $Fc\gamma RIII$ KO mice, Samuelsson et al. showed that IVIg reduced platelet clearance through the concurrent inhibition of $Fc\gamma RIII$ with the upregulation of $Fc\gamma RIIB$ (Samuelsson et al., 2001; Teeling et al., 2001). Additionally, IVIg was shown to be ineffective at treating ITP in $Fc\gamma RIIB$ KO mice (Samuelsson et al., 2001; Crow et al., 2003). However, another study found that IVIg specifically downregulated activating $Fc\gamma RIIIA$ on $CD11c^+$ DCs, which had no effect on $Fc\gamma RIIB$ expression (Siragam et al., 2006).

In accordance with this theory that IVIg upregulates $Fc\gamma RIIB$ as part of its mechanism, there is one model for the mechanism of action of IVIg that continues to be discussed, despite it being refuted multiple times (Nimmerjahn and Ravetch, 2008b; Yoshikatsu et al., 2006). This model was developed by Ravetch et al. who proposed that the $Fc\gamma$ sialylated fraction of IgG is responsible for the immunomodulatory properties of IVIg (Baerenwaldt et al., 2010; Nimmerjahn and Ravetch, 2008a; Schwab and Nimmerjahn, 2013; Schwab et al., 2014). This model proposed that a small fraction (10%) of total IVIg sialylated in the $Fc\gamma$ domain, engages the CD209 receptor on regulatory macrophages and DCs, which causes them to release IL-33 (Schwab et al., 2014; Anthony et al., 2011; Anthony et al., 2008a; Anthony et al., 2008b). IL-33 is a Th2 polarizing cytokine that further promotes the release of IL-4 from basophils at sites of inflammation. IL-4 acts on macrophages to upregulate the expression of $Fc\gamma$ RIIB, which lowers inflammation (Schwab et al., 2014). This model proposes that the subsequent upregulation of $Fc\gamma$ RIIB on effector cells raises their threshold for activation, thus allowing for a reduction in inflammation (Samuelson et al 2001; Schwab et al., 2014).

However, this model is severely flawed in that many groups have proven that there is absolutely no requirement for the sialylated fraction of IVIg to be present for it to ameliorate ITP and many other autoimmune diseases such as RA (Guhr et al., 2011; Käsermann et al., 2012; Leontyev et al., 2012; von Gunten et al., 2014). Additionally, many other groups have shown that there is no requirement for $Fc\gamma RIIB$, IL-33, IL-4, or basophils (Campbell et al., 2014; Leontyev et al., 2012; Leontyev et al., 2018). This model is illustrated in **Figure 1.1**.

Figure 1.1 Mechanism of Action of Sialylated IVIg.

Adapted from Schwab, I., and Nimmerjahn, F. (2013). Intravenous immunoglobulin therapy: how does IgG modulate the immune system? Nature Reviews Immunology *13*, 176-189. (Schwab and Nimmerjahn, 2013). <u>https://doi.org/10.1038/nri3401</u>



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In auto-Ab-dependent ITP and K/BxN arthritis, the anti-inflammatory activity of IVIg is dependent on IgG glycovariants rich in terminal sialic acid residues, the C-type lectin receptor DC-SIGN-related protein 1 (SIGNR1) and FcγRIIB. Whereas the IVIg-dependent suppression of ITP is independent of the spleen, basophils and Th2-type cytokines, the amelioration of RA requires an intact splenic architecture, basophils, IL-4 and IL-33. Ultimately, both pathways result in the upregulation of FcγRIIB expression and decreased expression of activating FcγRs, which leads to reduced platelet phagocytosis in ITP and a lower level of tissue inflammation in K/BxN arthritis. In a paper published by our lab in 2014, we looked at cytokine induction by IVIg using a multiplex cytokine assay with both BALB/c and C57BL/6 mice with ITP. We showed that IL-33 was produced following IVIg treatment in BALB/c mice, but not in C57BL/6 mice (Leontyev et al., 2014). This supports another study that has also shown that IL-33 is not a prominent player in the mechanism of IVIg action (Maddur et al., 2017). Additionally, high amounts of IL-4 were produced in both mouse strains after treatment with IVIg, even though previous studies showed that IL-4 and common gamma chain-deficient mice with ITP were successfully treated with IVIg (Crow et al., 2007). In addition to IL-4, both IL-11 and granulocyte–macrophage colony-stimulating factor (GM-CSF) were produced significantly following IVIg treatment in both BALB/cJ and C57BL/6 mice, with IL-11 reaching nanogram levels (Leontyev et al., 2014).

More recently, our lab further investigated cytokines in the mechanism of IVIg and we showed that IL-4, and GM-CSF were not involved in the mechanism of action of IVIg in ITP when IVIg was able to treat ITP in IL-4 and GM-CSF KO mice (Lewis et al., 2018). In the same paper, we also showed that when mice were depleted of their basophils, they were still susceptible to ITP induction and were successfully treated with IVIg. This was the first piece of contradictory evidence to refute Ravetch's model showing that basophils are not important for the mechanism of action of IVIg in ITP. This evidence was corroborated by another study from our lab showing that basophils are not needed for the treatment of RA by IVIg in the K/BxN mouse model (Campbell et al., 2014). Lastly, our results with IL-11 were unclear. Even though IL-11 Receptor KO mice with ITP responded to IVIg similarly to WT mice, treatment of ITP WT mice given recombinant human IL-11 instead of IVIg showed an increase in platelet numbers and WT mice administered a neutralizing Ab to IL-11 showed a significant reduction in the ability of IVIg to ameliorate the ITP. To this date, it is still unclear which cytokines and if any cytokines contribute to the mechanism of action of IVIg and thus more studies need to be conducted to elucidate this.

There are additional mechanisms through which IVIg is proposed to work however, there is limited evidence for these theories and most of these theories have been refuted by subsequent publications. These mechanisms include the neutralization of pathogenic auto-Abs by antiidiotype Abs present within IVIg preparations (Berchtold et al., 1989; Rossi et al., 1989). However, this was later refuted by Crow et al. who showed that IVIg adsorbed against the Ab used to induce ITP in mice protected against passively induced ITP, indicating that Abs with anti-idiotype activity present in IVIg are not necessary for its effective treatment of ITP (Crow et al., 2001).

Immunomodulation by IVIg has been proposed to work through the upregulation of Tregs (Dammacco et al., 1986; De Groot et al., 2008; Delfraissy et al., 1985; Ephrem et al., 2008; Macey and Newland, 1990). This was corroborated by Aslam et al. who showed that mice with ITP treated with IVIg had increased numbers of Tregs (Aslam et al., 2012). The inhibition of complement-dependent cytotoxicity effects (Mollnes et al., 1998) and the subsequent clearance of cells (Basta et al., 1989), as well as induction of auto-Ab-clearance via the neonatal Fc receptor (FcRn) have been proposed (Hansen and Balthasar, 2002). The involvement of FcRn in the mechanism of IVIg action in ITP was later refuted by Crow et al., who showed that FcRn KO mice were susceptible to ITP and were able to be successfully treated by IVIg (Crow et al., 2011). Taken together, the mechanism of action of IVIg is complicated and it likely works differently depending on what autoimmune model is used.

1.5.1 Disadvantages of IVIg

IVIg is generally considered to be a safe drug because it has minimal side effects and most of these side effects are benign, with chills and headaches being most common, whereas more serious adverse events such as, thrombosis and hemolysis, are very rare (Branch, 2015; Branch et al., 2018; Provan et al., 2010). IVIg also has a very favourable safety profile because it is immunomodulatory rather than immunosuppressive.

Despite this, there are serious challenges associated with the manufacturing of this drug and with the application of this drug. First, the manufacturing of IVIg requires highly specialized production facilities, which are very expensive to maintain (Zuercher et al., 2011). Patients who receive IVIg are at risk of contracting blood borne pathogens, particularly newly emerging infections, so careful attention must be paid to pathogen safety. Not only is the cost of these production facilities expensive, but IVIg itself is very expensive because its supply is dependent on the availability and the collection of human plasma. With an increase in the demand of IVIg to treat a variety of autoimmune and inflammatory conditions and a decrease in the supply of

human plasma donations, the price of IVIg has drastically increased to pay for the high doses required for immunomodulation (Glauser, 2014).

1.5.2 Replacements for IVIg

The numerous challenges associated with IVIg production and its application has provided an incentive to develop an alternative therapy with similar efficacy but with improved characteristics. This particularly applies for indications where the effector mechanism(s) of IVIg are dependent on the Fc of IgG. Most of the proposed effector mechanisms of IVIg in autoimmune and inflammatory diseases are thought to be mediated by the Fc of IgG.

Accordingly, many researchers have investigated plasma-derived Fcs as a replacement for IVIg. A therapeutic role for these fragments in autoimmune diseases have been suggested in experimental models of RA and ITP (Campbell et al., 2014; Samuelsson et al., 2001). Additionally, monomeric Fc derived from the plasma of donors successfully treated primary ITP in children (Debre et al., 1993) and similar efficacy was shown in Kawasaki disease (Hsu et al., 1993). However, there were issues that arose from the small molecular weight of these plasma derived Fcs, such as rapid clearance from the body. Additionally, these potential replacements did not resolve the issues associated with obtaining human plasma.

Researchers turned to the development of recombinant Fcs that they could structure into oligomers with multiple Fcs to overcome these issues of rapid clearance and to hopefully promote higher efficacy at lower doses. This alternative would also preclude the use of human plasma.

Many strategies have been used to multimerize the Fc of IgG in order to produce polyvalent Fc molecules (Bosques and Manning, 2016; Czajkowsky et al., 2012; Zuercher et al., 2016). First, stradomers were made through the fusion of the human IgG2 hinge region to human IgG1 Fc or to mouse IgG2a Fc. This led to the production of stradomerized Fcs that bound FcγRs with high avidity (Jain et al., 2012). These stradomers demonstrated therapeutic efficacy in animal models of ITP, RA, inflammatory neuropathy and myasthenia gravis (Jain et al., 2012; Niknami et al.;

Thiruppathi et al., 2014), Importantly, efficacy was achieved at approximately 20-40-fold lower doses than IVIg.

A trivalent multimer was also made and compared to multimers with increasing and decreasing valencies (Ortiz et al., 2016). It was found that any molecule with a valency greater than 3 bound $Fc\gamma Rs$ with high avidity and triggered activating signal transduction pathway(s), whereas any molecule with a valency of 3 or less bound $Fc\gamma Rs$ with high avidity without triggering activating signals (Ortiz et al., 2016). In this study, the trimer was called M230 and it was made by replacing the F(ab')2s with two additional IgG1 Fcs. Association of the fragments was stabilized by 'knobs-into-holes' technology developed by Ridgway et al. in 1996 (Ridgeway et al., 1996). Results from both the collagen-induced arthritis (CIA) and the collagen antibody-induced arthritis (CAIA) models showed that M230 was more efficacious than IVIg at 10-fold lower doses (Washburn et al., 2015). In the ITP mouse model, it was most effective as it raised platelet counts at 100-fold lower doses than IVIg (Ortiz et al., 2016). This study addressed the importance of monitoring adverse event profiles of multimeric Fc molecules. Like IgG aggregates, Fc multimers can crosslink Fc γ Rs and lead to subsequent activation. The consequences of this would be a severe inflammatory response, which would ultimately be counterintuitive to the objective of the Fc.

A hexameric Fc molecule called HexaGard was developed using technology developed by Smith et al. (Smith et al., 1995) who showed that the addition of the C-terminal 18 aa tail-piece of IgM lead to hexamerization of IgG. This technology was applied to hexamerize human IgG1 Fcs to form HexaGard (Czajkowsky et al., 2015). Hexagard showed high avidity for FcγRs and for CD-209 and showed efficacy in a mouse model of ITP. However, future studies on Hexagard were discontinued because it appeared to have unwanted side effects, such as the activation of complement through its enhanced avidity to C1q, which lead to C5b-9 deposition (Czajkowsky et al., 2015).

In addition to Hexagard, multiple hexameric-Fc fusion proteins were engineered to be tested in multiple *in vitro* and *in vivo* systems to determine the consequences of multi-valent $Fc\gamma R$ engagement (Blundell et al., 2017). The first hexameric-Fc was composed of an IgG4 and

IgG1 entitled γ 4eng-hexameric-Fc. The second hexameric-Fc was a mutant version of this protein containing L234F/P331S mutations to reduce platelet and complement activation, entitled γ 1eng-hexameric-Fc. Finally, the third hexameric-Fc was another mutant which had F234L/F296Y mutations introduced to increase its ability to block Fc γ R dependant phagocytosis, entitled γ 4eng-F234L F296Y hexameric-Fc (Qureshi et al., 2017).

The major findings of this study were that the hexameric-Fcs bound avidly to $Fc\gamma Rs$, and this interaction caused the rapid internalisation of these hexameric-Fcs and the subsequent degradation of activating but not inhibitory $Fc\gamma Rs$. This effect was prolonged and occurred in both *in vitro* and *in vivo* systems. This effect disrupted $Fc\gamma R$ function and lead to the potent inhibition of phagocytosis. The hexameric-Fcs were further tested in a mouse model of ITP and found to be effective at protecting platelets from phagocytic degradation for a prolonged period. This study highlighted the therapeutic utility for hexameric-Fcs in diseases involving $Fc\gamma R$ -mediated phagocytosis, such as ITP. However, this study also highlighted the potential challenges for the clinical development of hexameric-Fc based therapeutics because of their rapid clearance in *in vivo* systems.

More recently, much attention has been given to a recombinant human IgG1 Fc multimer, GL-2045. Gliknik designed GL-2045 as a human version of their mouse stradomer, M-045 (Jain et al., 2012; Niknami et al., 2013; Thiruppathi et al., 2014; Zuercher et al., 2016; Zuercher et al., 2019). GL-2045 is composed of the human IgG1 Fc with its C-terminus fused to the human IgG2 hinge region to facilitate the formation of stradomers (Sun et al., 2017; Zhou et al., 2017). When this molecule was first designed, it was shown to be a potent inhibitor of complement activation (Sun et al., 2017). However, the more recent paper published by the same group focused on its potential as a replacement for IVIg for the treatment of certain autoimmune diseases because of its ability to efficiently mimic the anti-inflammatory and immunomodulatory properties of Ig Fc aggregates (Zhang et al., 2019).

The paper showed that GL-2045 was more efficient at blocking the binding of immune complexes (ICs) from patients with RA to FcγRs and it was more efficient at inhibiting phagocytosis in comparison to IVIg. Additionally, GL-2045 efficiently ameliorated ITP in a mouse model and both prevented and suppressed arthritis in the CIA mouse model. GL-2045

also reduced the systemic levels of potent inflammatory cytokines such as IL-6 and IL-Now, Gliknik and Pfizer have entered into an exclusive worldwide licencing agreement, which allows Pfizer to develop and, if approved by applicable regulatory authorities, commercialize GL-2045 for the treatment of certain autoimmune diseases. As of 2018, GL-2045 has been in phase 1 clinical trials. A summary of the recombinant Fc proteins that are currently under investigation as of 2019 are summarized in **Table 1.2**.

Table 1.2 Recombinant Fcs Under Clinical Investigation.

Modified from Zuercher, A.W., Spirig, R., Morelli, A.B., and Käsermann, F. (2016). IVIg in autoimmune disease- Potential next generation biologics. Autoimmunity Reviews *15*, 781-785. (Zuercher et al., 2016). <u>https://doi.org/10.1016/j.autrev.2016.03.018</u>

Molecule	Description	Preclinical Data	Indications	Stage	Institution
GL2045;	Fc multimerized by	Animal models:	CIDP (Orphan	Clinic Phase I	Gliknik/Pfizer
Stradomer TM	IgG2 hinge	CIA, ITP, MG	Drug Status		
		In-vitro: FcγR	Granted, US)		
		avidity ↑	MG, RA		
HexaGard™	IgG1 Fc with IgM	In-vitro: FcγR	Autoimmune	Preclinical	Liverpool
	tailpiece and	avidity ↑; no	or	studies	School of
	mutations	binding to FcRn	inflammatory	discontinued	Tropical
	[L309C/H310L]		disease		Medicine
M230	IgG1 Fc based	Animal models:	Autoimmune	Clinic Phase 1	Momenta
	trimer	arthritis, ITP	disease		Pharmaceuticals and CSL Ltd.
		In-vitro:			
		ADCP ↓, FcγR			
		avidity ↑			

1.5.3 Fc-µTP-L309C as a Potential Replacement for IVIg

CSL Behring designed 2 hexameric recombinant Fc molecules entitled Fc- μ TP and Fc- μ TP-L309C to replace their IVIg product, Privigen, and their SCIg product, Hizentra (Spirig et al., 2018). Fc- μ TP was generated by fusing the 18 aa residues (PTLYNVSLVMSDTAGTCY) of the human IgM tailpiece to the C-terminus of either a WT human IgG1 Fc, Fc- μ TP, or a variant with a point mutation changing the Leucine residue at position 309 to a Cysteine residue, Fc- μ TP-L309C. The same technology that was used to create Hexagard was also used to create Fc- μ TP and Fc- μ TP-L309C (Qureshi et al., 2017).

This resulted in the formation of two recombinant Fc hexamers, with the point mutations in Fc- μ TP-L309C providing a more stable structure than Fc- μ TP, because of the formation of disulphide bonds between the Fc molecules. Both Fc- μ TP-L309C and Fc- μ TP were predominantly present in hexameric form (approximately 85%), however, there were lower order species present for both recombinant Fcs and higher order structures, most likely dimers of the hexameric molecule, present for Fc- μ TP-L309C. However, because of its more stable structure, the entirety of my thesis is focused on on Fc- μ TP-L309C. The biochemical characterizations of Fc- μ TP-L309C and Fc- μ TP are illustrated in **Table 1.3** and **Figure 1.2**.

Table 1.3 Biochemical Characteristics of Fc-µTP-L309C and Fc-µTP.

Modified from Spirig, R., Campbell, I.K., Koernig, S., Chen, C.G., Lewis, B.J., Butcher, R., Muir, I., Taylor, S., Chia, J., Leong, D, et al. (2018). rIgG1 Fc hexamer inhibits antibodymediated autoimmune disease via effects on complement and FcγRs. The Journal of Immunology 200, 2542-2553. (Spirig et al., 2018). <u>https://doi.org/10.4049/jimmunol.1701171</u>

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rFc	Technique	%Monomer	%Dimer	% Trimer	% Hexamer	% Multimer
Molecule						
Fc-µTP	SEC-MALS	13 (73 kDa)		2 (168 kDa)	84 (355 kDa)	
	AF4-MALS	10(60 kDa)			87 (305 kDa)	3 (491 kDa)
Fc-µTP-	SEC-MALS		4 (114 kDa)	4 (211 kDa)	84 (383 kDa)	8 (745 kDa)
L309C	AF4-MALS	2 (62 kDa)			83 (327 kDa)	15 (592 kDa)

Figure 1.2 Biochemical Characteristics of Fc-µTP-L309C and Fc-µTP.

Adapted from Spirig, R., Campbell, I.K., Koernig, S., Chen, C.G., Lewis, B.J., Butcher, R., Muir, I., Taylor, S., Chia, J., Leong, D, et al. (2018). rIgG1 Fc hexamer inhibits antibodymediated autoimmune disease via effects on complement and FcγRs. The Journal of Immunology 200, 2542-2553. (Spirig et al., 2018). https://doi.org/10.4049/jimmunol.1701171

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(A) Schematic diagram of Fc-mTP and Fc-mTP-L309C hexamer structures. Light gray bars represent presumed disulphide bonds. (B) SDS-PAGE of Fc-mTP (left) and Fc-mTP-L309C (right) rFc multimers. Molecular mass markers in kDa are shown. (C) SEC and (D) A4F with MALS of Fc-mTP (left panels) and Fc-mTP-L309C (right panels). Chromatograms show the normalized UV (280 nm) signals, and the bold lines are the molecular mass (in kDa) of material eluted at the time indicated.

The potential advantages of replacing IVIg with these molecules are evident. First, they allow for the reproducible manufacturing of high amounts of recombinant product with consistent quality using processes that are independent of plasma supply. A reduction in the need for plasma would provide massive benefits for the Canadian health care system because IVIg is expensive to produce and it is supplied to patients free of charge. Importantly, the potential for higher specific activity may allow for significantly lower doses for patients such that they could be administered subcutaneously from the comfort of one's home. This could drastically increase the quality of life for patients who receive regular IVIg infusions.

1.5.4 IVIg in Rheumatoid Arthritis (RA)

Plasma-derived IgG products are manufactured with chromatographic purification processes and are available in formulations suitable for i.v. or for subcutaneous (s.c.) application (Danieli et al., 2014). IVIg/SCIg has been increasingly used for the treatment of patients with chronic or acute autoimmune and inflammatory diseases, such as primary ITP, Guillain–Barré syndrome, Kawasaki disease, chronic inflammatory demyelinating polyneuropathy, myasthenia gravis and several other rare diseases (Gelfand, 2012). Additionally, IVIg is used off-label in many other diseases and is currently under evaluation for the treatment of RA (Katz-Agranov et al., 2015).

However, there is a limited amount of research performed on the therapeutic efficacy of IVIg in RA (Kanik et al., 1996; Maksymowych et al., 1996; Muscat et al., 1995; Vaitla and McDermott, 2010). The human trials that were done to evaluate this were not controlled and were performed in the pre-biologic era thus, the results were equivocal (Kanik et al., 1996; Maksymowych et al.,

1996; Muscat et al., 1995; Vaitla and McDermott, 2010). Additionally, the patient cohort for these studies was small, with some studies having less than 20 patients enrolled (Prieur et al., 1990; Tumiati et al., 1992). A high-dose IVIg protocol of 1-2 g/kg of IVIg per month for a minimum of 6 months is critical for effectively treating systemic autoimmune diseases (Sewell and Jolles, 2002). However, these studies used lower doses of 400 mg/kg of IVIg without any long-term follow-up (Prieur et al., 1990; Tumiati et al., 1992).

With the limited amount of studies performed on the therapeutic efficacy of IVIg in RA (Kanik et al., 1996; Maksymowych et al., 1996; Muscat et al., 1995; Vaitla and McDermott, 2010), and with the increasing amount of evidence that IVIg and other recombinant Fcs work to treat RA in various mouse models (Jain et al., 2012; Mekhaiel et al., 2011; Niknami et al., 2013; Ortiz et al., 2016; Qureshi et al., 2017; Ridgway et al., 2017; Spirig et al., 2018; Thiruppathi et al., 2014; Washburn et al., 2015; Zhou et al., 2017), we decided to examine the efficacy of IVIg/SCIg and Fc-µTP-L309C in a mouse model of RA. The next portion of the introduction will be focused on this disease.

1.6 RA

RA is a chronic inflammatory autoimmune disease that primarily affects the joints of the hands and feet but can affect other joints and commonly involves systemic complications (Smolen et al., 2016). RA is a form of polyarthritis that affects the joints symmetrically and it is characterized by synovitis and synovial hyperplasia, cartilage and bone erosion, as well as systemic inflammation (Scott et al., 2010; Stoffer et al., 2016). This chronic inflammation and progressive tissue destruction leads to irreversible structural deformities and the eventual loss of function of the affected joints (Alarcon et al., 2015).

1.6.1 Etiology of RA

Although the cause(s) of RA is not fully understood, several factors have strongly suggested that genetics and certain environmental exposures increases one's risk of developing RA.

1.6.1.1 Genetic Risk Factors

There is an increased risk of developing RA if you have a family history of RA (Frisell et al., 2016). The strongest genetic link between family members resides in a set of alleles within the HLA genes called the shared epitope (SE) (Bowes and Barton, 2008; Holoshitz, 2010; Imboden, 2009; Gregersen et al., 1987; Gonzalez-Gay et al., 2002; Raychaudhuri et al., 2012). The SE accounts for approximately 40% of the genetic association with RA especially in anticitrullinated peptide Ab (ACPA)- positive disease; ACPAs are common auto-Abs generated in RA (Van der Woude et al., 2009). The SE encodes aa sequences that predict structural similarities in the HLA peptide-binding groove. The SE resulted from the observation that several alleles of the HLA-DRB1 gene increased the risk of developing RA and furthermore the severity of RA (de Almeida et al., 2011; Gregersen et al., 2005). The SE is in the HLA-DR peptide-binding groove and it affects both peptide binding to APCs and antigen presentation to T cells (de Almeida et al., 2011; Gregersen et al., 1987; Huizinga et al., 2005).

Many other genetic risk factors for RA have been identified by genome-wide association studies (GWAS) (Okada et al., 2014). More than 100 loci have been identified that may increase a person's risk of developing RA (Eyre et al., 2012; Frisell et al., 2013; Frisell et al., 2016; Kim et al., 2017; Messemaker et al., 2015; Stahl et al., 2012). One of the strongest associations is with a polymorphism in the protein tyrosine phosphatase, non-receptor type 22 (PTPN22) gene that is thought to lower the threshold of activation for T cells (Rieck et al., 2007; Stanford and Bottini, 2014; Zhang et al., 2011). Recent studies have also suggested that this polymorphism may lead to hypercitrullination, which could alter self-proteins such that they are recognized as foreign. because of the interaction between PTPN22 and peptidylarginine deiminase (PADI), an enzyme responsible for citrullination (Chang et al., 2015; Chang et al., 2016).

PADI4, is an enzyme that post translationally modifies arginine into citrulline. Several mutations in this enzyme have been found to be associated with ACPA-positive RA. These mutations cause hypercitrullination of certain peptides, which causes an immune response against these proteins (Messemaker and Huizinga, 2015; Plenge et al., 2005; Viatte et al., 2013; Yamada et al., 2003). In addition to hypercitrullination, there are other epigenetic modifications that are associated with RA such as, hypermethylation (Bottini and Firestein, 2013). This hypermethylation has been shown to promote a more aggressive phenotype for fibroblast-like synoviocytes (FLSs) and has been associated with more severe RA (Maeshime et al., 2016; Whitaker et al., 2013).

There are also other genetic mutations that cause a smaller risk of developing RA, and these are mainly involved in signalling pathways for cytokines and immune checkpoints. Specific mutations in CTLA4, signal transducer and activator of transcription (STAT)-4, IL-6, CC chemokine receptor (CCR)-6, tumour necrosis factor (TNF) receptor-associated factor 1, and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) have been shown by several studies to confer an increased risk (Deane et al., 2017).

1.6.1.2 Environmental Risk Factors

Smoking tobacco is one of the greatest environmental risk factors for developing RA and it is now thought to be restricted to ACPA-positive patients with the SE (Chang et al., 2014; Kallberg et al., 2011). Smoking, in combination with the SE, can increase one's risk by more than 20-fold (Kallberg et al., 2011). Smokers with RA generally have higher levels of inflammation, systemic complications and a worse prognosis (Chang et al., 2014; Kallberg et al., 2011; Sokolove et al., 2016). The increased risk associated with smoking might be due to epigenetic modifications, as smoking was shown to cause hypomethylation of certain genes, whereas disease-modifying antirheumatic drug (DMARD) treatment induced hypermethylation of the same genes (Svendsen et al., 2016).

Silica exposure is an environmental risk factor for RA, particularly for ACPA-positive RA (Stolt et al., 2010; Turner and Cherry, 2000). The silica in dust has been linked to autoimmunity in firefighters and other emergency responders during the September 11 attacks and in Malaysian women working in the textile industry (Too et al., 2016; Webber et al., 2015).

The association between periodontal disease and RA is mediated by the oral microbiota, particularly *Porphyromonas gingivalis* (*P. gingivalis*) and *Aggregatibacter actinomycetemcomitans* (Aa) (Kharlamova et al., 2016; Konig et al., 2016; Laugisch et al., 2016; Lundberg et al., 2010; Wegner et al., 2010). These bacteria citrullinate host proteins through the endogenous expression of their PADI4, which provokes a breach of tolerance to citrullinated proteins (Konig et al., 2016; Wegner et al., 2010).

Besides oral microbiota, there has been a significant interest in the relationship between gut microbiota and RA. The diversity of the gut microbiome has been shown to be decreased in individuals with RA in comparison to individuals without RA (Forbes et al., 2016). As similar trend has been found in other autoimmune diseases as well (Gianchecchi and Fierabracci, 2019). Additionally, bacteria in the phylum, Actinobacteria, are not commonly found in the gut microbiome of the general population, however they are found in abundance in people with RA (Chen et al., 2016). In the colon, *Prevotella copri* species were enriched in people newly diagnosed with RA (Scher et al., 2013; Zhang et al., 2016) and this enrichment was associated with certain HLA-DR mutations (Pianta et al., 2017).

Lastly, viral infections have been suggested to trigger RA via molecular mimicry such as with parvovirus B19, Chikungunya virus, and Epstein–Barr virus (EBV) (Balandraud et al., 2004; Gasque et al., 2016; Nacjute et al., 2016; Tan and Smolen, 2016).

1.6.2 Diagnosis of RA

The typical age of onset for RA is between 30 and 60 years of age but early onset of the disease is possible. (Alamanos and Drosos 2005; Alamanos et al., 2006; Silman and Pearson 2002). RA is currently diagnosed based on the 2010 American College of Rheumatology (ACR)/ European League Against Rheumatism (EULAR) classification criteria, which consider the number of active joints, symptom duration, serology, and acute-phase reactants (Aletaha et al. 2010; Kay and Upchurch, 2012; Neogi et al. 2010). The disease is scored based on these 4 criteria, and a cumulative score of 6/10 or higher is required for an RA diagnosis (Aletaha et al. 2010; Kay and

Upchurch, 2012; Neogi et al. 2010). The 2010 ACR/EULAR classification criteria are further described in **Table 1.4**. Prior to 2010 classification criteria, the 1987 ACR criteria were used for RA diagnosis, however this system lacked sensitivity and the ability to determine early onset RA. (Arnett et al. 1988; Van der Linden et al., 2011).

Table 1.4 2010 ACR/EULAR Classification Criteria.

Modified from Aletaha, D., Neogi, T., Silman, A.J., Funovits, J., Felson, D.T., Bingham III, C.O., Birnbaum, N.S., Burmester, G.R., Bykerk, V.P., Cohen, M.D., et al. (2010). 2010 Rheumatoid arthritis classification criteria: An American College of Rheumatology/European League Against Rheumatism collaborative initiative. Arthritis and Rheumatism *62*, 2569-2581. (Aletaha et al., 2010). <u>http://dx.doi.org/10.1136/ard.2010.138461</u>

Criteria	Grade
Joint involvement and distribution	
1 large joint	0
2-10 large joints	1
1-3 small joints	2
4-10 small joints	3
>10 joints (at least 1 small joint)	5
Symptom duration	
< 6 weeks	0
≥ 6 weeks	1
Serology	
ACPA negative and RF negative	0
ACPA low-positive or RF low-positive	2
ACPA high-positive or RF high-positive	3
Acute-phase reactants	
Normal CRP and normal ESR	0
Abnormal CRP or abnormal ESR	1

Disease activity can be effectively assessed by the Clinical Disease Activity Index (CDAI) or the Simplified Disease Activity Index (SDAI) (Felson et al., 1995; Felson et al., 2011; Smolen et al., 2016). The SDAI and CDAI consider several variables such as, swollen and tender joint counts, patient global assessment, or patient perspective, and C-reactive protein (CRP). These variables are assessed by questions with a 0-10 response given by either the physician or the patient (Aletaha et al., 2012; Aletaha et al., 2005; Smolen et al., 2003). Clinical remission, which is defined as a state of no, or minimal disease activity, is the main target in RA and corresponds to a CDAI score of ≤ 2.8 or an SDAI score of ≤ 3.3 (Anderson et al., 2011).

Another measure of disease activity in RA is the Disease Activity Score (DAS) and its modification using the 28-joint count assessment method (DAS28) (Prevoo et al., 1995). The DAS28 measures disease activity by calculating the number of swollen and tender joints, erythrocyte sedimentation rate (ESR) or CRP, and RA activity self-reported visual analogue scale score (Prevoo et al., 1995). This score is limited by the fact that it does not consider erosions, extra-articular manifestations, joint structure deformities, or disability and that ESR and CRP scores are heavily weighted (Anderson et al., 2011; Fleischmann et al., 2015; Makinen et al., 2005; Thiele et al., 2013). The disease activity measures used for RA are further described in **Table 1.5**.

Table 1.5 Disease Activity Measures Used for RA.

Modified from Smolen, J.S., Alehata, D., Barton, A., Burmester, G.R., Emery, P., Firestein, G.S., Kavanaugh, A., McInnes, I.B., Solomon, D.H., Strand, V., et al. (2018). Rheumatoid arthritis. Nature Reviews Disease Primers *4*, 18001. (Smolen et al., 2018). https://doi.org/10.1038/nrdp.2018.1

Scoring System and	Disease activity states				
formula	Remission	Low disease	Moderate	High	
		disease activity	disease activity	disease activity	
SDAI (SJC28+TJC28+PGA+EGA+CRP)	≤3.3	>3.3-11	>11-26	>26	
CDAI (SJC28+TJC28+PGA+EGA)	≤2.8	>2.8–10	>10-22	>22	
DAS (Complex formula including the	≤1.6	>1.6-2.4	>2.4-3.7	>3.7	
Ritchie index, SJC44, ESR and GH)					
DAS28 (Complex formula including the	≤2.6	>2.6-3.2	>3.2–5.1	>5.1	
TJC28, SJC28, ESR (or CRP) and GH)					

The ACR improvement criteria are used to determine response to treatment and the criteria rely on improvement compared with baseline in the core set of disease activity measures such as, swollen and tender joint counts, patient global assessment, physician global assessment, patient pain assessment, physical function or quality of life score and ESR/CRP (Felson et al., 1995). An improvement of 20% (ACR20) implies a 20% improvement in tender or swollen joint counts and a 20% improvement in at least three out of the five criteria is achieved. ACR50 and ACR70 are the same instruments with improvement levels defined as 50% and 70% respectively (Aletaha et al., 2012).

The Health Assessment Questionnaire Disability Index is another commonly used measure (Maska et al., 2011). It is a measure of disability calculated based on functional assessment by the patient (Maska et al., 2011). The main limitation of this scale is that it is an indirect and subjective measure of disease severity (Boini and Guillemin, 2001; Pincus, 2006).

Radiological measures are not subjective, and they focus on joint damage. The Sharp/van der Heijde Score measures erosions in 44 joints and joint space narrowing in 42 joints (Van der Heijde et al., 1999). The Scott Modification of the Larsen Method is another measure of radiological damage that examines erosions and joint destruction in hands, wrists, and feet (Edmonds et al., 1999). The limitations of these methods are that they do not consider factors other than joint damage and they rely on specialists to determine each measurement (Boini and Guillemin, 2001; Pincus, 2006).

1.6.3 Epidemiology of RA

The prevalence of RA in the western world is 1–2% and is approximately 1% worldwide (Rudan et al., 2015). The incidence rate of RA is 20-50 per 100,000 people per year (Alamanos et al., 2006). These rates vary based on ethnicity and geographical location with lower prevalence in Southern European (0.3-0.7%) and developing countries (0.1-0.5%) (Alamanos et al., 2006). The risk of arthritis increases with age and arthritis is more common among women than men. (Alamanos and Drosos 2005; Silman and Pearson, 2002). These sex-based differences are common among various autoimmune diseases such as, SLE and multiple sclerosis (Desai and Brinton, 2019).

1.6.4 Symptoms of RA

Individuals present with pain, swelling and stiffness in a symmetrical pattern, predominantly affecting the smaller joints of the hands and feet (Imboden, 2009). Typically the proximal interphalangeal joints (PIPJs) and the metacarpophalangeal joints (MCPJs) on the hands are affected, while the distal interphalangeal joints (DIPJs) are spared (Monach et al., 2004). If left untreated, there is cumulative damage to the articular cartilage and subchondral bone erosion, which eventually leads to irreversible joint destruction, structural deformities and loss of function of the affected joints (Alarcon et al., 2015). Classic RA deformities involving the digits are the boutonnière (irreversible flexion at the PIPJs and hyperextension at the DIPJs), swan neck (irreversible hyperextension at the PIPJs and flexion at the DIPJs) and Z-thumb (flexion at the MCPJs and hyperextension at the interphalangeal joints to 90°) deformities (Monach et al., 2004). These changes result from irreversible damage to the tendons and the joint capsule.

RA is a comorbid disease with cardiovascular disease being the most common comorbidity (Dougados et al. 2014; Symmons and Gabriel 2011). Sjogren's syndrome, diabetes, osteoporosis, pulmonary diseases, infections, mental health conditions, and malignancies are additional comorbidities posing significant risks for RA patients. Many of these conditions can result from treatment, environmental risk factors, or chronic inflammation (Dougados et al. 2014; Symmons and Gabriel 2011). As a result, RA is associated with premature death as patient lifespan can be

decreased by 10 to 15 years (van den Hoek et al., 2017). However, this is heavily influenced by disease severity and disability (Alamanos et al., 2006; Brooks, 2006). Effective control over disease activity with modern therapies has decreased mortality rates substantially (Listing et al., 2015).

1.6.5 Prognostic Markers of RA

Rheumatoid factor (RF), an Ab specific for the Fc portion of IgG, is a serological marker for RA (Mc Ardle et al., 2015). It is associated with a more severe disease with extra-articular manifestations and significant joint damage (Nell et al., 2005; Scott et al., 2013; Syversen et al., 2008). However, RF is also present in the serum of patients with other autoimmune diseases and so it lacks specificity for RA (Ingegnoli et al., 2013).

More recently, ACPAs were found to be a more specific serological marker for RA (Whiting et al., 2010). ACPA-positive patients also have a more aggressive disease and more significant joint damage (Lindqvist et al., 2005; Robinson et al., 2013). ACPAs often appear prior to disease onset and they remain stable over time. These also do not appear in the healthy individuals or in people with other autoimmune disease and so they are a better prognostic marker than RF (Forslind et al., 2004; Kastbom et al., 2004; Rönnelid et al. 2005).

Magnetic resonance imaging (MRI) and ultrasound are used to assess radiological outcomes. Power Doppler assessment of synovial inflammation using ultrasound has also been found to predict radiographic progression in both early RA and established RA patients (Freeston et al., 2010). The presence of bone marrow edema detected through MRI in early RA is predictive of subsequent joint damage (Haavardsholm et al., 2008; Hetland et al., 2009; Palosaari et al., 2006).

In addition to accounting for approximately 40% of disease heritability, HLA-DRB1 alleles have been found to have a reproducible association with worse disease outcome for RA patients (Gonzalez-Gay et al., 2002).

1.7 Pathogenesis of RA

The pathogenesis of RA is very complex (Catrina et al., 2011; Firstein and McInnes, 2017; McInnes and Schett, 2011; Smolen et al., 2018; Smolen et al., 2016). As was previously mentioned, GWAS studies have identified many genetic loci that are involved in disease pathogenesis (Okada et al., 2014). The strongest genetic link was made between HLA-DRB1 alleles, which are implicated in T-cell recognition of autoreactive peptides (Okada et al., 2014). Genetic links between post-translational modification enzymes such as PADI and cell signalling pathways are also important and are thought to alter immune checkpoints (Okada et al., 2014).

Environmental exposures such as smoking and silica dust operate on this genetic background to promote disease (Catrina et al., 2016; Källberg et al., 2011). A lack of diversity in bacteria in the gastrointestinal tract could also cause long-term effects on immune regulation and tolerance, and *P. gingivalis* or Aa in the oral mucosa have been proposed to promote disease by affecting host protein citrullination (Konig et al., 2016; Scher et al., 2013).

In early RA there is a breach in tolerance, which most likely occurs at mucosal sites. This results in the production of auto-Abs that recognise a range of post-translationally modified proteins that have either been citrullinated, carbamylated, or acetylated (Trouw et al., 2017).

The process in which this autoreactivity leads to the severe infiltration of immune cells into the joints is poorly understood. Articular localisation might arise from trauma to the joints or to the blood vessels surrounding the joints. Another theory involves the activation of periarticular osteoclasts by circulating auto-Abs, which results in enhanced bone resorption (Harre et al., 2012; Krishnamurthy et al., 2016). These osteoclasts can release TNF- α and IL-8 that recruit immune cells to the joints and promotes inflammation (Harre et al., 2012; Krishnamurthy et al., 2016).

Once the synovial lesion is established it contains large numbers of infiltrating immune cells and aggressive synovial fibroblasts that eventually form a pannus, which is an abnormal layer of fibrovascular tissue that covers the joints and leads to cartilage and bone erosion (Zvaifler and Firestein, 1994). The interplay between cells, cytokines and chemokines that influence this disease process are very complex and will be described in the sections below.

1.7.1 APCs in RA

Monocytes and macrophages are found in the synovial membrane of patients with RA (Burmester et al., 1983). Macrophage colony-stimulating factor, (M-CSF) causes these cells to mature and exit the bone marrow and then to traffic into the synovium (Cornish et al., 2009). Macrophages are central mediators of synovitis by releasing inflammatory cytokines such as TNF, IL-1, and IL-6 and reactive oxygen species (ROS) and matrix degrading enzymes (Kinne et al., 2007). Macrophages in the RA synovium are polarized to an M1 phenotype (Quero et al., 2017).

It has also been shown that there is a skewing of M1/M2 monocytes in ACPA-positive RA patients and that this phenomenon enhances osteoclastogenesis (Fukui et al., 2017; Okamoto et al., 2017). Proinflammatory cytokines such as TNF, IL-6, IL-1 β , and IL-17 that polarize monocytes into an M1 phenotype can promote the differentiation of these monocytes into osteoclasts (Pettit et al., 2006). ACPA can also enhance NF-kB activity and TNF- α production in monocytes and macrophages by binding surface-expressed citrullinated proteins (Lu et al., 2010).

The accumulation of DCs in the articular cavity has also been reported (Zvaifler et al., 1985). It has been proposed that these DCs promote T cell activation and differentiation however, little research has gone into investigating this cell type.

1.7.2 Neutrophils and Mast Cells in RA

Neutrophils are found in the synovial fluid of RA patients (Quayle et al., 1997). They are thought to be involved in both the triggering of arthritis and in the promotion of inflammation by releasing prostaglandins, matrix degrading enzymes, and ROS (Cascão et al., 2010). It has been proposed that infectious agents such as aA and *P. gingivalis*, which cause periodontal disease, work together with neutrophils to trigger arthritis. Pathogen aA can secrete leukotoxin A that forms pores in neutrophil membranes, which leads to the release of citrullinated autoantigens at mucosal sites that perpetuates auto-Ab production (Konig et al., 2016). The PADI of *P*.

gingivalis can citrullinate host proteins and induce ACPA production. In turn, ACPAs can induce neutrophil extracellular trap (NET) formation (NETosis) which perpetuates the provision of citrullinated autoantigens (Khandpur et al., 2013; Wegner et al., 2010).

Mast cells can produce high levels of vasoactive amines, cytokines, chemokines, and proteases, through FcγR activation upon engagement with ICs or ACPAs (Hueber et al., 2010; Nigrovic and Lee, 2007; Suurmond et al., 2015).

1.7.3 Osteoclasts in RA

Bone erosion happens in RA as a result of increased osteoclastogenesis that can be induced by IC or ACPA engagement on Fc γ Rs expressed on osteoclasts (Harre et al., 2012). Cytokines such as M-CSF, TNF- α , IL-1, IL-6 and IL-17 can also promote osteoclast differentiation and activation and can promote bone resorption through the increased expression of receptor activator of NF- κ B ligand (RANKL) (Gravallese et al., 1998; Schett et al., 2008; Schett and Teitelbaum, 2009). When osteoclasts are activated in the joint, they can degrade cartilage and bone. Destruction of these tissues leads to deep resorption pits, which are filled by inflammatory cells that eventually leads to permanent joint damage and deformity (Karmakar et al., 2010).

1.7.4 Synovial Fibroblasts in RA

The synovium is made up of bone marrow-derived macrophages and synovial fibroblasts (FLSs) (Edwards, 1994). FLSs maintain homeostasis in the joint by secreting hyaluronic acid and lubricin for joint lubrication and load bearing function. In RA, the dysfunction of FLSs leads to a hyperplastic synovium, which eventually leads to the formation of the pannus (Zvaifler and Firestein, 1994). FLSs gain an aggressive phenotype, which is thought to be caused by aberrant DNA methylation patterns (Ai et al., 2016; Pap et al., 2000). It is not clear how this happens or if it happens inside or outside of the joint. This aggressive phenotype causes them to proliferate, lose their contact inhibition, release inflammatory cytokines and matrix metalloproteinases, which all cause joint destruction (Muller-Ladner et al., 1997; Filer et al., 2013). These FLSs

secrete cytokines and chemokines into the joint microenvironment to promote their survival and the accumulation of immune cells (Filer et al., 2006). Additionally, FLSs have been shown to alter their gene expression such that they become resistant to apoptosis, which also contributes to their survival during pannus formation (Aupperle et al., 1998; Schett et al., 1998).

1.7.5 Tumour Necrosis Factor (TNF) in RA

The role of TNF in RA has been elucidated with TNF inhibitors, which were initiated as a therapy because of prior studies that showed that TNF had a pro-inflammatory role in leukocyte activation, matrix metalloproteinase production, angiogenesis and pain (Charles et al., 1999; Feldmann and Maini, 2001; McInnes and Schett, 2007). Some of the common TNF inhibitors are molecules or monoclonal Abs that bind and sequester TNF such as, etanercept, infliximab, and adalimumab (Feldmann, 2002). Early studies used joint tissue biopsies from RA patients that were given TNF inhibitors to show that they influenced synovial fibroblast activation, angiogenesis, cytokine and chemokine expression, and activation of regulatory pathways (Buch et al., 2008; Cañete et al., 2009; Gerlag and Tak, 2008; Herenius et al., 2011; Izquierdo et al., 2004; Klaasen et al., 2009; Lindberg et al., 2010; Tak et al., 1996). Transcriptional and metabolomic studies have revealed that TNF also plays a major role in bone metabolism (Kapoor et al., 2013; Siebert et al., 2017). Many clinical trials suggest that TNF has a central role in RA pathogenesis due to its pleiotropic effects (Lipsky et al., 2000; Moreland et al., 1999; Van Vollenhoven et al., 2012).

1.7.6 Interleukin (IL)-6 in RA

Similar studies were done with IL-6 inhibitors in RA. Many research groups have focused on the transcriptional changes that are mediated upon inhibition of IL-6 receptor with tocilizumab, the most common monoclonal Ab targeting IL-6 receptor (Scott, 2017; Smolet et al., 2008). Various studies have identified that IL-6 and IFN signalling pathways in peripheral blood may operate in parallel in RA (Sanayama et al., 2014). Studies of the effects of IL-6 in the synovium are less understood however, inhibition of IL-6 receptor function has been shown to reduce the expression of T-cell activation markers and chemokines (Ducreux et al., 2014). The concentration of IL-6 in the synovium has been shown to be an important marker of inflammation in patients treated with IL-6 receptor inhibitors because it correlates with responses to IL-6 receptor inhibition (Wright et al., 2015). These studies suggest that IL-6 can regulate T cell activation and migration and various inflammatory signalling pathways in RA.

1.7.7 T Cells in RA

The role of T cells in the disease pathogenesis of RA is controversial (McInnes and Schett, 2011). Even though there are strong genetic links with HLA-DRB1 alleles costimulatory pathways in patients with RA, the depletion or functional modification of T cells using ciclosporin, anti-CD4Abs, anti-CD5Abs, or alemtuzumab, a monoclonal Ab that binds to CD52 on T cells, have not shown therapeutic efficacy in clinical trials (Keystone, 2002). However, abatacept, a co-stimulation inhibitor of T cells that binds CTLA-4, has renewed interest in targeting T cells in RA. Robust clinical responses that were like those achieved with TNF inhibitors, were achieved with abatacept (Smolen et al., 2016). Abatacept reduced interactions between T cells and APCs, and it reduced inflammatory cytokine production and osteoclastogenesis (Buch et al., 2009; Choy, 2009; Cutolo et al., 2016; Kanbe et al., 2013; Wenink et al., 2012). Joint tissue biopsies from RA patients have shown that abatacept reduces the cellular infiltration of B cells, which highlights the cross-talk between synovial B and T cells (Gazeau et al., 2017; Scarsi et al., 2014).

1.7.8 B Cells in RA

The involvement of B cells in RA pathogenesis is strongly suggested by the auto-Ab response that produce ACPAs and RF. ACPAs are Abs directed against citrullinated proteins and RF is an Ab directed against the Fc portion of IgG (Derksen et al., 2017). The formation of these auto-Abs is associated with both genetic and environmental risk factors for RA, such as the SE and smoking (Derksen et al., 2017). These auto-Abs can be detected many years before disease onset in some patients and they also have an important role in perpetuating inflammation (Derksen et al., 2017). Recent studies have shown that the presence of ACPAs have been linked to severe bone erosions in RA through its effects on osteoclasts (Harre et al., 2012). RF seems to be less specific to RA and is found in other systemic autoimmune diseases such as SLE and mixed connective tissue disease (Ingegnoli et al., 2013). Findings from biopsy studies have shown that B cells can form ectopic lymphoid follicles in the joints of RA patients for a local source of auto-Abs (McInnes and Schett, 2011).

Rituximab is a B cell depleting Ab that targets CD20 on B cells, but it has no effect on plasma cells (Ab-secreting cells) because plasma cells do not express CD20 (Shaw et al., 2003). Studies with Rituximab suggest that it works best in seropositive patients (Chatzidionysiou et al., 2007). Although it reduces auto-Ab titers, its anti-inflammatory effect occurs before this reduction and its is likely to work through an alternative mechanism that is involved in T and B cell cross-talk (Cohen and Keystone, 2015). Rituximab has been shown to affect cytokine and chemokine production by B cells and synovial biopsies have shown that it also affects macrophages and T cells (Bresnihan et al., 2017; Das et al., 2014; Gutierrez-Roelens et al., 2011; Kavanaugh et al., 2008; Lund, 2008; Rosengren et al., 2011; Rosengren et al., 2008; Teng et al., 2009; Thurlings et al., 2010; Walsh et al., 2008; Wunderlich et al., 2017).

1.8 Treatments for RA

The treat-to-target strategy is used to treat RA, whereby the regular assessment of disease activity drives disease management in order to achieve treatment targets and optimize patient outcomes (Grigor et al., 2004; Klarenbeek et al., 2011; Smolen, 2016; Smolen et al., 2016;

Verstappen et al., 2007). The goal of this strategy is to achieve disease remission or very low disease activity. Disease remission prevents joint damage and the progression of joint damage if it has already occurred. Any new treatment should convey at least a 50% improvement in disease activity within 3 months, and the treatment target should be reached within the subsequent 3 months (Aletaha et al., 2006; Smolen et al., 2010). At this point, the treating physician should consider tapering therapeutics. If this does not occur, the treatment is reassessed and adjusted accordingly. Even though this strategy has been recommended by the ACR, EURLAR and the Asia Pacific League of Associations for Rheumatology, it is rarely implemented by physicians (Lau et al., 2015; Singh et al., 2015; Smolen et al., 2016). Physicians struggle to implement this strategy because time and resources are constrained, and patient adherence is low (Aletaha et al., 2006; Kuusalo et al., 2015; Pascual-Ramos et al., 2009; Schoenthaler et al., 2012; Smolen et al., 2010).

1.8.1 Disease Modifying Anti-Rheumatic Drugs (DMARDs)

DMARDs are required to slow the progression of and interfere with the inflammatory process involved in RA (Smolen and Steiner, 2003). Synthetic DMARDs are small chemical drugs that can be further classified as conventional synthetic DMARDs, and targeted synthetic DMARDs. The mode(s) of action of conventional synthetic DMARDs are generally unknown, whereas targeted synthetic DMARDs target specific molecules within cells (Smolen and Steiner, 2003).

Some of the common conventional synthetic DMARDs are methotrexate, sulfasalazine, hydroxychloroquine and leflunomide. Some of the common targeted synthetic DMARDs are Janus Kinase (JAK) inhibitors, such as baricitinib, which inhibits JAK 1 and JAK 2 and tofacitinib, which inhibits JAK 1, JAK 2 and JAK 3.

Biological DMARDs are usually monoclonal antibodies that target soluble extracellular and cell membrane-associated proteins with high specificity (Smolen and Steiner, 2003). Some of the common biological DMARDs are adalimumab, which targets TNF, tocilizumab, which targets IL-6 receptor, abatacept, which targets CD80 and CD86 on T cells, and rituximab, which targets CD20 on B cells.

Although conventional synthetic DMARDs are generally well tolerated, some of the common adverse effects associated with them are, nausea, vomiting, abdominal pain, increase in liver enzymes, oral ulcers, and leukocytopenia (Ramiro et al., 2014; Ramiro et al., 2017; Ruderman et al., 2012).

Biological DMARDs have a good benefit-to-risk profile, however there is a higher risk of developing infections. This risk is influenced by smoking, concomitant glucocorticoid treatment, age and comorbidities (Burmester et al., 2013a). There are more serious adverse events associated with biological DMARDs such as, an increased risk of tuberculosis reactivation with TNF inhibitors, a risk of gastrointestinal perforations with tocilizumab and, very rarely, an increased risk of progressive multifocal leukoencephalopathy with rituximab treatment (Burmester et al., 2013b; Molloy et al., 2017; Strangfeld et al., 2017).

JAK inhibitors have a similar adverse effect profile as biological DMARDs, except for an increased risk of herpes zoster virus reactivation in certain populations (Dhillon, 2017; Kubo et al., 2016; Ramiro et al., 2014; Ramiro et al., 2017; Winthrop, 2017).

1.8.2 Glucocorticoids and Nonsteroidal Anti-inflammatory Drugs

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used in RA to relieve pain and swelling by inhibiting prostaglandin synthesis by cyclooxygenase enzymes (Smolen et al., 2016). However, they cannot prevent or inhibit joint damage. Some of the common NSAIDs are ibuprofen and naproxen, which are very safe with gastrointestinal events being the most common adverse effect (Crofford, 2013; Scheiman, 2016).

Glucocorticoids have disease-modifying activity, but their adverse effects preclude long-term use. Glucocorticoids reduce inflammation rapidly, so they are usually given for a limited period together with conventional synthetic DMARDs until the latter have exerted their effects (Smolen et al., 2016). Some of the common glucocorticoids are prednisone and prednisolone. The long-term use of glucocorticoids can have a broad spectrum of serious adverse effects, such as skin atrophy, osteoporosis, disturbed glucose tolerance, hypertension, elevated intraocular pressure, cataract development and a higher risk of infections (Strehl et al., 2016). Thus, glucocorticoids

are usually given at moderate doses in the initial treatment phase with rapid tapering or later when flares occur at the lowest possible dose for the shortest time period possible.

1.8.3 First Line Therapies for RA

The first line therapy for RA is treatment with methotrexate plus short-term glucocorticoids (Smolen et al., 2014; Smolen et al., 2017). Methotrexate should be rapidly escalated to the optimal dose (25mg once weekly) with folic acid (1 mg daily) to prevent side effects associated with folic acid deficiency.

Glucocorticoids should be given orally at a low dose for a maximum of 6 months until methotrexate exhibits its effects Alternatively, higher doses of glucocorticoids can be administered intramuscularly or intravenously at the start of methotrexate treatment or during a disease flare-up (van der Goes et al., 2010; Verschueren et al., 2015). A higher dose of oral glucocorticoids can be used for patients with a more severe or progressive disease (Verschueren et al., 2015). Glucocorticoids must be tapered or stopped within the first 6 months to prevent adverse effects associated with long-term glucocorticoid use (del Rincón et al., 2014; Pincus et al., 2013; Smolen et al., 2016).

The combination of methotrexate plus glucocorticoids is the standard first line therapy for RA patients because it leads to remission in ~25% of patients within 6 months. Additionally, this therapy gives additive structural protection when compared with methotrexate alone (Chatzidionysiou and Sfikakis, 2019; Wassenberg et al., 2005). Also, studies have shown that there is no added advantage to combining this therapy with one or two other conventional synthetic DMARDs (de Jong et al., 2014; Verschueren et al., 2015).

When the use of methotrexate is precluded, alternative conventional synthetic DMARDs can be used, however methotrexate has shown to optimize the efficacy of biological DMARDs (Singh et al., 2015; Smolen et al., 2013).

1.8.4 Second Line Therapies for RA

Patients who do not enter remission after first line therapies are treated differently depending on their prognostic factors such as, auto-Ab presence, high disease activity, or early radiographic joint damage (Smolen et al., 2014; Smolen et al., 2017). Those without prognostic factors should receive another conventional synthetic DMARD as monotherapy, again with a short course of glucocorticoids (Smolen et al., 2006). Those who have adverse prognostic markers or who have failed two courses with conventional synthetic DMARDs should receive a biological DMARD (Smolen et al., 2014; Smolen et al., 2017). If that fails, they should receive a targeted synthetic DMARD (Kiely et al., 2011).

1.8.5 Non-responders

Primary non-responders are patients who never showed an adequate response to a therapy and secondary non-responders are patients whose response to treatment diminished (Emery et al., 2015; Smolen et al., 2016). In non-responders, another biological DMARD or a targeted synthetic DMARD is recommended, although the response rate generally decreases with multiple drug exposures. For patients who are non-responders to biological DMARDs, they are more likely to respond to targeted synthetic DMARDs (Smolen et al., 2016). Interestingly, it has been shown that secondary non-responders to TNF inhibitors respond well to molecules with the same mode of action but different immunogenicity (Emery et al., 2015).

1.9 Animal Models of RA

Animal models have been used extensively in studies of RA pathogenesis. Several mouse models have progressed our understanding of the immunopathogenesis of RA and have contributed to the development of several therapies. These models include the induced arthritis models such as CIA and CAIA, and spontaneous arthritis models such as the TNF- α -transgenic mouse model, IL-1 receptor antagonist KO mouse model, and the K/BxN serum transfer mouse model. These mouse models will be described in detail below.

1.9.1 Induced Models of RA

The CIA mouse model is the gold standard *in vivo* model for RA because its pathological features highly resemble those in humans (Asquith et al., 2009; Holmdahl et al., 2002; Kannan et al., 2005; Myers et al., 1997; Trentham et al., 1977). CIA is a form of polyarthritis with synovial inflammation, cartilage destruction and bone erosion (Brand et al., 2003; Bevaart et al., 2010; Holmdahl et al., 2002). In CIA, susceptible mouse strains such as DBA/1, B10.Q and B10.RIII DBA/1, are immunized against type II collagen (CII), usually chick, in complete Freund's adjuvant (Brand et al., 2007; Inglis et al., 2008; Rosloniec et al., 2010; Trentham et al., 1977). The clinical signs of arthritis usually appear 3-5 weeks after immunization (Brand et al., 2007; Inglis et al., 2008). The pathogenesis of CIA is dominated by Th1 and Th17 cells providing B cell help such that auto-Abs to CII are made (Mauri et al., 1996; McNamee et al., 2015; Svensson et al., 1998).

The CAIA mouse model represents the effector phase of arthritis (Mitamura et al., 2007; Nandakumar et al., 2004). A cocktail of anti-CII monoclonal Abs are administered to induce arthritis in mice (Nandakumar and Holmdahl, 2006; Stuart and Dixon, 1983). Lipopolysaccharide (LPS) is used to enhance the incidence and severity of the arthritis. There are many susceptible mouse strains that can be used for this model and the clinical signs of arthritis generally appears a few days after Ab administration (Khachigian, 2003; Stuart and Dixon, 1983). ICs initiate inflammation by activating the classical complement pathway or by engaging FcγRs on immune cells (Banda et al., 2012; Banda et al., 2009; Banda et al., 2007; Hietala et al., 2004; Holmdahl et al., 1986; Kagari et al., 2003; Mougdil et al., 2011; Nandakumar et al., 2005; Rowley et al., 2008).

1.9.2 Spontaneous Models of RA

Human TNF-α-transgenic mice spontaneously develop arthritis (Keffer et al., 1991; Li and Schwartz, 2003). After 3–4 weeks of age, synovial hyperplasia and inflammatory cell infiltrates are observed in the joint and at around 10 weeks of age the mice exhibit pannus formation, cartilage destruction and bone erosion (Li and Schwartz, 2003). FLSs are the main drivers of

arthritis in this model, rather than immune cells (Aidinis et al., 2003). This model showed researchers the clinical significance of TNF in the pathogenesis of arthritis and was instrumental in the development of TNF inhibitors (Keffer et al., 1991; Williams et al., 2005).

IL-1 receptor antagonist KO mice also develop spontaneous arthritis. Interestingly, these mice generate antibodies against CII, double stranded DNA and IgM RF (Horai et al., 1998; Horai et al., 2000). After 5 weeks of age, synovial inflammation ensues and at 8 weeks of age mice experience higher rates of morbidity. Given the fact the anti-IL-1 β therapies such as Anakinra, a molecule targeting the human IL-1 receptor antagonist, have only worked in a minor subset of RA patients, this mouse model is not commonly used to study this disease (Guo et al., 2018).

1.9.3 K/BxN Serum Transfer Model of RA

In 1996, the K/BxN serum transfer model of arthritis was discovered by the Mathis and Benoist laboratory (Kouskoff et al., 1996). The model was a product of serendipity whereby they crossed the T cell receptor (TCR) transgenic KRN mice (on a C57BL/6 background) that recognize a bovine ribonuclease peptide (RNase 43–56) presented by I-Ak MHC class II molecule with non-obese diabetic (NOD) mice. To their surprise, the F1 generation, called K/BxN mice, developed severe arthritis at 4-5 weeks of age (Kouskoff et al., 1996).

Arthritis progression in K/BxN mice is driven by the activation of T cells expressing the KRN TCR that recognizes a self-peptide bound to the NOD-derived MHC class II I-Ag7 molecule on APCs. The peptide recognized by the K/BxN TCR is glucose-6-phosphate isomerase (G6PI) (Matsumoto et al., 1999), a cytosolic glycolytic enzyme catalyzing the inter-conversion of d-glucose-6-phosphate to d-fructose6-phosphate (Ditzel, 2004). Pathogenic auto-Abs to G6PI are created when activated T cells give help tom B cells through CD40:CD40-ligand (L) engagement (Ditzel, 2004; Korganow et al., 1999; Kouskoff et al., 1996; Mangialaio et al., 1999).

The serum from K/BxN mice contain anti-G6PI auto-Abs and thus can be transferred to a variety of mouse strains, including B and T cell deficient mice, to induce arthritis (Ji et al., 2001; Korganow et al., 1999). This arthritis lasts for ~2-4 weeks however, repeated administration of the serum can prolong the arthritis (Korganow et al., 1999; Monach et al., 2007a; Monach et al.,

2007b). Since its discovery in 1996, this model has been used extensively to dissect several important effector pathways of arthritis *in vivo* and these will be discussed in significant detail in the sections outlines below.

1.9.3.1 Immune Complexes (ICs) in the K/BxN Serum Transfer Model

G6PI is present in the cytoplasm of cells and in the circulation. It can also be found on the surface of articular cartilage through noncovalent interactions (Matsumoto et al., 2002). It has been hypothesized that anti-G6PI Abs bind directly to extracellular G6PI and subsequently form ICs on the articular cartilage surface (Corr et al., 2002; Maccioni et al., 2002; Wipke et al., 2004). Anti-G6PI Abs from serum have been shown to localize specifically to the distal joints in the front paws and hind ankles, where they can remain for up to 24 hours (Wipke et al., 2002). Importantly, it has been hypothesized that ICs in the serum increase vascular permeability specifically in the distal joints through activation of $Fc\gamma RIII$ on neutrophils (Bindstadt et al., 2006; Nimmerjahn et al., 2007). Neutrophils release vasoactive mediators upon activation that increase the vascular permeability of the joints to allow ICs and other Abs to enter the joint space. Mast cells can also engage ICs with their $Fc\gamma Rs$, causing them to degranulate, which has been hypothesized to perpetuate this cycle (Bindstadt et al., 2006). Once ICs and anti-G6PI Abs are in the joint space, they can bind to G6PI present on the articular cartilage surface to trigger an inflammatory cascade. The initiation of arthritis in the K/BxN serum transfer model is further illustrated in **Figure 1.3**.

Figure 1.3 The Initiation Phase of the K/BxN Serum Transfer Model.

Adapted from Christensen, A.D., Haase, C., Cook, A.D., and Hamilton, J.A. (2016). K/BxN Serum-Transfer Arthritis as a Model for Human Inflammatory Arthritis. Frontiers in immunology 7, 213. (Christensen et al., 2016). © 2016 Christensen, Haase, Cook and Hamilton. https://www.frontiersin.org/articles/10.3389/fimmu.2016.00213/full https://creativecommons.org/licenses/by/4.0/


A summary of some of the literature on the early stages of K/BxN serum transfer progression, leading to the formation of anti-G6PI-G6PI ICs in the joint is depicted. (1) In the blood, the anti-G6PI Abs bind to G6PI and form ICs. (2) On neutrophils, the ICs bind to FcγRs triggering the release of vasoactive mediators and the local increase in vascular permeability, thus allowing the ICs and anti-G6PI Abs to enter the perivascular tissue in the joint. (3) In the perivascular tissue, the ICs bind to FcγRs on mast cells causing them to degranulate, resulting in enhances vascular permeability. (4) ICs, anti-G6PI Abs, nonspecific Abs and serum proteins enter the joint cavity, where (5) anti-G6PI Abs bind to G6PI expressed on the cartilage surface.

1.9.3.2 FcyRs in the K/BxN Serum Transfer Model

FcγRs are the link between Abs and the innate immune system in the K/BxN serum transfer model (Corr et al., 2002). The role of the different FcγRs in the K/BxN serum transfer model was explored using KO mice. FcγR KO mice were protected from arthritis, demonstrating the crucial role of FcγRs in this model (Ji et al., 2002). More specifically, FcγRI KO mice developed arthritis normally, whereas FcγRIII KO mice developed reduced arthritis with delayed onset (Boross et al., 2008; Corr et al., 2002; Ji et al., 2002). This is likely because FcγRIII engagement with ICs on neutrophils and mast cells is required for the release of inflammatory cytokines, ROS, and vasoactive amines, which play an important role in inducing arthritis (Ravetch and Bolland, 2001).

The importance of FcγRIV was demonstrated by showing that the blockade of FcγRIV with monocloncal Abs prevented the development of arthritis (Seeling et al., 2013). Additionally, it has been demonstrated that mice with a specific deletion of FcγRIV on osteoclasts were protected from arthritis and bone erosion induced by K/BxN serum (Mancardi et al., 2011; Negishi-Koga et al., 2015; Seeling et al., 2013). FcγRIIB, the only inhibitory FcγR, was shown to have an immunosuppressive role in this model as FcγRIIB KO mice experienced enhanced disease progression after transfer with K/BxN serum (Boross et al., 2008; Bruhns et al., 2003; Corr et al., 2002).

1.9.3.3 Neutrophils in the K/BxN Serum Transfer Model

Neutrophils are arguably one of the most important effector cells in the induction and in the continuation of arthritis in the K/BxN serum transfer model. This was demonstrated by showing that mice depleted of neutrophils did not develop arthritis after serum transfer and mice depleted of neutrophils after arthritis onset had their disease reversed (Wipke et al., 2001). It was proposed that arthritis was prevented in these mice by blocking neutrophil migration into the joint (Wang et al., 2012). The importance of neutrophils was further supported by the fact that

Gfi-1 KO mice, mice without mature neutrophils, were resistant to the development of arthritis (Monach et al., 2010).

Leukotriene B4 (LTB4) and its receptor, leukotriene B4 receptor 1 (BLT1), were important in elucidating the role of neutrophils in the K/BxN serum transfer model. In response to LTB4 binding to BLT1, neutrophil chemotaxis is induced (Haribabu et al., 2000; Sezin et al., 2017; Tager et al., 2000). It was suggested that neutrophils recruit other neutrophils to the joints in an autocrine manner through the LTB4-BLT1 axis (Chen et al., 2006; Kim et al., 2006). Additionally, the release of LTB4 from neutrophils is dependent on the engagement of complement component 5a receptor (C5aR) with C5a (Sadik et al., 2012).

IL-1 β is apro-inflammatory cytokine released by neutrophils and it promotes cartilage destruction and the release of neutrophil-activating chemokines such as CXC chemokine L1 (CXCL1), CXCL5 and CC chemokine L9 (CCL9) from endothelial cells in the synovium (Chou et al., 2010; Jacobs et al., 2010; Ritzman et al., 2010). Sadik et al., also showed that Fc γ R engagement with ICs is necessary for the release of IL-1 β from neutrophils (Sadik et al., 2012). This work was corroborated by other studies that have shown that the deletion of spleen tyrosine kinase (Syk), which is phosphorylated to induce Fc γ R activation, in neutrophils was able to prevent arthritis upon serum transfer (Elliott et al., 2011; Németh et al., 2018).

It is important to note that the activation of C5aR and FcγRs occur independently from one another. This phenomenon is unique to the K/BxN serum transfer model because other models of auto-Ab induced inflammation generally require sensitization of immune cells with C5a to promote FcγR activation (Nigrovic et al., 2010; Shushakova et al., 2002; Tusuboi et al., 2011).

1.9.3.4 Monocytes and Macrophages in the K/BxN Serum Transfer Model

Macrophages are also a major player in this model of arthritis. Depletion studies in the K/BxN serum transfer model have shown that arthritis does not occur without macrophages and that when macrophages are reconstituted to macrophage depleted mice, arthritis ensues (Solomon et al., 2005). The most important study that has elucidated the role of macrophages in the K/BxN serum transfer model was done by Misharin et al. They showed that non-classical (Ly6C⁻)

monocytes were recruited to the joint when synovial inflammation occurred, where they gave rise to M1 macrophages that initiated arthritis (Misharin et al., 2014). Additionally, there were tissue-resident M2 macrophages in the joint that maintained joint integrity by resolving inflammation (Misharin et al., 2014). They also showed that macrophages that were originally recruited to the joints expressed both M1 and M2 genes however, there was a shift towards an M2 phenotype where they, together with the tissue-resident macrophages, resolved the arthritis (Misharin et al., 2014; Seeling et al., 2013). Taken together, this data suggests that circulating non-classical monocytes are recruited to the joint in the initiation phase of arthritis, where they are involved in both the development and resolution of arthritis.

1.9.3.5 Mast Cells in the K/BxN Serum Transfer Model

The role of mast cells in the K/BxN serum transfer model is controversial. The first studies done to investigate mast cells in the K/BxN serum transfer model were done in mice genetically deficient in mast cells (Feyerabend et al., 2011). Serum from K/BxN mice was transferred into two different strains of mast cell deficient mice and both strains displayed no signs of arthritis and the transfer of mast cells into these strains restored their arthritis (Lee et al., 2002). However more recently, it was proposed that mast cells may be dispensable for the development of arthritis. Another mast cell deficient strain induced by the Cre-Lox system were not resistant to arthritis (Feyerabend et al., 2011).

It was also shown that mast cells could be activated by ICs through $Fc\gamma RIII$, which led to release of IL-1 β and tryptase/heparin complexes, which were shown to induce the expression of the neutrophil chemoattractants in cultured FLSs (Nigrovic et al., 2007; Shin et al., 2009). These studies were corroborated when arthritis was suppressed by preventing the degranulation of mast cells with salbutamol, a common vasodilator (Kneilling et al., 2007).

1.9.3.6 Natural Killer Cells in the K/BxN Serum Transfer Model

A role for natural killer (NK) cells in the K/BxN serum transfer model was suggested by the observation that arthritis was attenuated in two strains of NK cell deficient mice (Kim et al., 2006). Furthermore, when these mice were reconstituted with NK cells from WT mice but not from $Fc\gamma R$ KO mice, arthritis was restored (Kim et al., 2006). This study reported that NK cells may also engage ICs through $Fc\gamma RIII$ to promote arthritis (Kim et al., 2006).

1.9.3.7 Complement in the K/BxN Serum Transfer Model

The activation of complement is a key driver of arthritis in the K/BxN serum transfer model. It was discovered using mice deficient in complement proteins that are unique to each pathway that the alternative pathway is critical for the development of arthritis (Binstadt et al., 2009; Ji et al., 2002; Solomon et al., 2002). The alternative pathway is activated by ICs, which leads to the production of C3 and deposition of C3 on arthritic joints to drive inflammation. (Binstadt et al., 2009; Ji et al., 2009; Ji et al., 2009; Ji et al., 2002; Monach et al., 2007). Although the importance of C3 in this model has been demonstrated, more recent studies have shown that C5, the cleavage product of C3, is the central mediator of arthritis in this model (Auger et al., 2012; Hobday et al., 2014).

The importance of C5 in this model was established using C5 KO mice, which were resistant to arthritis (Ji et al., 2002). In a complementary study, similar results were obtained with an anti-C5 monoclonal Ab that both prevented and treated arthritis (Ji et al., 2002). C5 is cleaved by C5 convertase to produce the potent anaphylatoxin, C5a. Again, C5a and its receptor were necessary for arthritis development as mice deficient in both components were resistant to arthritis (Ji et al., 2002). C5a functions as a chemoattractant and it is proposed to activate neutrophils to release chemokines and increase vascular permeability in the joint space (Ditzel et al., 2004).

1.9.3.8 Proinflammatory Cytokines in the K/BxN Serum Transfer Model

The role of cytokines in the K/BxN serum transfer model has been elucidated by using mice that were deficient in TNF, IL-1 and IL-6, or their receptors. Mice deficient in TNF were not resistant to arthritis (Ditzel et al., 2004; Ji et al., 2002b; Kyburz et al., 2000). IL-6 was also shown to be completely indispensable in this model (Ji et al., 2002b; Kyburz et al., 2000; Lamacchia et al., 2012).

IL-1 receptor deficient mice, however, were resistant to disease, demonstrating a critical role for IL-1 α and or β (Ji et al., 2002b;). IL-1 receptor antagonist could be used as a treatment for arthritis in K/BxN mice as their inflammation was resolved upon treatment (Choe et al., 2003; Martin et al., 2017).

The IL-17 family of cytokines have also been implicated in RA and in many other autoimmune diseases (Corr et al., 2011; Gaffen, 2009; Kuwabara et al., 2017; Lubberts, 2015; Martin et al., 2013; Tabarkiewicz et al., 2015; Yang et al., 2014). The roles of these cytokines have been investigated in the K/BxN serum transfer model however, the results are controversial. First, it was shown that administration of an anti-IL-17A monoclonal Ab did not exert any effect on arthritis progression in the K/BxN serum transfer model (Pfeifle et al., 2017). Then it was shown that mice deficient in IL-17 receptor subunit A were protected from the serum-induced arthritis, and this study was confirmed by results that showed that IL-17A KO mice exhibited reduced arthritis (Jacobs et al., 2009).

More recently, the focus has shifted to the gut microbiota suggesting that it regulates arthritis progression independent of Th17 cells, which are major producers of IL-17, because antibiotic treatment inhibited arthritis in IL-17–deficient mice (Block et al., 2016). However, a different study showed that the opposite was true and that in fact neutralization of IL-17 prevented arthritis development in specific-pathogen-free K/BxN mice resulting from a direct effect of this cytokine on B cells to inhibit germinal center formation and auto-Ab production (Wu et al., 2010).

1.9.3.9 Cartilage Loss and Bone Erosion in the K/BxN Serum Transfer Model

IL-1β is the primary driver of cartilage loss in the K/BxN serum transfer model. This was first described in IL-receptor KO mice, which were resistant to arthritis and showed no signs of cartilage destruction (Ji et al., 2002b; Joosten et al., 1999). Macrophage migration inhibitory factor (MIF) is also an important regulator of cartilage loss as MIF KO mice were resistant to arthritis and cartilage destruction in the K/BxN serum transfer model (Chen et al., 2014; Sing et al., 2013).

Osteoclasts are activated in the inflamed synovia and erode bone. Osteoclasts are activated via RANKL and it was shown that RANKL KO mice were protected from bone erosion after transfer of K/BxN serum (Pettit et al., 2001). Interestingly, a more recent study was published showing that TNF and IL-6 are capable of inducing RANKL-independent osteoclastogenesis through an alternative pathway. These TNF/IL-6 generated osteoclasts were able to resorb mineralized tissue *in vitro* and this osteoclastogenesis was directly influenced by IC engagement FcγRIV on osteoclasts (O'brien et al., 2016; Seeling et al., 2013).

In addition to cartilage destruction, MIF has also been shown to influence bone erosion. MIF KO mice transferred with arthritic K/BxN serum exhibited markedly reduced bone erosion in comparison to WT mice. It was also shown *in vitro* that MIF facilitated RANKL-induced osteoclastogenesis (Sing et al., 2013).

The complexities of the effector phase of the K/BxN serum transfer model are summarized in **Figure 1.4**.

Figure 1.4 The Effector Phase of the K/BxN Serum Transfer Model.

Adapted from Christensen, A.D., Haase, C., Cook, A.D., and Hamilton, J.A. (2016). K/BxN Serum-Transfer Arthritis as a Model for Human Inflammatory Arthritis. Frontiers in immunology 7, 213. (Christensen et al., 2016). © 2016 Christensen, Haase, Cook and Hamilton. https://www.frontiersin.org/articles/10.3389/fimmu.2016.00213/full https://creativecommons.org/licenses/by/4.0/



(1) The alternative complement pathway is activated by the ICs, leading to C3 cleavage and eventually to the generation of C5a. Subsequently, C5a activates neutrophils *via* C5aR, which leads to their release of LTB4. (2) Activation of neutrophils by the LTB4/BLT1 interaction and (3) by Fc γ Rs leads to the release of IL-1 β , which then induces neutrophilattracting chemokines, for example, CXCL1, CXCL5, and CCL9, from resident tissue cells. Additionally, neutrophils participate in their own recruitment by releasing the CXCL2 and to a lesser extent, CCL3. (4) IL-1 β , and other pro-inflammatory cytokines, mediates the release of G-CSF locally in the joint, leading to neutrophil mobilization. (5) Leukocyte, for example, neutrophil, recruitment into the joint is facilitated by their LFA-1 binding to its ligands (ICAM1, ICAM2, and JAM-A) expressed on the activated vascular endothelium; Ly6C⁻ blood monocytes are also recruited. (6) Cartilage loss in the inflamed joint is mediated by, for example, IL-1 β and macrophage-derived macrophage MIF. (7) Bone erosion upon osteoclast activation by RANKL/RANK interaction and release of MIF; MMP8 can protect against bone erosion and arthritis. (8) Both TNF and the prostaglandin, PGI₂, are produced in the joint and either directly or indirectly mediate pain.

1.10 Rationale, Hypotheses and Specific Aims

1.10.1 Rationale

High dose IVIg/SCIg has been increasingly used for the treatment of patients with chronic or acute autoimmune and inflammatory diseases, such Kawasaki disease and ITP. In addition, IVIg is currently under evaluation for many other diseases such as RA. The use of this therapy in North America has grown substantially over the past decade and this has burdened the Canadian Blood Services operating budget. IVIg/SCIg is pooled from the blood of thousands of donors and manufactured via chromatographic processes to formulate a highly purified product. Its manufacture requires highly specialized production facilities that are very expensive. Moreover, its supply is dependent on the availability and the collection of human plasma and it is subject to some natural variability. These challenges associated with growing product demand, production and availability have provided incentives to develop recombinant proteins as potential alternatives to IVIg/SCIg. CSL Behring has developed a recombinant hexameric Fc protein called, Fc-µTP-L309C. We compared its therapeutic efficacy to that of IVIg/SCIg to determine if it was a viable alternative to IVIg/SCIg for the treatment of certain autoimmune diseases.

The potential advantages of replacing IVIg with Fc- μ TP-L309C are evident. First, they may allow reproducible manufacturing of a homogenous, recombinant product with high lot-to-lot consistency using processes that are independent of plasma supply. A reduction in the need for plasma would provide massive benefits for the Canadian health care system because IVIg is expensive to produce and it is supplied to patients free of charge. Importantly, the potential for higher specific activity may allow for a significantly lower effective dose and injections of lower amounts of protein. This could drastically increase the quality of life for patients who receive regular IVIg infusions because this recombinant product replacement would likely be administered subcutaneously from the comfort of one's home. Additionally, the mechanism of IVIg is still unknown and investigating the mechanism of Fc- μ TP-L309C may help us to elucidate the mechanism of IVIg.

1.10.2 Hypotheses

1. Fc- μ TP-L309C will be more efficacious than IVIg at treating ITP in a mouse model. There is substantial evidence showing that other recombinant Fc multimers with similar structural properties to Fc- μ TP-L309C, can treat ITP in a mouse model at 10-1000-fold lower doses than IVIg. The Fc γ R blockade is proposed to be part of their mechanism of action.

2. IVIg will work to ameliorate RA in the K/BxN serum transfer model and in the endogenous K/BxN model if it is given at immunomodulatory doses. Most of the research to date on the efficacy of IVIg in human RA is not done with immunomodulatory doses of IVIg. Also, all of the animal studies to date have used the K/BxN serum transfer model of RA, instead of treating K/BxN mice endogenously. Thus, Fc- μ TP-L309C will be more efficacious than IVIg at ameliorating RA in the K/BxN serum transfer model or in the endogenous K/BxN mouse model due to its ability to avidly bind Fc γ Rs. Fc γ R activation by ICs are one of the dominant inflammatory pathways in the K/BxN serum transfer model of RA.

3. The protective effects of Fc- μ TP-L309C will be mediated by a collaboration of different mechanisms of action as its effects can be exerted on various arms of the immune system.

1.10.3 Specific Aims

1. To determine if Fc- μ TP-L309C can ameliorate ITP in a passive mouse model more effectively than IVIg/SCIg.

2. To determine if IVIg/SCIg and Fc-µTP-L309C can ameliorate RA in the K/BxN serum transfer model and in the endogenous K/BxN mouse model.

3. To elucidate the mechanism of Fc- μ TP-L309C in both mouse models with a focus on the endogenous K/BxN model because it is a better model of autoimmunity.

Chapter 2

Increased efficacy of Fc-μTP-L309C compared to IVIg to ameliorate ITP in a passive mouse model is due to the internalization and degradation of FcγRIII on monocytes

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2.1 Abstract

Fc-µTP-L309C is a potential replacement for IVIg for the treatment of autoimmune diseases, such as ITP. Fc-µTP-L309C is a hexamer composed of the of the IgM µ-tailpiece fused to the Cterminus of human IgG1 Fc. The point mutation at position 309 stabilizes Fc-µTP-L309C and facilitates avid binding to FcyRs. The mechanism through which IVIg ameliorates ITP is unknown however, it is thought to involve the blockade of FcyRs. Recently, it has been shown that Fc-µTP-L309C is more efficacious than IVIg at ameliorating Ab-mediated autoimmune diseases through its effects on FcyRs. We compared the ability of Fc- μ TP-L309C with that of IVIg to inhibit phagocytosis in an in vitro assay and indeed, Fc-µTP-L309C was a more potent inhibitor having an IC₅₀ of 0.03 μ g/ml in comparison to IVIg with an IC₅₀ of 350 μ g/ml. In a passive mouse model of ITP, we showed that Fc-µTP-L309C recovered platelet counts to a higher degree than IVIg at 10-fold lower doses. However, when Fc-µTP-L309C was administered intraperitoneally, the mice experienced a drop in body temperature. We circumvented this response by administering Fc-µTP-L309C subcutaneously. We hypothesised that the efficacy of Fc-µTP-L309C was due to the avid binding of FcyRs, which is not exhibited by a mainly monomeric IVIg. Using confocal microscopy, we showed that $Fc_{\mu}TP-L309C$ is internalized by monocytes whereas, IVIg remains on the cell surface. Subsequently, we showed that these $Fc\gamma RIII$ is degraded via western blot, which we believe perturbs cellular homeostasis.

2.2 Introduction

IVIg is a major replacement therapy for primary immunodeficiency, (Ballow, 2002; Buckley and Schiff, 1991; Jolles et al., 2017) and is a first line treatment for diseases such as ITP (Bierling and Godeau, 2004; Bussel, 1989; Bussel 2006; Fehr et al., 1982; Imbach et al., 1989; Tarantino, 2006). Although both $F(ab')_{2}$ - and Fc-dependent mechanisms have been suggested to be involved in the immunomodulatory effects of this therapy, research in the field has emphasized that the IgG Fc fragment is crucial for its anti-inflammatory properties. One of the proposed Fc-dependent mechanisms includes the blockade of activating $Fc\gamma Rs$ and this has been proposed as the mechanism of action of IVIg in ITP (Bussel, 2000).

Today, IVIg is pooled from the blood of thousands of donors and manufactured via chromatographic processes to formulate a highly purified, polyclonal IgG product that is suitable for i.v. or s.c. applications (Danieli et al., 2014). Even though the use of SCIg has increased the patient convenience associated with IVIg treatment, its manufacture still requires highly specialized production facilities with a focus on pathogen safety (Martin et al., 2013; Zuercher et al., 2011). Moreover, its supply is dependent on the availability and the collection of human plasma and it is subject to some natural variability. These challenges associated with growing product demand, production and availability have provided incentives to develop various Fc constructs as potential alternatives to IVIg/SCIg for diseases where its mechanism has been suggested to be Fc-dependent (Jain et al., 2012; Niknami et al., 2013; Spirig et al., 2018; Thiruppathi et al., 2014; Washburn et al., 2015; Zhou et al., 2017).

Indeed, various Fc multimers have been produced that show enhanced efficacy compared to IVIg for amelioration of disease in animal models, such as ITP (Jain et al., 2012). In a recently published paper, we reported a recombinant human IgG1 Fc, Fc- μ TP-L309C, which was produced by fusing the 18 aa IgM tailpiece to the C-terminus of a variant human IgG1 Fc with a point mutation at position 309 (Spirig et al., 2018). This point mutation facilitates the stabilized hexamerization of this molecule through the formation of disulphide bonds and it increases the avidity of Fc- μ TP-L309C to Fc γ Rs (Ridgway et al., 1996). We used the monocyte monolayer assay (MMA) to determine whether this increased avidity influences the ability of Fc- μ TP-

L309C to inhibit phagocytosis in comparison to IVIg, which is primarily in monomeric form and thus exhibits a lower avidity for Fc γ Rs. Additionally, we decided to compare the therapeutic efficacy of Fc- μ TP-L309C to IVIg in a mouse model of ITP because the blockade of Fc γ Rs has been a proposed mechanism of action for IgG products in ITP (Bussel, 2000; Jain et al., 2012). However, the intraperitoneal (i.p.) administration of Fc- μ TP-L309C caused an anaphylactic response in the mice and this was prevented by the s.c. administration of Fc- μ TP-L309C.

In our work reported herein, we demonstrated that Fc- μ TP-L309C is a good inhibitor of Fc γ Rmediated phagocytosis in the MMA and that it also shows functional efficacy as it raises platelet counts in a mouse model of ITP more effectively than SCIg. We decided to investigate whether Fc- μ TP-L309C was simply blocking Fc γ Rs, or whether it was further internalizing and degrading Fc γ Rs as this has been proposed as a mechanism by which higher order multimers perturb cellular homeostasis to ameliorate inflammation (Qureshi et al., 2017).

2.3 Materials and Methods

2.3.1 Mice

C57BL/6 (F, 6 weeks old) and BALB/c (F, 6 weeks old) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were kept under a natural light-dark cycle, maintained at 22 \pm 4°C, and fed with standard diet and water ad libitum. All experiments were performed after animal use protocols (AUP 1788) were approved by the University Health Network Animal Research Committee in Toronto.

2.3.2 Biological Reagents

The rat monoclonal anti-mouse GPIIb (CD41; clone MWReg30, rat IgG1 λ) Ab used to induce ITP was purchased from BD PharMingen (Mississauga, ON). Privigen 10% IVIg, Hizentra 20% SCIg, and Fc- μ TP-L309C were from CSL Behring AG (Bern, Switzerland). Rabbit anti-sheep IgG used to opsonize sheep RBCs in the MMA were purchased from Thermo Fisher Scientific (Waltham, MA). Polyclonal sheep RBCs were purchased from Innovative Research Inc. (Toronto, ON).

2.3.3 Cell Isolation and Cell Culturing

Mouse blood was obtained in accordance with the University Health Network Animal Research Committee (Toronto, ON). All methods were carried out in accordance with relevant guidelines and regulations. Mouse blood was obtained via cardiac puncture and PBMCs were also isolated from peripheral blood by density gradient centrifugation. RAW264.7 (RAW) cells (American Type Culture Collection, Manassas, VA) were maintained in RPMI 1640 (Sigma-Aldrich, St. Louis, MO) 10% fetal bovine serum (FBS) (Sigma-Aldrich), 4 mM L-glutamine (Sigma-Aldrich), penicillin (100 U/ml) (Sigma-Aldrich), and streptomycin (100 U/ml) (Sigma-Aldrich) (complete RPMI).

2.3.4 MMA

The MMA was used to monitor the ability of IVIg or Fc-µTP-L309C to block FcyR-mediated phagocytosis, as described previously with minor modifications (Branch et al., 1984; Tong et al., 2016). Briefly, RAW cells and mouse PBMCs were counted and seeded into an 8-well tissue culture chamber slide (LAB-TEK CHAMBER, Nalge Nunc Int., Rochester, NY), and incubated for 1 hour at 37° C, in a 5% atmosphere of CO₂. Meanwhile sheep RBCs were opsonized with rabbit anti-sheep IgG, respectively (1 hr, 37° C, 5% CO₂). After cell adherence, the supernatant containing non-adherent cells was removed and the cells were treated with either IVIg or FcµTP-L309C and incubated again for 1 hr at 37°C, 5% CO₂. Opsonized sheep RBCs (suspended in 5% FBS/RPMI 1640) were then added to the tissue culture chamber slide, and incubated (1 hr, 37 °C, 5% CO₂). After incubation, the slides were vigorously washed and fixed in 100% methanol and then mounted in elvanol (Tong et al., 2016). Phagocytic events were manually quantified using a phase contrast microscope (40X) (Type DFC345 Fx, Leica Microsystems, Richmond Hill, ON) and an average phagocytic index (PI) of the test (with IVIg or Fc-µTP-L309C) or control (without inhibitors) was obtained by dividing the number of phagocytosed sheep RBCs by the number of monocytes or RAW cells and multiplying by 100 as previously described (Branch et al., 1984; Tong et al., 2016). Percent inhibition was determined by subtracting the PI_{control} from PI_{test} divided by PI_{test}, multiplying by 100 (Branch et al., 1984; Tong et al., 2016).

2.3.5 ITP Mouse Model

A dose-escalation mouse model of passive platelet Ab (MWReg30) induced ITP was used, as described previously (Katsman et al., 2010). Briefly, mice were injected i.p. with 68 µg/kg of MWReg30 on days 0 and 1, followed by increasing doses of an additional 34 µg/kg each subsequent day. It has been well established that under these conditions, platelet nadir is achieved on day 2 and maintained until day 5 (Katsman et al., 2010; Leontyev et al., 2012; Leontyev et al., 2014). Platelets in whole blood samples were quantified daily using a calibrated flow cytometer (FACSCalibur, BectonDickinson, Franklin Lakes, NJ) as previously described (Katsman et al., 2010). For treatment of ITP, IVIg or SCIg were given i.p. or s.c. respectively, 2

hours after platelet Ab administration on day 2 of the experiment at 2 g/kg for BALB/c and at 2.5 g/kg for C57BL/6 mice, as optimized previously (Leontyev et al., 2012; Leontyev et al., 2014). Fc-µTP-L309C was compared to IVIg and SCIg and was also given i.p. or s.c. 2 hours after platelet Ab administration on day 2 of the experiment at 200 mg/kg, 100 mg/kg, and 50 mg/kg. Human serum albumin (HSA) was used as a protein control at the same doses as IVIg/SCIg.

2.3.6 Adverse Responses

Body temperature was used to assess the occurrence of systemic shockinduced by different routes of administration of IVIg, SCIg and Fc-µTP-L309C. Briefly, mice were injected i.p. with 2 g/kg of IVIg or s.c. with 2 g/kg of SCIg. Additionally, mice were injected i.p. or s.c. with 200 mg/kg of Fc-µTP-L309C. Body (rectal) temperature was monitored at 0, 15, 30, 45, and 60 minutes and at 2, 3, 4, 5, and 6 hours post injection using Thermocouple Thermometer, model TK-610B (Harvard Apparatus, USA). HSA was used as a protein control at the same doses as IVIg/SCIg.

2.3.7 Analysis of Fc-µTP-L309C Internalization

Mouse blood was obtained via cardiac puncture and PBMCs were also isolated from peripheral blood by density gradient centrifugation. Fc-µTP-L309C and IVIg were fluorescently tagged using an Ab labelling kit (Thermo Fisher Scientific, Alexa Fluor[®] 488 Ab Labeling Kit) according to the manufacturer's instructions. PBMCs were prepared by cytospin to allow them to adhere to microscope slides. PBMCs were then incubated at 4 °C or 37 °C for 30 minutes with either Fc-µTP-L309C or IVIg. PBMC's were then washed and fixed with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) for 10 minutes, before further labelling. Monocytes were labelled with a rat anti-mouse F4/80 Ab (clone A3-1, Bio-Rad, Hercules, CA), followed by a goat anti-rat IgG (H+L) antibody conjugated to Alexa Fluor[®] 555 (Thermo Fisher Scientific). Nuceli were labelled with DAPI (Thermo Fisher Scientific). Cells were washed again in PBS before imaging. Images were acquired using a Leica SP8 Confocal microscope with a 63X objective and appropriate excitation wavelengths and emission filters. Laser power and

acquisition settings were kept constant between image acquisitions of different conditions. Images shown are representative of cells from three independent experiments.

2.3.8 Western Blotting

RAW cells were plated in 6-well plates and incubated with Fc-µTP-L309C for 3 hours. Cell lysates were prepared using Triton X-100 containing a mammalian protease inhibitor cocktail, PIC003 (Bioshop Canada Inc, Burlington, ON). Cell lysates were analysed by immuno-blotting using an Ab against FcγRIII (clone R002, Sino Biological, Wayne, PA). Ponceau S staining was done for total protein normalization.

2.3.9 Statistical Analysis

Statistical analyses were performed using a computer software Prism (GraphPad Software, Version 6.0, San Diego, CA). Data are presented as mean \pm standard deviation (SD).

2.4 Results

2.4.1 Fc-µTP-L309C is a better inhibitor of FcγR-mediated phagocytosis in comparison to IVIg

To compare the abilities of IVIg and Fc- μ TP-L309C to inhibit Fc γ R-mediated phagocytosis, we used the MMA to generate an average PI for each drug at varying concentrations. We took the inverse of the average PI to generate a % inhibition for each drug at each concentration. Using curve fitting software from GraphPad Prism, the absolute IC₅₀ (the concentration at which 50% inhibition is observed) of each drug was determined. The IC₅₀ of IVIg was 350 µg/ml (Figure 2.1A), whereas the IC₅₀ of Fc- μ TP-L309C was 0.03 µg/ml (Figure 2.1B) using RAW cells. The IC₅₀ of IVIg was 5600 µg/ml (Figure 2.1C), whereas the IC₅₀ of Fc- μ TP-L309C was 0.05 µg/ml (Figure 2.1C) using mouse PBMCs.

2.4.2 Fc-µTP-L309C provides therapeutic benefit in a mouse model of ITP when given intraperitoneally

The therapeutic effect of Fc- μ TP-L309C was tested in a mouse model of ITP (Figure 2.2A). Mice were treated with a single dose of Fc- μ TP-L309C (200 mg/kg) given intraperitoneally when the platelet nadir was attained 2 days after initiating Ab-mediated depletion. Treatment with Fc- μ TP-L309C resulted in increased platelet numbers, which were higher at days 3 and 4 than for mice treated with a 10-fold larger dose of IVIg (2000 mg/kg) given intraperitoneally (Figure 2.2A).

2.4.3 Intraperitoneal injection of Fc-µTP-L309C but not subcutaneous delivery triggers body temperature decreases in mice

We examined whether Fc- μ TP-L309C induced a temperature drop in mice after different routes of administration (Figure 2.2B). Administration of Fc- μ TP-L309C at 200 mg/kg via i.p. injection triggered a rapid drop in the body temperature of mice, which was fully recovered by 6 hours (Figure 2.2B). However, we observed that in each consecutive experiment, the temperature drop induced by i.p. injection of Fc- μ TP-L309C was less pronounced (this experiment was repeated 3 consecutive times). A similar decrease in body temperature was absent when mice were treated with 200 mg/kg of Fc- μ TP-L309C given s.c. Treatment with 2 g/kg of IVIg given i.p. and treatment with 2 g/kg of SCIg given s.c. did not induce a temperature drop (Figure 2.2B).

2.4.4 Fc-µTP-L309C provides therapeutic benefit in a mouse model of ITP when given subcutaneously

The therapeutic effect of Fc- μ TP-L309C was tested in a mouse model of ITP (Figure 2.2C). Mice were treated with a single dose of Fc- μ TP-L309C (200 mg/kg) given subcutaneously when the platelet nadir was attained 2 days after initiating antibody-mediated depletion. Treatment with Fc- μ TP-L309C resulted in increased platelet numbers, which were higher at days 3 and 4 than for mice treated with a 10-fold larger dose of SCIg (2000 mg/kg) (Figure 2.2C).

2.4.5 Internalisation and degradation of Fcγ-receptors following Fc-μTP-L309C engagement

To test the effect of Fc- μ TP-L309C on Fc γ Rs, we incubated mouse PBMCs with a fluorescently labelled Fc- μ TP-L309C either at 4 °C or 37 °C for 30 minutes. The cells incubated at 37 °C showed a considerable number of intracellular vesicles containing Fc- μ TP-L309C as well as some surface Fc- μ TP-L309C (Figure 2.3A). In contrast, the PBMCs labelled at 4 °C showed significant plasma membrane co-localization of Fc- μ TP-L309C with F4/80 on monocytes (Figure 2.3A). The same experiments were performed with fluorescently labelled IVIg. We observed significant co-localisation of IVIg with F4/80 on monocytes at both 4 °C and 37 °C (Figure 2.3B). To test the fate of the Fc γ Rs in this system, we performed Western-blotting for Fc γ RIII after exposure to Fc- μ TP-L309C for 3 hours at 37 °C. Time 0 was used as a control as degradation induced by Fc- μ TP-L309C was apparent after 1 hour (data not shown). We observed significant degradation of the activating, Fc γ RIII after exposure to Fc- μ TP-L309C (Figure 2.3C).



Figure 2.1. IC50s of IVIg and Fc-µTP-L309C.

A. The IC₅₀ curve of IVIg is shown to have an absolute IC₅₀ of 350 µg/ml. Triplicate data was expressed as mean \pm standard deviation (SD) as error bars. **B.** The IC₅₀ curve of Fc-µTP-L309C is shown to have an absolute IC₅₀ of 0.03 µg/ml. Triplicate data was expressed as mean \pm standard deviation (SD) as error bars. **C.** This table shows the IC₅₀ of IVIg, which has an IC₅₀ of 5600 µg/ml, using mouse monocytes in comparison to Fc-µTP-L309C, which has an IC₅₀ of 0.05 µg/ml, using mouse monocytes.



В



- ← Fc-µTP-L309C (200 mg/kg) i.p.
- ■·· Fc-µTP-L309C (200 mg/kg) s.c.
- ▲ IVIg (2000 mg/kg) i.p.
- ▼ SClg (2000 mg/kg) s.c.
- + HSA i.p.



Figure 2.2 Therapeutic effect of Fc-µTP-L309C in experimental ITP.

A. Adapted from Spirig, R., Campbell, I.K., Koernig, S., Chen, C.G., Lewis, B.J., Butcher, R., Muir, I., Taylor, S., Chia, J., Leong, D, et al. (2018). rIgG1 Fc hexamer inhibits antibodymediated autoimmune disease via effects on complement and FcγRs. The Journal of Immunology 200, 2542-2553. (Spirig et al., 2018). <u>https://doi.org/10.4049/jimmunol.1701171</u>. Dose escalation of MWReg30 maintains platelet nadir over time. Treatment with a single i.p. dose of Fc-µTP-L309C at day 2 results in alleviation of ITP comparable with IVIg. Shown are the mean platelet values in the blood; data show the mean (± SD) of platelet counts (Fc-µTP-L309C, n = 5; IVIG and PBS, n = 3). **B.** Mice were treated with 200 mg/kg of Fc-µTP-L309C given i.p. or s.c, or they were treated with 2 g/kg of IVIg (i.p.) or SCIg (s.c.) at time 0, 24, and 48 hours. Body (rectal) temperature was measured at 0, 15, 30, 45, and 60 minutes and at 2, 3, 4, 5, and 6 hours post each injection with a thermometer. Shown are the average body temperatures; error bar indicates the range of temperatures (mean ± SD; n= 3 per treatment group). This experiment was repeated 3 independent times. **C.** Treatment with a single s.c. dose of Fc-µTP-L309C (200 mg/kg, 100 mg/kg, or 50 mg/kg) or SCIg (2500 mg/kg) on day 2 in C57BL/6J mice with ITP. Shown are mean PLT values in the blood; error bars indicate the range of PLT counts (mean \pm SD; n = 6 for each treatment group). Similar results were obtained in two independent experiments.





37 °C

В





Fc-µTP-L309C/IVIg F4/80 DAPI



70kD-55kD-40kD-

Ponceau S

Figure 2.3 Internalization of Fc-µTP-L309C and subsequent degradation of activating FcyRIII.

A. PBMCs were incubated with AF488-conjugated Fc- μ TP-L309C or IVIg (**B**) at either 4 °C or 37 °C for 30 minutes, followed by incubation with AF555-conjugated F4/80 to label monocytes. Cells were then fixed and imaged by confocal microscopy. Representative images taken from one of three independent experiments is shown. **C.** RAW cells were incubated with Fc- μ TP-L309C for 3 hours. Cell lysates were analyzed by Western blotting using antibodies against Fc γ RIII. Two representative blots from three independent experiments are shown.

2.5 Discussion

We showed that Fc- μ TP-L309C was a better inhibitor of Fc γ R-mediated phagocytosis in comparison to IVIg in the MMA as shown by their IC₅₀s. These results were corroborated by our other studies showing that the hexamerization of IgG-Fc lead to a dramatic increase in its binding avidity to Fc γ Rs and inhibition of phagocytosis by THP1 cells (Spirig et al., 2018). This finding is not unique to Fc- μ TP-L309C, as it is also exhibited by other Fc multimers such as the StradomerTM (Jain et al., 2012), the Hexa Fc (HexagardTM) (Mekhaiel et al., 2001; Smith et al., 1995; Sorensen et al., 1996), and the Fc3Y (Ortiz et al., 2016). However, our studies are the first to show such a difference in efficacy to inhibit the phagocytosis of opsonized RBCs.

We decided to compare the therapeutic efficacy of Fc- μ TP-L309C and IVIg in a mouse model of ITP. Fc- μ TP-L309C was more effective at ameliorating ITP in comparison to IVIg at 10-fold lower doses. However, many studies have shown that the hexamerization of Fcs has been associated with unwanted negative effects such as nonspecific Fc γ R signaling leading to intracellular activation (Ortiz et al., 2016; Qureshi et al., 2017). Specifically, a previous study showed that Fc multimers with two or three Fcs bound multiple Fc γ Rs with high avidity but did not elicit Fc γ R signaling or calcium flux in monocytes (Ortiz et al., 2016). However, Fc multimers with five or more domains triggered dose-dependent Fc γ R-mediated signaling and calcium flux as well as rapid internalization of the Fc multimer via Fc γ RII (Ortiz et al., 2016).

Given the fact that Fc-µTP-L309C is a hexamer, we decided to investigate whether Fc-µTP-L309C triggered any adverse events in vivo before we tested its efficacy in our mouse models. We used temperature drop as a surrogate marker for adverse events such as systemic shock (Khodoun et al., 2013; Strait et al., 2002), and we analyzed whether Fc-µTP-L309C caused a temperature drop in mice after peritoneal and subcutaneous injections. We used both IVIg (i.p.) and SCIg (s.c.) as controls because the IgG present within them is mainly in monomeric form. Although Fc-µTP-L309C induced a temperature drop in mice when it was injected intraperitoneally, it did not induce a temperature drop when it was injected subcutaneously. Thus, it appears s.c. administration circumvented this adverse event that was observed with i.p.

injection and so the s.c. route of injection became our preferred route of administration of Fc- μ TP-L309C.

Seeing as though our focus was shifted to s.c. administration, we decided to compare the therapeutic efficacy of Fc- μ TP-L309C and SCIg in a mouse model of ITP. As expected, Fc- μ TP-L309C was more effective at raising platelet counts in mice with ITP in comparison to SCIg at 10-fold lower doses. These results corroborated our recent findings, as well as others that have shown the increased therapeutic efficacy of other Fc multimers over IVIg in other mouse models of ITP (Jain et al., 2012; Qureshi et al., 2017; Zuercher et al., 2011).

The engagement of Fc γ Rs by higher order Fc multimers has been associated with their internalization (Mellman et al., 1984; Qureshi et al., 2017) To test the effect of Fc- μ TP-L309C on Fc γ Rs, we incubated mouse PBMCs with a fluorescently-labelled hexameric-Fc either at 4 °C or 37 °C for 30 minutes. The cells incubated at 37 °C showed a considerable number of intracellular vesicles containing Fc- μ TP-L309C as well as some surface Fc- μ TP-L309C, suggesting the hexameric-Fc was efficiently internalised. In contrast, the PBMCs labelled at 4 °C showed significant plasma membrane of Fc- μ TP-L309C with considerable co-localisation with F4/80 on monocytes, indicating the hexameric Fc had remained at the cell surface at this temperature. The same experiments were performed with fluorescently labelled IVIg. We observed significant co-localisation of IVIg with F4/80 on monocytes at both 4 °C and 37 °C indicating that IVIg remained at the cell surface at both temperatures.

To test the fate of the Fc γ Rs in this system, we performed Western-blotting for Fc γ RIII after exposure to Fc- μ TP-L309C for 3 hours at 37 °C. We observed significant degradation of themactivating Fc γ RIII after contact with Fc- μ TP-L309C. We believe that receptor internalisation and degradation could disrupt cellular homeostasis and Fc γ R function for a prolonged period. We believe that this accounts for the efficacy of Fc- μ TP-L309C exhibited in our mouse model of ITP however, this may pose challenges for further *in vivo* testing of Fc- μ TP-L309C and other Fc-based therapeutics if the Fc multimers are rapidly cleared from the system. We did not investigate the effects of Fc- μ TP-L309C on Fc γ RIIb however, it would be interesting to see whether Fc γ RIIb is degraded after exposure to Fc- μ TP-L309C. Other studies have proposed that FcγRIIb is spared from degradation after exposure to Fc multimers because of an evolutionary response to re establish immune homeostasis after prolonged exposure to ICs (Boonak et al., 2013; Mousavi et al., 2007; Qureshi et al., 2017; Zhang et al., 2010).

Chapter 3

Using the K/BxN mouse model of endogenous, chronic, rheumatoid arthritis for the evaluation of potential immunoglobulin-based therapeutic agents

Adapted from Lewis, B.J., Ville, J., Blacquiere, M., Cen, S., Spirig, R., Zuercher, A.W., Käsermann, F. and Branch, D.R. (2019). Using the K/BxN mouse model of endogenous, chronic, rheumatoid arthritis for the evaluation of potential immunoglobulin-based therapeutic agents, including IVIg and Fc-µTP-L309C, a recombinant IgG1 Fc hexamer. BMC immunology *20*, 44. (Lewis et al., 2019).

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3.1 Abstract

High-dose IVIg, and more recently, SCIg, are used to treat a variety of autoimmune diseases; however, there are challenges associated with product production, availability, access and efficacy. These challenges have provided incentives to develop a human recombinant Fc as a more potent alternative to IVIg and SCIg for the treatment of autoimmune diseases. Recently, a recombinant human IgG1 Fc hexamer called Fc-µTP-L309C, was shown to be more efficacious than IVIg in a variety of autoimmune mouse models. We bred K/BxN mice and first investigated the ability of IVIg to treat endogenous, chronic arthritis using a dose escalation model. We then compared the abilities of SCIg and Fc-µTP-L309C to treat endogenous, chronic arthritis. Finally, we investigated the abilities of IVIg, SCIg and Fc-µTP-L309C to prevent arthritis in K/BxN mice and we used the K/BxN serum transfer model to corroborate these results. Amelioration of endogenous, chronic arthritis was achieved in K/BxN mice when treated with high and frequent doses of IVIg, SCIg and Fc-µTP-L309C. However, Fc-µTP-L309C was efficacious at 10-fold lower doses that IVIg/SCIg. Also, arthritis could be prevented when Fc-µTP-L309C was given prior to onset of the arthritis in both the endogenous model and in the serum transfer model. When we examined the histological scores of hematoxylin and eosin (H&E) stained ankle sections of mice treated with Fc-µTP-L309C, our results confirmed our clinical findings. FcµTP-L309C significantly reduced inflammatory cell infiltrate and tissue destruction. Our results show that Fc-µTP-L309C is a powerful treatment for the prevention and amelioration of severe, chronic arthritis in a true autoimmune mouse model of RA. Thus, the K/BxN endogenous arthritis model should be useful for testing potential therapeutics for RA.

3.2 Introduction

Plasma derived IgG is a major replacement therapy for primary immunodeficiency (Ballow, 2002; Buckley and Schiff, 1991; Jolles et al., 2017) and is a first line treatment for diseases such as ITP (Bierling and Godeau, 2004; Bussel, 1989; Bussel 2006; Fehr et al., 1982; Imbach et al., 1989; Tarantino, 2006), chronic inflammatory demyelinating polyneuropathy (Tackenberg et al., 2010) and Kawasaki disease (Burns et al., 1998; Oates-Whitehead et al., 2003). Additionally, it is used to treat a variety of other autoimmune and inflammatory diseases and neurologic disorders in combination with other therapies or when traditional therapies fail (Alabdali et al., 2014; Ballow, 2011; Brandt and Gershwin, 2006; Etxioni and Pollack, 1989; Kaveri et al., 1991; Nimmerjahn and Ravetch, 2008; Prins et al., 2007; Pyne et al., 2002; Stangel et al., 1998).

Although both F(ab')2- and Fc-dependent mechanisms have been suggested to be involved in the anti-inflammatory effects of this therapy, research in the field has emphasized that the IgG Fc is crucial for its immunomodulatory properties (Nimmerjahn and Ravetch, 2008). Some of the proposed Fc-dependent mechanisms include the blockade of activating Fc γ Rs (Bussel, 2000), the requirement for the FcRn (Hansen and Balthasar, 2002; Li et al., 2005), the expansion of regulatory T cell populations (De Groot et al., 2008), the upregulation of the inhibitory receptor Fc γ RIIB (Samuelsson et al., 2001) and the modulation of DC activity (Kapur et al., 2017; Sigaram et al., 2006).

Today, plasma derived IgG is pooled from the blood of thousands of donors and manufactured via chromatographic processes to formulate a highly purified, polyclonal IgG product that is suitable for i.v. or s.c. applications (Danieli et al., 2014; Zuercher et al., 2011). Even though the use of SCIg has increased the patient convenience associated with IVIg treatment, its manufacture still requires highly specialized production facilities with a focus on pathogen safety (Martin et al., 2013; Zuercher et al., 2011). Moreover, its supply is dependent on the availability and the collection of human plasma and it is subject to some natural variability. These challenges associated with growing product demand, production and availability have provided incentives to develop various Fc constructs as potential alternatives to IVIg/SCIg for diseases where its mechanism has been suggested to be Fc-dependent (Bussel, 2000; De Groot et al., 2008; Hansen

and Balthasar, 2002; Kapur et al., 2017; Li et al., 2005; Nimmerjahn and Ravetch, 2008; Samuelsson et al., 2001; Sigaram et al., 2006).

Various Fc multimers have been produced that show enhanced efficacy compared to IVIg for amelioration of disease in animal models, such as ITP and RA (Jain et al., 2012; Mekhaiel et al., 2011; Niknami et al., 2013; Qureshi et al., 2017; Spirig et al., 2018; Thiruppathi et al., 2014; Washburn et al., 2015; Zhou et al., 2017). In a recently published paper, we reported a recombinant human IgG1 Fc, Fc- μ TP-L309C, which was produced by fusing the 18 aa IgM tailpiece to the C-terminus of a variant human IgG1 Fc with a point mutation at position 309 (Spirig et al., 2018). This point mutation facilitates the stabilized hexamerization of this molecule through the formation of disulphide bonds and distinguishes this recombinant hexamer from others reported (Spirig et al., 2018). We showed that Fc- μ TP-L309C has high binding avidity for Fc γ Rs and could suppress CIA and CAIA and ameliorate ITP in mouse models when given therapeutically at 10-fold lower doses than IVIg (Spirig et al., 2018).

With the limited amount of studies performed on the therapeutic efficacy of IVIg in RA (Kanik et al., 1996; Maksymowych et al., 1996; Muscat et al., 1995; Prieur et al., 1990; Pyne et al., 2002; Tumiati et al., 1992; Vaitla and McDermott, 2010) we decided to examine whether IVIg could ameliorate arthritis in an endogenous, chronic mouse model of RA. The K/BxN serum-transfer model is an established mouse model of RA that recapitulates the effector phase of the disease and has been examined for potential treatment efficacy of IVIg by several investigators (Anthony et al., 2008a; Anthony et al., 2008b; Campbell et al., 2014; Kaneko et al., 2006). However, K/BxN mice, themselves, that spontaneously generate a true autoimmune-mediated arthritis, have never been used as a therapeutic tool to investigate the efficacy of RA therapeutic agents, including IVIg.

K/BxN mice are generated using KRN mice expressing a TCR transgene for G6PI peptide in the context of IAg7 MHC class II (Ditzel et al., 2004). To generate K/BxN mice, KRN are bred with NOD/Lt mice expressing the MHC class II haplotype IAg7 that is required to interact with the TCR transgene. This mating results in the generation of auto-Abs to G6PI (Ditzel et al., 2004; Korganow et al., 1999; Mangialaio et al., 1999; Matsumoto et al., 1999). G6PI is present on the articular cartilage and thus, ICs form to drive the activation of various immune cells (Ditzel et al., 2004).

al., 2004; Korganow et al., 1999; Mangialaio et al., 1999; Matsumoto et al., 1999). Since the sera of K/BxN mice contain pathogenic auto-Abs to G6PI, it can be transferred into naïve mice and arthritic manifestations occur a few days to weeks later (Anthony et al., 2008a; Anthony et al., 2008b; Campbell et al., 2014; Kaneko et al., 2006). However, serum recipients develop arthritis in the absence of the adaptive immune system, which poses serious limitations for examining the intricacies of the human disease. Thus, instead of a serum-transfer model, we used the endogenous K/BxN mice to better recapitulate the autoimmune-mediated RA; the resulting immune-mediated mechanism would include the influence of T-cells, antigen presenting cells and B-cells; a true autoimmune condition.

Because no one has previously shown that IVIg could ameliorate the endogenous chronic arthritis in this mouse model, we used a dose escalation and frequency of dosing approach to determine if IVIg had any efficacy in this RA model. We also investigated the therapeutic efficacy of SCIg and Fc- μ TP-L309C in this same model to determine efficacy, if any, and whether any of these agents could serves as a potential therapeutic alternative for the treatment of RA.

3.3 Materials and Methods

3.3.1 Mice

KRN TCR transgenic mice on a C57BL/6 background were obtained from The Jackson Laboratory, a kind gift from C. Benoist (Harvard Medical School, Boston, MA). NOD/Lt mice were purchased from The Jackson Laboratory. Arthritic mice were obtained by crossing KRN mice (F, 6 weeks old) with NOD/Lt (M, 6 weeks old) mice to produce K/BxN mice expressing both the TCR transgene KRN and the MHC class II molecule I-Ag7. BALB/c (F 6, weeks old) were purchased from The Jackson Laboratory. Mice were kept under a natural light-dark cycle, maintained at 22 ± 4 °C, and fed with standard diet and water ad libitum. All experiments were performed after animal use protocols (AUP 1788) were approved by the University Health Network Animal Research Committee in Toronto.

3.3.2 Biological Reagents

Privigen 10% IVIg, Hizentra 20% SCIg, and Fc-µTP-L309C, respectively, were from CSL Behring AG. HSA was from the Canadian Blood Services (Toronto, ON). H&E was from Sigma-Aldrich.

3.3.3 K/BxN Serum Transfer Arthritis

Severely arthritic adult K/BxN mice were bled and the sera was pooled. BALB/c mice were injected i.p. with 200 μ l of pooled sera on days 0 and 2 as indicated in the figure legends. The volume of sera was chosen based on *in vivo* titration of pooled sera. Mice were given treatment on day 2 with either an i.p. injection of 2 g/kg of IVIg, a s.c. injection of 2 g/kg of SCIg or a s.c. injection of 200 mg/kg of Fc- μ TP-L309C as indicated in the figure legends. HSA was used as a protein control.
3.3.4 Arthritis prevention in the K/BxN mouse model of endogenous, chronic arthritis

K/BxN mice, prior to onset of arthritis, at 21 days of age, were treated by i.p. injections of 2 g/kg of IVIg or s.c. injections of 2 g/kg of SCIg or s.c. injections of 200 mg/kg of Fc- μ TP-L309C. Treatments were given on days 1, 3, 5, 7, 11, 13, 15, 17, 19, and 21, as indicated in the figure legends. HSA was used as a protein control.

3.3.5 Arthritis treatment in the K/BxN mouse model of

endogenous, chronic arthritis

To investigate the ability of IVIg to ameliorate chronic arthritis, K/BxN mice with high clinical scores of 9 or greater were treated by i.p. injections of either 1 g/kg, 2 g/kg, or 4 g/kg of IVIg as indicated in the figure legends on days 1, 3, 5, 7, 9, and 11. The lowest dose of IVIg that showed efficacy was 2 g/kg and this dose was selected to compare to SCIg while a titration of Fc-µTP-L309C was performed using s.c. injections of Fc-µTP-L309C at 200 mg/kg, 100 mg/kg, or 50 mg/kg. Final comparisons were done using 2 g/kg IVIg and SCIg and 200 mg/kg Fc-µTP-L309C. Treatments were administered on days 1, 3, 5, 7, 9, and 11. HSA was used as a protein control.

3.3.6 Arthritis scoring

The clinical scores and the hind paw widths of the mice were monitored daily over the course of each experiment. The development of arthritis was assessed daily, and the severity of arthritis was scored for each paw on a 3-point scale, in which 0 = normal appearance, 1 = localized edema/ erythema over one surface of the paw, 2 = edema/ erythema involving more than one surface of the paw, 3 = marked edema/erythema involving the whole paw. The scores of all four paws were added for a composite score, with a maximum score of 12 per mouse. Ankle thickness of the hind paws was measured in millimeters (mm) at the widest point (the malleoli) with the legs fully extended with digital calipers (Manostat, Herisau, Switzerland).

3.3.7 Histology of arthritic joints

Mice were euthanized and the left rear paws were fixed in 10% neutral-buffered formalin, decalcified, and embedded in paraffin. Sagittal tissue sections were stained with H&E and scored blinded to the treatment groups. Ankle joints were scored for exudate (presence of inflammatory cells within the joint space), synovitis (degree of synovial membrane thickening and inflammatory cell infiltration), and tissue destruction (cartilage and bone erosion and invasion), each out of 5 (0 = normal, 1 = minimum, 2 = mild, 3 = moderate, 4 = marked, and 5 = severe), and these were tallied for a total score out of 15.

3.3.8 Statistical analysis

Statistical tests were performed using GraphPad Prism 8 for Windows software. Analyses of differences between sample groups were performed using the tests indicated in the figure legends. Data shown are mean \pm standard deviation (SD), unless otherwise stated. P < 0.05 was considered statistically significant.

3.4 Results

3.4.1 IVIg can treat chronic inflammatory arthritis

It is controversial as to whether IVIg can be used in the treatment of RA; although, only low doses, not immunomodulatory doses, have been examined (Kanik et al., 1996; Maksymowych et al., 1996; Muscat et al., 1995; Prieur et al., 1990; Pyne et al., 2002; Tumiati et al., 1992; Vaitla and McDermott, 2010). Previously, in the CIA and CAIA mouse models, we showed that Fc- μ TP-L309C could ameliorate the arthritis using a 10-fold lower dose than IVIg (Spirig et al., 2018). However, the K/BxN mouse model is a very robust chronic arthritis model and is considered to be a better model of human RA (Christensen et al., 2016; Ditzel et al., 2004; Korganow et al., 1999; Mangialaio et al., 1999; Matsumoto et al., 1999; Monach et al., 2007; Monach et al., 2008); thus, we wanted to test efficacy of IVIg, SCIg and Fc- μ TP-L309C in this endogenous, chronic mouse model of RA and evaluate whether this particular mouse model could be useful to evaluate future potential treatments for RA.

We first used a dose-escalation and frequency of dosing approach to determine if there was any effect on endogenous RA and, if so, an optimal dose and dosing schedule at which IVIg exhibited therapeutic efficacy in the treatment of arthritis in K/BxN mice. First, we determined whether there was an optimal dosing of IVIg that would reduce the clinical scores and paw swelling of K/BxN mice with chronic arthritis. We administered 6 treatments with IVIg given i.p. at doses of 1 g/kg, 2 g/kg and 4 g/kg over the course of this experiment. Treatments were administered on days 1, 3, 5, 7, 9, and 11. Clinical scores and hind paw widths of the mice were monitored and it was shown that the clinical scores and paw widths of the mice treated with multiple doses of 4 and 2 g/kg of IVIg (Figure 3.1A & B) were significantly reduced in comparison to mice that were treated with multiple doses of 1 g/kg dose of HSA. However, the clinical scores and paw widths of mice that were treated with multiple doses of 1 g/kg of IVIg (Figure 3.1A & B) were not reduced in comparison to mice that were given the same g/kg dose of HSA. Further modifying the dosing regimen was not optimal as the mice tended to rebound with their arthritis with reducing or stopping the dosing (data not shown). As the dosing with 2 g/kg or 4 g/kg gave

similar amelioration of the arthritis, we decided that an optimal dose of IVIg could be administered using 2 g/kg every other day.

3.4.2 Fc-µTP-L309C is more efficacious than IVIg/SCIg at treating chronic arthritis

After demonstrating that IVIg could effectively ameliorate chronic arthritis at 2 g/kg with doses administered every other day, we next wanted to investigate whether SCIg and Fc- μ TP-L309C could do the same or better. We administered 6 s.c. treatments of SCIg at 2 g/kg or 6 s.c. treatments of Fc- μ TP-L309C at 200 mg/kg, 100 mg/kg or 50 mg/kg over the course of this experiment. We started with a dose of 200 mg/kg for Fc- μ TP-L309C because of our previous work showing this to be an optimal dose for amelioration of ITP, CIA and CAIA (Spirig et al., 2018).

Treatments were administered on days 1, 3, 5, 7, 9, and 11. We monitored the clinical scores and hind paw widths of the mice and found that the clinical scores and paw widths of the mice treated with multiple doses of 2 g/kg of SCIg (Figure 3.2 A & B) were reduced in comparison to mice that were given the same g/kg dose of HSA. However, we also found that the clinical scores and the paw widths of mice treated with multiple doses of 200 mg/kg of Fc-µTP-L309C (Figure 3.2 A & B) were significantly reduced in comparison to mice that were given 2 g/kg SCIg or the same g/kg dose of HSA. Fc-µTP-L309C exhibited a dose-response as even doses of 50 mg/kg and 100 mg/kg showed a trend for efficacy with 200 mg/kg being the most efficacious. Even though SCIg could ameliorate arthritis with the same dosing scheme as IVIg, Fc-µTP-L309C exhibited greater therapeutic efficacy than both IVIg and SCIg in this model at 10-fold lower doses.

Histological evaluation confirmed our clinical findings, with Fc- μ TP-L309C significantly reducing disease (inflammatory cell infiltrate and tissue destruction) in the ankle joints of mice given 6 s.c. injections of Fc- μ TP-L309C in comparison to mice treated with HSA (Figure 3.2 C & D). Balb/c mice showed no signs of disease and were used as a negative control (Figure 3.2 C & D).

3.4.3 Fc-µTP-L309C, IVIg and SCIg can prevent arthritis in both the endogenous and in the serum transfer K/BxN model

As a complimentary study to our investigation of the therapeutic efficacy of IVIg, SCIg and Fc- μ TP-L309C in the K/BxN model, we wanted to investigate whether these Ig-based agents could also prevent arthritis. The first approach we used to examine this was to treat 21-day old K/BxN mice with either 11 i.p. injections of 2 g/kg of IVIg or s.c. injections of SCIg or 11s.c. injections of 200 mg/kg of Fc- μ TP-L309C. The clinical scores of the mice were monitored over the course of this experiment. Injections were administered on days 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, and 21. We found that the clinical scores did not significantly increase in mice treated with 2 g/kg of IVIg (Figure 3.3 A) or SCIg (Figure 3.3 A) or with 200 mg/kg of Fc- μ TP-L309C (Figure 3.3 A) in comparison to mice treated with HSA. However, the increase in clinical scores of mice treated with Fc- μ TP-L309C. Prophylaxis using SCIg appeared to not be as efficacious as either IVIg or Fc- μ TP-L309C appeared to completely prevent onset of arthritis, bringing clinical scores to baseline levels. This again highlights the therapeutic efficacy of Fc- μ TP-L309C over IVIg and SCIg.

The second approach we used was to examine the prophylactic effect of these immunoglobulinbased agents on the severity of K/BxN serum transfer-induced arthritis. We used Balb/c mice for these experiments treated with IVIg, SCIg and Fc- μ TP-L309C. Similar results were found in this model as in the endogenous model (Figure 3.3 B). However, in this model IVIg appeared to be less able to provide prophylaxis early on but all agents resulted in the inhibition of progression of the arthritis by day 9-10.

Histological evaluation confirmed our clinical findings, with Fc- μ TP-L309C significantly preventing disease onset (inflammatory cell infiltrate and tissue destruction) in the ankle joints of mice given 6 s.c. injections of Fc- μ TP-L309C in comparison to mice treated with HSA (Figure 3.2 C & D).



Figure 3.1 Therapeutic effect of IVIg in experimental RA.

The clinical scores (**A**) and paw width measurements (**B**) are shown for mice treated with multiple doses of 4 g/kg, 2 g/kg or 1 g/kg of IVIg, using HSA-treated mice as a control. Arrows indicate treatment given on days 1, 3, 5, 7, 9, and 11. Shown are the average clinical scores and the average paw width measurements (mm); error bars indicate range of clinical scores/range of paw widths (mean \pm SD; n =4-5 for each treatment group). **P<0.001 2 g/kg IVIg vs. HSA and ***P<0.0001 4 g/kg IVIg vs. HSA.



С

Fc-µTP-L309C treated

HSA treated





BALB/c





Figure 3.2 Therapeutic effect of Fc-µTP-L309C in experimental RA.

The clinical scores (**A**) and paw width measurements (**B**) are shown for mice treated with multiple doses of 2 g/kg of SCIg or with multiple doses of 200 mg/kg, 100 mg/kg, or 50 mg/kg of Fc- μ TP-L309C, using HSA-treated mice as a control. Injections were given on days 1, 3, 5, 7, 9, and 11. Shown are the average clinical scores and the average paw width measurements (mm); error bars indicate range of clinical scores/range of paw widths (mean ± SD; n = 5 for each treatment group). ***P<0.0001 200 mg/kg Fc- μ TP-L309C vs. 2000 mg/kg SCIg, 100 mg/kg Fc- μ TP-L309C vs. HSA, 2000 mg/kg SCIg vs. HSA, 50 mg/kg Fc- μ TP-L309C vs. HSA. The representative H&E-stained sections of ankle joints (**C**) and the histological scores (**D**) of BALB/c mice, HSA-treated mice and, Fc- μ TP-L309C-treated mice after 6 s.c. injections are shown here. Data show the mean (±SD) histological scores of joints (n =6) at day 11 of disease. ***p<0.001, compared with HSA-treated, Kruskal–Wallis with Dunn test.





C indicates cell infiltration, T indicates tissue destruction, and S indicates sites of synovial inflammation.



D

Figure 3.3 Prevention of arthritis with Fc-µTP-L309C treatment.

The clinical scores (**A**) are shown for mice treated with 11 injections of IVIg, SCIg or Fc- μ TP-L309C. Injections were given on days 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, and 21. Shown are the average clinical scores; error bars indicate range of clinical scores (mean ± SD; n = 6 for each treatment group). ***P<0.0001 2000 mg/kg IVIg vs. HSA, 2000 mg/kg SCIg vs. HSA and 200 mg/kg Fc- μ TP-L309C vs. HSA. Similar results were obtained in two independent experiments. The clinical scores (**B**) are shown for BALB/c mice given i.p. injections of 200 μ l of arthritic serum on days 0 and 2, indicated by ^, that were treated with 2 g/kg of IVIg or SCIg or with 200 mg/kg of Fc- μ TP-L309C on day 2, indicated by arrow, in comparison to mice treated with HSA. Shown are the average clinical scores; error bars indicate range of clinical scores (mean ± SD; n = 5 for each treatment group). ***P<0.0001 2000 mg/kg IVIg vs. HSA, 2000 mg/kg SCIg vs. HSA and 200 mg/kg Fc- μ TP-L309C vs. HSA. The representative H&E-stained sections of ankle joints (**C**) and the histological scores (**D**) of HSA-treated mice and, Fc- μ TP-L309C-treated mice after 11 s.c. injections are shown here. Data show the mean (±SD) histological scores of joints (n

=10) at day 21 of disease. ***p<0.001, compared with HSA-treated mice, Kruskal–Wallis with Dunn test.

3.5 Discussion

The value of IVIg in the treatment of RA has not been extensively evaluated. The human trials that have been done to evaluate this were small and not well controlled, and the results were equivocal (Kanik et al., 1996; Maksymowych et al., 1996; Muscat et al., 1995; Prieur et al., 1990; Pyne et al., 2002; Tumiati et al., 1992; Vaitla and McDermott, 2010). The patient cohorts for these studies were small, with some studies having less than 20 patients enrolled (Kanik et al., 1996; Maksymowych et al., 1996). A high-dose IVIg protocol of 1-2 g/kg of IVIg per month for a minimum of 6 months is critical for effectively treating systemic autoimmune diseases (Sewell and Jolles, 2002). However, these studies used lower than immunomodulatory doses of IVIg, used different doses and dosing schedules without any consistent long-term follow-up (Kanik et al., 1996; Maksymowych et al., 1996; Muscat et al., 1995; Prieur et al., 1990; Tumiati et al., 1992; Vaitla and McDermott, 2010). Because there is no scientific basis for the effect of IVIg in RA, mouse models are used to investigate its effect. IVIg has been shown to ameliorate arthritis in the CIA, CAIA and the K/BxN serum transfer models (Bruhns et al., 2003; Campbell et al., 2014; Patel et al., 2011; Schwab et al., 2012).

Many researchers have used the K/BxN serum transfer model to study arthritis whereby the inflammatory response in the serum recipients happens in the absence of the adaptive immune system (Anthony et al., 2008a; Anthony et al., 2008b; Campbell et al., 2014; Kaneko et al., 2006). Although this model is a useful tool to understand how auto-Abs drive the progression of arthritis by interacting with downstream components of the innate immune system, there are some major differences between the serum transfer model and the human disease. These include differences in Ab specificities and function, and differences in immune cells that drive pathogenesis (Matsumoto et al., 1999; Korganow et al., 1999). Considering this, we decided to treat K/BxN mice with severe arthritis endogenously because we, and others, believe that the immune cells involved in driving the inflammatory response in K/BxN mice more tightly mimic those experienced in the human seropositive disease (Ditzel, 2004; Monach et al., 2007; Monach et al., 2008).

We are the first to use Ig-based agents to try and treat the spontaneous generation of RA in the K/BxN mouse model. Therefore, we first used a multiple dose and frequency of dosing approach to determine if there was any effect on the RA and, if so, the optimal treatment regimen where IVIg was effective. We used an established clinical scoring system to determine when the mice had reached a high level of arthritis, which resulted in a clinical score of 10-12 per mouse. Upon reaching a high clinical score, we then treated these mice with 4 g/kg, 2 g/kg and 1 g/kg of IVIg, 2 times per week. With this dosing scheme, we did not observe any decrease in clinical scores or amelioration of arthritis (data not shown). Next, we treated these mice with the same doses of IVIg, administered every other day. With this dosing scheme, we found that the mice that were given 2g/kg and 4 g/kg doses showed a similar, significant decrease in their clinical scores and in their paw measurements in comparison to mice given HSA. This is something that has not been shown before in this mouse model of arthritis, however it is comparable to results found in the K/BxN serum-transfer model (Campbell et al., 2014; Washburn et al., 2015) and in the more commonly known arthritis models such as the CAIA and CIA models (Spirig et al., 2018), demonstrating the efficacy of IVIg in mouse models of arthritis (Anthony et al., 2008a; Anthony et al., 2008b; Kaneko et al., 2006; Lee et al., 2014; Ohmi et al., 2016; Spirig et al., 2018). Importantly, the efficacy of IVIg in the CAIA model is dependent on the Fc of IVIg (Campbell et al., 2014). This supports our rationale for testing Fc-µTP-L309C in the K/BxN model.

Upon establishing the efficacy of IVIg in the K/BxN mouse model, we wanted to compare its effects with those of Fc- μ TP-L309C and SCIg. Both i.v. and i.p. injection of IVIg have been used previously without any evidence of a difference in efficacy. Because i.p. is easier and more volume can be injected, we used i.p. in our studies. We observed that SCIg and IVIg were equally effective at ameliorating endogenous arthritis and that Fc- μ TP-L309C was more effective than both SCIg and IVIg at 10-fold lower doses. It should be noted that Fc- μ TP-L309C also exhibited higher therapeutic efficacy when given intraperitoneally in both the CIA and in the CAIA mouse models of arthritis (Spirig et al., 2018). In our case, to better insure we would see an effect, we used high doses of Fc- μ TP-L309C to be very short (Spirig et al., 2018). Additionally, it has been proposed that subcutaneous absorption of biotherapeutics is relatively slow and mostly incomplete (Richter and Jacobsen, 2014). These factors also pose potential

issues in developing dosing schemes to test the efficacy of Fc- μ TP-L309C to treat human diseases.

Lastly, as a complementary study to our work in the endogenous model, we wanted to know if IVIg, SCIg or Fc- μ TP-L309C had any prophylactic activity. We used both the endogenous model and the serum transfer model to investigate this. For the endogenous model we treated mice with either IVIg, SCIg, or Fc- μ TP-L309C at weaning age before arthritis had developed. In both models, we found that both IVIg and SCIg prevented disease onset equally in these mice except for a small rebound at the start of the treatment cycle in the serum transfer model. Fc- μ TP-L309C however, exhibited complete prevention without any rebound at the start of the treatment cycle.

In summary, we are the first group to show that K/BxN mice, a model for human RA, having endogenous, chronic, severe arthritis can be effectively treated with IVIg, SCIg and with a recombinant protein, Fc-µTP-L309C. This model is a close representation of human autoimmune RA that is superior to the passive Ab-induced serum-transfer model of arthritis, which models only the effector phase of arthritis and does not involve the adaptive immune system. Our work demonstrates the utility of using the K/BxN mouse for evaluating potential therapeutic agents for treatment of RA. Although the efficacy of both IVIg and recombinant Fcs have yet to be confirmed in human studies, our results show that perhaps using higher doses and more frequent dosing in human RA could have benefit. Perhaps Ig-based therapies would show efficacy if used to treat certain subgroups of patients, or to treat patients with comorbidities that preclude them from using first line therapies, or to treat patients in whom other therapies are contraindicated. Importantly, the therapeutic effects of both IVIg and Fc-µTP-L309C could be achieved with s.c. routes of administration. The s.c. administration of IgG products has become increasingly attractive in recent years due to patient convenience (home administration) and better systemic tolerability. Considering that lower doses are needed for Fc-µTP-L309C to exhibit therapeutic efficacy in comparison to IVIg with the s.c. route of administration, Fc-µTP-L309C, given subcutaneously, could serve as a possible replacement therapy for IVIg/ SCIg in certain autoimmune diseases.

Chapter 4

Fc-µTP-L309C Reduces Antibody Production and Deposition in the Endogenous K/BxN Mouse Model of RA

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4.1 Abstract

Ab-mediated autoimmune diseases can generate ICs that are not cleared from the circulation and become highly pathogenic by engaging $Fc\gamma Rs$, activating complement and inducing a local inflammatory response. Arthritis in K/BxN mice is provoked by pathogenic auto-Abs to G6PI, which is a ubiquitously expressed enzyme that is present in cells, in the circulation and on the articular cartilage. When the G6PI auto-Abs deposit on the articular cartilage of K/BxN mice, arthritis ensues due to the activation of various components of the innate immune system.

Recent studies have investigated the *in vivo* efficacy of recombinant Fc protein-based therapeutics. Many of the recombinant Fc proteins that have been produced have shown to have a protective effect in mouse models of arthritis, such as the K/BxN serum transfer model. Fc- μ TP-L309C is a recombinant human IgG1 Fc with a point mutation at position 309 fused to the human IgM tail-piece to form a hexamer. We have previously shown that Fc- μ TP-L309C effectively prevents and ameliorates arthritis in the K/BxN serum transfer model and in the endogenous K/BxN model through an unknown mechanism. We decided to investigate the ability of Fc- μ TP-L309C to reduce Ab production and deposition on the articular cartilage of K/BxN mice.

First, we showed that Ab levels in K/BxN mice are reduced upon repeated injection of Fc- μ TP-L309C. We showed that this was not because of an effect of Fc- μ TP-L309C on FcRn, which can recycle pathogenic auto-Abs to promote inflammation. We further investigated this by looking at the effect of Fc- μ TP-L309C on B cells. We showed that Fc- μ TP-L309C does not bind to Fc γ RIIb on B cells to prevent Ab production. However, we demonstrated that Fc- μ TP-L309C decreased Ab production by B cells and reduced the subsequent deposition of auto-Abs on the articular cartilage potentially through a local increase in TGF- β and FoxP3⁺ Tregs in the joints.

Thus, Fc-µTP-L309C may provide a therapeutic benefit for Ab-mediated autoimmune diseases through its indirect effects on B cells.

4.2 Introduction

There is a need for therapies for Ab-mediated autoimmune diseases that affect Ab production itself. Ab-mediated autoimmune diseases can generate ICs that are not cleared from the circulation and become highly pathogenic by engaging Fc receptors, activating complement and inducing a local inflammatory response (Theofilopoulos and Dixon, 1980). The endogenous K/BxN mouse model of RA is a useful tool to understand how auto-Abs drive the progression of arthritis by interacting with downstream components of the innate immune system.

K/BxN mice are generated using KRN mice expressing a TCR transgene for G6PI peptide in the context of IAg7 MHC class II expressed by NOD/Lt mice (Ditzel et al., 2004; Matsumoto et al., 1999). The inflammatory response in the model is driven by auto-Abs against the ubiquitously expressed self-antigen, G6PI, which is present in cells, in the circulation and on the articular cartilage (Kouskoff et al., 1996). When the G6PI auto-Abs deposit on the articular cartilage of K/BxN mice, arthritis ensues due to the formation of ICs that can activate various components of the innate immune system (Wipke et al., 2004). Although the role of auto-Abs in the pathogenesis of RA remains controversial, the form of arthritis that K/BxN mice exhibit is like that experienced by humans. As in humans, the disease is chronic, progressive, and symmetrical, and it exhibits all the classical histological features such as, leukocyte invasion, synovitis, pannus formation, cartilage, and bone destruction (Korganow et al., 1994).

Recent studies have investigated the *in vivo* efficacy of recombinant Fc protein-based therapeutics. Various approaches for controlled multimerization of Fc to form polyvalent molecules have been explored (Zuercher et al., 2016). Fusion of the human IgG2 hinge region to human IgG1 Fc or mouse IgG2a Fc led to expression of multimerized Fcs that bound Fc γ R with high avidity (Jain et al., 2012). These molecules demonstrated therapeutic efficacy in animal models of arthritis with doses as low as ~50 mg/kg body weight.

Using an alternative strategy, Ortiz et al. (Ortiz et al., 2016) studied Fc multimers of increasing valency and identified molecules that bound $Fc\gamma Rs$ with high avidity without triggering activating signals. A trivalent molecule termed Fc3Y showed protection in mouse models of arthritis (Ortiz et al., 2016).

Finally, a hexameric Fc molecule showed increased binding to $Fc\gamma Rs$ and effectively interfered with $Fc\gamma R$ function (Qureshi et al., 2017). Additionally, this molecule was also very effective at treating animal models of arthritis.

A hexameric recombinant Fc multimer called Fc- μ TP-L309C was developed by CSL Behring. Fc- μ TP-L309C was produced by fusing the 18 aa IgM tailpiece to the C-terminus of a variant human IgG1 Fc with a point mutation at position 309 (Spirig et al., 2018). This point mutation facilitates the stabilized hexamerization of this molecule through the formation of disulphide bonds and distinguishes this recombinant hexamer from others reported (Spirig et al., 2018). We showed that Fc- μ TP-L309C has high binding avidity for FcRs and could suppress arthritis in the CIA, CAIA, and in the K/BxN mouse models (Lewis et al., 2019; Spirig et al., 2018). We decided to investigate the ability of Fc- μ TP-L309C to reduce Ab production and deposition on the articular cartilage of K/BxN mice.

We showed that Ab levels in K/BxN mice are reduced upon repeated injection of Fc- μ TP-L309C and that the subsequent deposition of Abs on articular cartilage was reduced. This was presumably due to a local increase in TGF- β and FoxP3⁺ Tregs in the joints of K/BxN mice.

4.3 Materials and Methods

4.3.1 Mice

KRN TCR transgenic mice on a C57BL/6 background were obtained from The Jackson Laboratory, a kind gift from C. Benoist. NOD/Lt mice were purchased from The Jackson Laboratory. Arthritic mice were obtained by crossing KRN mice (F, 6 weeks old) with NOD/Lt (M, 6 weeks old) mice to produce K/BxN mice expressing both the TCR transgene KRN and the MHC class II molecule I-Ag7. FcRn KO (M and F, 6 weeks old) mice and BALB/c (F 6, weeks old) mice were purchased from The Jackson Laboratory. Mice were kept under a natural lightdark cycle, maintained at 22 ± 4 °C, and fed with standard diet and water ad libitum. All experiments were performed after animal use protocols (AUP 1788) were approved by the University Health Network Animal Research Committee in Toronto.

4.3.2 Biological Reagents

Hizentra 20% SCIg, Fc-µTP-L309C, and Fc-µTP-L309C-H310L were from CSL Behring AG. HSA was from the Canadian Blood Services. Recombinant mouse G6PI was provided as a kind gift from R. Holmdahl (Karolinska Institutet, Stockholm, Sweden).

4.3.3 Anti-G6PI ELISA

5 μg/ml of recombinant mouse G6PI in PBS was coated on ELISA microtiter plates overnight at 4°C. Mouse peripheral blood was diluted using 10-fold serial dilutions (1:3 to 1:3000000) as described previously (Monach et al., 2008). Anti-G6PI Abs in the peripheral blood were detected with alkaline phosphatase conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories, Baltimore, PA). The concentration of the anti-G6PI Abs in the peripheral blood was determined using rabbit anti-mouse G6PI (clone A11695; Antibodies Online, Limerick, PA).

4.3.4 K/BxN Serum Transfer Arthritis

Severely arthritic adult K/BxN mice were bled and the sera was pooled. FcRn KO mice were injected i.p. with 200 μ l of pooled sera on days 0 and 2 as indicated in the figure legends. The volume of sera was chosen based on *in vivo* titration of pooled sera. Mice were given treatment on day 2 with either a s.c. injection of 200 mg/kg of Fc- μ TP-L309C or a s.c. injection of 2000 mg/kg of SCIg as indicated in the figure legends. HSA was used as a protein control.

4.3.5 Arthritis treatment in the K/BxN mouse model of endogenous, chronic arthritis

K/BxN mice with high clinical scores of 9 or greater were treated by either s.c. injections of 200 mg/kg Fc- μ TP-L309C, s.c. injections of 200 mg/kg Fc- μ TP-L309C-H310L, or s.c. injections of 2000 mg/kg of SCIg as indicated in the figure legends on days 1, 3, 5, 7, 9, and 11. HSA was used as a protein control.

4.3.6 Arthritis scoring

The clinical scores of the mice were monitored daily over the course of each experiment. The development of arthritis was assessed daily, and the severity of arthritis was scored for each paw on a 3-point scale, in which 0 = normal appearance, 1 = localized edema/ erythema over one surface of the paw, 2 = edema/ erythema involving more than one surface of the paw, 3 = marked edema/erythema involving the whole paw. The scores of all four paws were added for a composite score, with a maximum score of 12 per mouse.

4.3.7 Binding of Fc-μTP-L309C to FcγRIIb on B Cells

Spleens were removed from K/BxN mice and placed in RPMI 1640 + 5% FCS on ice. The spleens were pushed through a fine mesh strainer (70- μ m cutoff) to form a single cell suspension and RBCs were lysed. The cells were washed and resuspended in PBS + 2% FCS for cell counts. B cells were enriched using the EasySep mouse B cell enrichment kit (Stemcell Technologies, Vancouver, BC). Cells were treated with 100 μ g/ml of Fc- μ TP-L309C for 30 mins at 37°C and stained for B220-APC (clone RA3-6B2, Biolegend, San Diego, CA) and Fc γ RIIb-PE (clone AT130-2, Thermofisher Scientific). Fc γ RIIb staining was analyzed by flow cytometry on a BD LSRFortessa (BD Biosciences, San Jose, CA), and the data were analyzed by using FlowJo software (Ashland, OR).

4.3.8 Anti-G6PI ELISpot

The spleens and popliteal lymph nodes from K/BxN mice that recieved 6 s.c. injections of 200 mg/kg of Fc- μ TP-L309C were isolated and placed in RPMI 1640 + 5% FCS on ice. Mice treated with HSA were used as a control. The spleens and popliteal lymph nodes were pushed through a fine mesh strainer (70- μ m cutoff) to form a single cell suspension. RBCs were lysed from the spleens. The cells were washed and resuspended in PBS + 2% FCS for cell counts. 96 well filter plates (MilliporeSigma, Burlington, MA) were coated with 5 μ g/ml of recombinant mouse G6PI overnight at 4°C. Spleen cells and popliteal lymph node cells were seeded onto the plate at 1,000,000 cells per well at 37°C overnight. The cells were washed from the plate and the wells were stained with goat anti-mouse IgG (Jackson Immunoresearch Laboratories) conjugated to HRP for 2 hours at 37°C. AEC (SK-4200, Vector laboratories, Burlingame, CA) was added into each well and colour development could occur for 9 minutes until the reaction was stopped with water. The plates were left to dry at room temperature overnight and the spots on the membrane were counted and analyzed using ImmunoSpot software (San Diego, CA).

4.3.9 Immunofluorescence

Dissected ankle joints from K/BxN mice that recieved 6 s.c. injections of 200 mg/kg of Fc-µTP-L309C were embedded in OCT, frozen in liquid nitrogen and mounted on a cryomicrotome support at -25°C. Mice treated with HSA were used as positive controls and BALB/c mice were used as negative controls. Sagittal sections (6-8 µm thick) were cut and transferred to an adhesive coated slide. Slides were stored at -80°C until use and then acetone-fixed for 1 minute and air dried for 30 minutes. The deposition of IgG was detected by Alexafluor488-conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories). Nuclei were counterstained with DAPI (Thermofisher Scientific). Representative images were taken using the LeicaSP8/STED Confocal microscope (Leica Camera AG, Wetzlar, Germany) and the images were analyzed using ImageJ (Bethseda, MD).

4.3.10 TGF-β and IL-10 ELISA

TGF- β and IL-1- levels from the joints of K/BxN mice that were treated with 6 s.c. injections of 200 mg/kg of Fc- μ TP-L309C were analyzed using a TGF- β ELISA kit (R&D Systems, Minneapolis, MN) and an IL-10 ELISA kit (R&D Systems). Mice treated with HSA were used as a control.

4.3.11 Joint Washes

Joint washes were performed as previously described (Campbell et al., 2016). Malleoli and surrounding soft tissue (excluding fat) were removed from both rear limbs and placed in RPMI 1640 + 5% FCS on ice for 60 min. The medium was then removed, centrifuged, and the supernatant (joint wash) stored at -20°C until subsequent analysis.

4.3.12 Effect of Fc-µTP-L309C on Tregs in the K/BxN Mice

The spleens, popliteal lymph nodes, and joints from K/BxN mice that received 6 s.c. injections of 200 mg/kg of Fc-µTP-L309C were isolated and placed in RPMI 1640 + 5% FCS on ice. Mice treated with HSA were used as a control. The spleens and popliteal lymph nodes were pushed through a fine mesh strainer (70-µm cut-off) to form a single cell suspension. RBCs were lysed from the spleens. The cells were washed and resuspended in PBS + 2% FCS for cell counts. Malleoli and surrounding soft tissue (excluding fat) were removed from both rear limbs and placed in RPMI 1640 + 5% FCS on ice for 60 min. The washed malleoli and cell pellets were combined for each individual mouse and digested for 30 min at 37°C with 1 mg/ml collagenase (CLS-1, 250 U/mg; Worthington Biochemical, Lakewood, NJ) and 0.1 mg/ml DNase I ($1U/\mu$ L; Thermo Fisher). The digests were strained (70-µm cut-off), washed, and resuspended in PBS + 2% FCS for cell counts. Tregs were enriched using the EasySep mouse Treg cell enrichment kit (Stemcell Technologies). Single-cell suspensions of spleens, popliteal lymph nodes and joint digests were resuspended in PBS containing 2% FCS. Cells were blocked with CD16/32 (BD Pharmingen, San Jose, CA) and stained with CD4-APC-Alexa 750 (clone RM4-5, Thermofisher Scientific, Waltham, MA), CD25-APC (clone PC61, Biolegend, San Diego, CA), FOXP3-FITC (clone FJK-16s, Thermofisher Scientific, Waltham, MA). A cascade yellow viability stain (Thermofisher Scientific, Waltham, MA) was used to distinguish between live and dead cells. For intracellular staining, cells were permeabilized with a FOXP3 permeabilization kit (Thermofisher Scientific). Cells were fixed with 4% PFA and staining was analyzed by flow cytometry on a BD LSRFortessa (BD Biosciences, San Jose, CA), and the data was analyzed by using FlowJo software (Ashland, OR).

4.3.13 Statistical Analyses

Statistical tests were performed using GraphPad Prism 8 for Windows software. Analyses of differences between sample groups were performed using the tests indicated in the figure legends. Data shown are mean \pm standard deviation (SD), unless otherwise stated. P < 0.05 was considered statistically significant.

4.4 Results

4.4.1 Fc-µTP-L309C lowers anti-G6PI Ab levels and ameliorates arthritis independent of FcRn

K/BxN mice that were at high clinical scores of 9-12, were given 6 s.c. injections on days 1, 3, 5, 7, 9, and 11 of either 200 mg/kg of Fc- μ TP-L309C or 2000 mg/kg of SCIg, and HSA was used as a protein control. The anti-G6PI Ab levels in the peripheral blood of these mice were measured on days 0, 2, 4, 6, 8, 10, and 12 by ELISA. The anti-G6PI Ab levels in the peripheral blood of K/BxN mice lowered significantly over time in mice that received 6 s.c. injections of 200 mg/kg of Fc- μ TP-L309C and in mice that received 6 s.c. injections of 200 mg/kg of SCIg in comparison to mice that received HSA (Figure 4.1 A).

FcRn KO mice were injected i.p. with 200 µl of arthritic sera on days 0 and 2. Mice were given treatment on day 2 with either a s.c. injection of 200 mg/kg of Fc-µTP-L309C or a s.c. injection of 2000 mg/kg of SCIg and HSA was used as a protein control. FcRn KO mice that were treated with 1 s.c. injection of 200 mg/kg of Fc-µTP-L309C or of 2000 mg/kg of SCIg did not develop arthritis, as indicated by an increase in clinical scores (Figure 4.1 B).

K/BxN mice with high clinical scores of 9-12 were treated by either s.c. injections of 200 mg/kg Fc- μ TP-L309C, s.c. injections of 200 mg/kg Fc- μ TP-L309C-H310L (H310L abrogates binding to FcRn), or s.c. injections of 2000 mg/kg of SCIg as indicated in the figure legends on days 1, 3, 5, 7, 9, and 11. HSA was used as a protein control. Fc- μ TP-L309C-H310 ameliorated arthritis to a similar degree as Fc- μ TP-L309C, as indicated by a decrease in clinical scores (Figure 4.1 C).

4.4.2 Fc-µTP-L309C reduces antibody production and deposition in K/BxN mice

Spleens were dissected from K/BxN mice and treated *ex vivo* with 100 μ g/ml of Fc- μ TP-L309C to analyze Fc γ RIIb staining on B cells. 99% of B cells from spleens treated with Fc- μ TP-L309C stained positive for Fc γ RIIb (Figure 4.2 A).

K/BxN mice that were at high clinical scores of 9-12, were given 6 s.c. injections on days 1, 3, 5, 7, 9, and 11 of 200 mg/kg of Fc- μ TP-L309C, and HSA was used as a protein control. The spleens and popliteal lymph nodes from these mice were isolated on day 12 and anti-G6PI Ab production from B cells in these organs were measured using ELISpot. There was significantly less anti-G6PI production in the spleens and popliteal lymph nodes in K/BxN mice that received 6 s.c. injections of 200 mg/kg of Fc- μ TP-L309C in comparison to mice that received HSA (Figure 4.2 B and C).

K/BxN mice that were at high clinical scores of 9-12, were given 6 s.c. injections on days 1, 3, 5, 7, 9, and 11 of 200 mg/kg of Fc- μ TP-L309C, and HSA treated mice and BALB/c mice were used as controls. The ankle joints of these mice were dissected on day 12 and stained for IgG deposition on the synovial lining using immunofluorescence microscopy. This deposition was quantified using ImageJ. There was significant IgG deposition on the synovial lining of the ankle joint of K/BxN mice that were treated with HSA (Figure 4.2 D and E). There was significantly less IgG deposition on the synovial lining of the ankle joints of K/BxN mice that were treated with 6 s.c. injections of 200 mg/kg of Fc- μ TP-L309C (Figure 4.2 D and E). There was no IgG deposition on the synovial lining of the ankle joints of BALB/c mice (Figure 4.2 D and E).

4.4.3 Tregs are increased and TGF- β and IL-10 are increased in K/BxN mice after Fc- μ TP-L309C treatment

K/BxN mice that were at high clinical scores of 9-12, were given 6 s.c. injections on days 1, 3, 5, 7, 9, and 11 of 200 mg/kg of Fc- μ TP-L309C, and HSA was used as a protein control. The spleens, popliteal lymph nodes, and ankle joints were collected on day 12 and analyzed for Tregs using flow cytometry. Tregs were increased in each organ in mice treated with 6 s.c. injections of 200 mg/kg of Fc- μ TP-L309C in comparison to mice treated with HSA (Figure 4.3 A).

K/BxN mice that were at high clinical scores of 9-12, were given 6 s.c. injections on days 1, 3, 5, 7, 9, and 11 of 200 mg/kg of Fc- μ TP-L309C, and HSA treated mice and BALB/c mice were used as controls. The ankle joints of these mice were dissected on day 12 and the synovial fluid was collected for an ELISA to measure TGF- β and IL-10 levels. TGF- β and IL-10 levels were higher in mice given 6 s.c. injections of 200 mg/kg of Fc- μ TP-L309C in comparison to mice treated with HSA however, these results were not significant for TGF- β (Figure 4.3 B).





Α





- SClg
- 📥 HSA



Figure 4.1 Fc-µTP-L309C reduces anti-G6PI Abs in K/BxN mice that is independent of FcRn.

Shown are the concentrations (**A**) of anti-G6PI Abs in the peripheral blood of K/BxN mice that were treated with 6 s.c. injections of either 200 mg/kg of Fc- μ TP-L309C or 2000 mg/kg of SCIg, and HSA was used as a protein control. Injections were done on days 1, 3, 5, 7, 9, and 11 and blood collection was done on days 0, 2, 4, 6, 8, 10, and 12. Shown are the average anti-G6PI concentrations measured in ng/ml and the error bars indicate the range of concentrations (mean ± SD, n = 8). ***p < 0.05, one-way ANOVA with Dunnett test, compared with HSA. The clinical scores (**B**) are shown for FcRn KO mice given i.p. injections of 200 µl of arthritic serum on days 0 and 2, indicated by ^, that were treated with 200 mg/kg of Fc- μ TP-L309C or with 2000 mg/kg of SCIg on day 2, indicated by arrow, in comparison to mice treated with HSA. Shown are the average clinical scores; error bars indicate range of clinical scores (**C**) are shown for K/BxN mice given 6 s.c. injections of 200 mg/kg of Fc- μ TP-L309C, 200 mg/kg of Fc- μ TP-L309C-H310, or with 2000 mg/kg of SCIg on days 1, 3, 5, 7, 9, and 11. Shown are the average clinical scores; error bars indicate scores; error bars indicate range of clinical scores (mean ± SD, n = 4). *** p <0.001 compared with HSA, Kruskal–Wallis with Dunn test.



В

Α





D



lgG



Ε

Figure 4.2 Fc-μTP-L309C does not bind to FcγRIIb on B cells but does reduce anti-G6PI Ab production by B cells and IgG deposits in joints of K/BxN mice.

Shown are the percentages (**A**) of B cells that stained positive for FcγRIIb from the spleens of K/BxN mice that were treated *ex vivo* with 100 µg/ml of Fc-µTP-L309C or with HSA. Shown are the average percentages; error bars indicate range of percentages (mean \pm SD, n = 8). The immunoglobulin secreting cells (ISCs)/10⁶ PBMCs (**B**) are shown for the spleens and popliteal lymph nodes of K/BxN mice that were given 6 s.c. injections of 200 mg/kg of Fc-µTP-L309C, and HSA was used as a protein control. Shown are the average ISC/10⁶ PBMC; error bars indicate range of ISC/10⁶ PBMC (mean \pm SD, n = 8). *** p <0.001 compared with HSA, Mann-Whitney test. A representative well image (**C**) from one mouse for each organ with each treatment is shown. Representative images (**D**) of ankle sections from K/BxN mice that were given 6 s.c. injections of 200 mg/kg of Fc-µTP-L309C, mice were used as controls. Ankle sections were stained with anti-IgG in red. (**E**) The average IgG deposition expressed as an integrated density (the product of area and mean gray value) is shown; error bars indicate the range of integrated densities of IgG depositions (mean \pm SD, n = 10). **** p <0.0001 compared with HSA, Kruskal–Wallis with Dunn test.

Α









Β

Figure 4.3 Fc-μTP-L309C increases Tregs and IL-10 and TGF-β levels in the joints of K/BxN mice.

Shown are the percentages (**A**) of Tregs from the spleens, popliteal lymph nodes, and ankle joints of K/BxN mice that were treated with 6 s.c. injections of 200 mg/kg of Fc- μ TP-L309C and HSA was used as a protein control. Shown are the percentages of Tregs; error bars represent the range of percentages (mean ± SD, n = 9) *** p<0.001, compared with HSA, Mann-Whitney test. (**B**) of TGF- β and IL-10 in the synovial fluid of K/BxN mice that were treated with 6 s.c. injections of 200 mg/kg of Fc- μ TP-L309C and HSA was used as a protein control. Injections were done on days 1, 3, 5, 7, 9, and 11 and synovial fluid collection was done on day 12. Shown are the average TGF- β and IL-10 concentrations measured in pg/ml; error bars indicate the range of concentrations (mean ± SD, n = 3). * p < 0.05, compared with HSA, Mann-Whitney test.

4.5 Discussion

We observed that upon repeated administration of Fc- μ TP-L309C in K/BxN mice, the anti-G6PI concentration decreased in the peripheral blood of K/BxN mice (Figure 4.1A). We hypothesized that this could happen through the binding of Fc- μ TP-L309C to FcRn or through some effect of Fc- μ TP-L309C on B cells such that Ab production is reduced. In the K/BxN serum transfer model, arthritis develops in normal mice following the transfer of anti-G6PI Abs that complex with endogenous G6PI (Ji et al., 2002). These ICs infiltrate joints, where they initiate an inflammatory cascade within minutes following transfer (Korganow et al., 1999; Maccioni et al., 2002; Wipke et al., 2004). This model is therefore instructive for the analysis of therapies that target the humoral response.

We first investigated the effects of Fc-µTP-L309C on FcRn. FcRn protects IgG from degradation and it extends the serum half-life of IgG by recycling it. This is especially important in IgG mediated autoimmune conditions where auto-Abs are propagated throughout the body by FcRn to enhance pathogenicity (Ghetie et al., 1996; Junghans and Anderson, 1996; Ward and Ober, 2009). Enhanced degradation of auto-Abs and alleviation of autoimmune symptoms have been demonstrated after treatment of animals with molecules competing for binding of IgG to FcRn (Patel et al., 2011), and such molecules are currently in clinical development (Sockolosky and Szoka, 2015)

We have previously shown that Fc- μ TP-L309C bound to human FcRn with high avidity, and so it may block recycling of IgG through FcRn, thereby increasing auto-Ab degradation (Spirig et al., 2018). Since arthritis in the K/BxN serum transfer model is dependent on the availability of pathogenic Abs and FcRn is the receptor primarily responsible for extending IgG's life span, we examined whether Fc- μ TP-L309C was able to ameliorate arthritis in FcRn KO mice using the K/BxN serum transfer model. It should be noted that FcRn KO mice are generally resistant to serum transfer induced arthritis. However, the protective effect of an FcRn-deficiency could be partially overcome, when larger doses (500 or 1,000 μ l) of K/BxN serum are transferred. In this study we found that transferring larger doses of K/BxN serum (1000 μ l) to FcRn KO mice did induce arthritis, however, Fc- μ TP-L309C was able to ameliorate arthritis in these mice (Figure 4.1B). As a complimentary study, we also showed that when the H310L mutation is introduced
to Fc- μ TP-L309C that abrogates binding to FcRn, this molecule (Fc- μ TP-L309C-H310L) is still able to ameliorate arthritis after repeated administration in K/BxN mice with high clinical scores (Figure 4.1C). These results show that Fc- μ TP-L309C is not critically dependant on FcRn for its ability to ameliorate arthritis in K/BxN mice and that instead it likely involves a collaboration of various other mechanisms of action.

We then investigated the effects of Fc-µTP-L309C on B cells. Arthritis progression in K/BxN mice is driven by activation of T cells expressing the KRN TCR that recognizes G6PI bound to the NOD-derived I-A^{g7} molecule on MHC class II APCs (Mangialaio et al., 1999). Activated T cells subsequently interact with B cells through TCR: Ag⁷–MHC class II molecules and CD40:CD40L engagement, thereby promoting polyclonal B-cell activation and T-helper cell-dependent production of disease-inducing IgGs (Ditzel, 2004; Korganow et al., 1999).

Fc γ RIIb is the only Fc γ R expressed on B cells and it mediates an inhibitory signal when bound by IgG to halt Ab production by B cells (Coggeshall, 1998; Smith and Clatworthy, 2010). We have previously shown that Fc-µTP-L309C bound to human Fc γ RIIb with high avidity, and so it may reduce Ab production by B cells, thereby decreasing the number of pathogenic auto-Abs available (Spirig et al., 2018). We did indeed show that B cells in the spleens and popliteal lymph nodes of K/BxN mice that had been repeatedly administered Fc-µTP-L309C, decreased anti-G6PI production and that there was subsequently less IgG deposition on the ankle joints of K/BxN mice (Figure 4.2 B and C) however, this was not due to Fc γ RIIb. In fact, Fc-µTP-L309C did not bind to Fc γ RIIb in K/BxN mice (Figure 4.2 A). This phenomenon was also observed with other agents and it may be consistent with an evolutionary mechanism to re-establish immune homeostasis following prolonged exposure to ICs (Mousavi et al., 2007; Zhang and Booth, 2010). This may have implications for potential clinical application of hexameric and other multivalent forms of Fc.

Tregs also play a major role in mediating the humoral response through the suppression of auto-Ab production by B cells (Fuijo et al., 2012). Nguyen et al. (Nguyen et al., 2007) reported a role for Tregs in Ab-induced arthritis at several levels. First, they showed that a Treg deficiency in K/BxN mice led to more accelerated aggressive arthritis with significantly earlier auto-Ab production. They also showed that Tregs accumulated in the inflamed joints of K/BxN serumtransferred C57BL/6 mice, which suggested that Tregs actively migrate to the site of Ab-induced inflammation and control the local inflammatory process. The main ways in which these Tregs control the local inflammatory response is through the secretion of IL-10 and TGF- β (Maloy et al., 2003). IL-10 has inhibitory effects on cells of the innate immune system through supressing pro-inflammatory cytokine and chemokine production by activated monocytes/macrophages and neutrophils (Moore et al., 2001), whereas TGF- β is more known for its effects on B cells such as its ability to suppress B cell survival, proliferation, differentiation into plasmablasts, and Ab secretion. Although there is some evidence that suggests that IL-10 can indirectly affect Ab secretion by B cells by modulating B cell metabolism (Komai et al., 2018), more studies are needed to determine whether this is true.

We found that the number of Tregs in the joints of K/BxN mice increased upon repeated administration of Fc-µTP-L309C and that the number of Tregs in the spleens and popliteal lymph nodes of K/BxN mice decreased upon repeated administration of Fc-µTP-L309C (Figure 4.3 A). Possibly, these Tregs from the spleens and popliteal lymph nodes were being recruited to the joints in response to Fc-µTP-L309C administration. In line with these results, we also found that the levels of IL-10 and TGF- β in the synovial fluid of these mice also increased upon repeated administration of Fc-µTP-L309C, presumably because of the increase in Tregs. This increase in Tregs upon repeated administration of Fc-µTP-L309C could be attributed to regulatory T cell epitopes (Tregitopes) in the Fc domain of IgG. There are several Tregitopes that have been identified within the Fc fragment of IgG that can activate and expand Tregs (De Groot et al., 2008). It has been suggested that the immunosuppressive activity of IVIg is due to Tregitopes in the Fc fragment of IgG because Treg populations are expanded after IVIg therapy (Ephrem et al., 2008; Kessel et al., 2007). We hypothesize that Fc-µTP-L309C is bound by FcyRs on APCs and internalized and degraded such that Fc-µTP-L309C can be processed into Tregitopes. These Tregitopes are presented to T cells on MHC class II such that they activate and expand Tregs to diminish the immune response and further reduce tissue damage and systemic effects.

We showed that $Fc-\mu TP-L309C$ ameliorates arthritis through its effects on the humoral immune system. $Fc-\mu TP-L309C$ should be further investigated as a potential therapeutic for Ab-mediated autoimmune diseases.

Chapter 5

The Effect of Fc-µTP-L309C on Neutrophils in the Endogenous K/BxN Mouse Model of RA

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5.1 Abstract

Neutrophils are a pivotal cell type in the K/BxN serum transfer model and play an essential role in the progression of arthritis. They are readily activated by ICs via their Fc γ Rs to release IL-1 β , which induces cartilage destruction. Neutrophils also release neutrophil-active chemokines to recruit themselves in an autocrine manner to perpetuate tissue destruction. Fc γ R-expression on neutrophils is of crucial importance for the recognition of ICs. We decided to investigate the effect(s) of Fc- μ TP-L309C on neutrophils because Fc- μ TP-L309C has high avidity to human Fc γ Rs.

We observed that K/BxN mice treated with Fc- μ TP-L309C had significantly less neutrophils in their ankle joints. We decided to investigate whether Fc- μ TP-L309C influenced neutrophil recruitment to the joints or whether Fc- μ TP-L309C induced neutrophil death in the joint. We also investigated the functional effects of Fc- μ TP-L309C on neutrophils in K/BxN mice.

We found that Fc-µTP-L309C had no effect on neutrophil recruitment into the joint, however there was some neutrophil cell death. We investigated whether Fc-µTP-L309C induced neutrophil cell death via respiratory burst however, the results were unclear.

When we investigated the functional effects of Fc- μ TP-L309C, we found that it prevented cartilage destruction in K/BxN mice by inhibiting IL-1 β release from neutrophils by blocking Fc γ RIII.

Although the effects of Fc- μ TP-L309C on neutrophil cell death are unclear, it is evident that Fc- μ TP-L309C affects the function of neutrophils by blocking Fc γ RIII. Fc- μ TP-L309C could be a potential therapeutic candidate for autoimmune and inflammatory conditions whereby neutrophils are the predominant cell type involved in the pathogenesis.

5.2 Introduction

Neutrophils are arguably one of the most important effector cells in the induction of arthritis in the K/BxN serum transfer model (Wipke et al., 2001). This has been supported by numerous studies that have shown that neutrophil depletion, or neutrophil deficient mice are resistant to arthritis (Daley et al., 2008; Monach et al., 2010; Wang et al., 2012). Fc γ R activation on neutrophils is necessary for the initiation and the progression of arthritis because it mediates the release of IL-1 β . IL-1 β is a crucial driver of cartilage destruction in the K/BxN serum transfer model (Ji et al., 2002b; Joosten et al., 1999). When IL-1 β is released into the joint, it promotes the release of neutrophil-activating chemokines such as CXCL1, CXCL5, and CCL9, from resident tissue cells in the synovium (Chou et al., 2010). This causes neutrophils to recruit themselves in an autocrine manner to perpetuate tissue destruction (Chou et al., 2010; Jacobs et al., 2010; Ritzman et al., 2010). Given the fact that Fc γ R engagement with ICs is crucial for mediating arthritis development, we decided to investigate the effect(s) of Fc- μ TP-L309C on neutrophils because Fc- μ TP-L309C has high avidity to Fc γ Rs.

Fc- μ TP-L309C is a hexameric recombinant Fc multimer. It was produced by fusing the 18 aa IgM tail-piece to the C-terminus of a variant human IgG1 Fc with a point mutation at position 309 (Spirig et al., 2018). This point mutation facilitates the stabilized hexamerization of this molecule through the formation of disulphide bonds (Spirig et al., 2018). We have previously shown that Fc- μ TP-L309C has high binding avidity for Fc receptors and could suppress arthritis in the CIA, CAIA, and the K/BxN mouse models (Spirig et al., 2018). We decided to investigate whether the mechanism of Fc- μ TP-L309C in the endogenous K/BxN model involves neutrophils.

We observed that K/BxN mice treated with Fc- μ TP-L309C had significantly less neutrophils in their ankle joints however, there was no effect on neutrophil chemotaxis. We observed equivocal results regarding the effects of Fc- μ TP-L309C

We decided to investigate whether Fc-μTP-L309C influenced neutrophil recruitment to the joints because the engagement of FcγRs on neutrophils with ICs leads to the autocrine recruitment of neutrophils (Chou et al., 2010; Jacobs et al., 2010; Ritzman et al., 2010). Although

we found that $Fc-\mu TP-L309C$ binds to $Fc\gamma RIII$ on neutrophils however, there was no effect on neutrophil chemotaxis.

Next, we decided to investigate whether Fc-µTP-L309C induced neutrophil death in the joints of K/BxN mice. It has been shown that IVIg can regulate the survival of human neutrophils (Schneider et al., 2017) and so Fc-µTP-L309C may exhibit a similar effect as it is composed of the Fc portion of IgG. Using the TUNNEL assay, we found that there was more neutrophil cell death in the joints of K/BxN mice treated with Fc-µTP-L309C in comparison to K/BxN mice with severe arthritis given HSA as a protein control however, the results were not significant. A complementary assay was done using flow cytometry and a similar pattern emerged. We used flow cytometry and luminometry to determine whether the cause of neutrophil death was through respiratory burst however, the results were equivocal.

When we investigated the functional effects of Fc- μ TP-L309C on neutrophils, we found that it prevented cartilage destruction in K/BxN mice through the inhibition of IL-1 β release by blocking Fc γ RIII. Although the effects of Fc- μ TP-L309C on neutrophil cell death are unclear, it is evident that Fc- μ TP-L309C affects the function of neutrophils by blocking Fc γ Rs. Fc- μ TP-L309C could be a potential therapeutic candidate for autoimmune and inflammatory conditions whereby neutrophils are the predominant cell type involved in pathogenesis.

5.3 Materials and Methods

5.3.1 Mice

KRN TCR transgenic mice on a C57BL/6 background were obtained from The Jackson Laboratory, a kind gift from C. Benoist. NOD/Lt mice were purchased from The Jackson Laboratory. Arthritic mice were obtained by crossing KRN mice (F, 6 weeks old) with NOD/Lt (M, 6 weeks old) mice to produce K/BxN mice expressing both the TCR transgene KRN and the MHC class II molecule I-Ag7. BALB/c (F 6, weeks old) mice were purchased from The Jackson Laboratory. Mice were kept under a natural light-dark cycle, maintained at $22 \pm 4^{\circ}$ C, and fed with standard diet and water ad libitum. All experiments were performed after animal use protocols (AUP 1788) were approved by the University Health Network Animal Research Committee in Toronto.

5.3.2 Biological Reagents

Hizentra 20% SCIg and Fc-µTP-L309C were from CSL Behring AG. HSA was from the Canadian Blood Services.

5.3.3 Arthritis treatment in the K/BxN mouse model of endogenous, chronic arthritis

K/BxN mice with high clinical scores of 9 or greater were treated by either s.c. injections of 200 mg/kg Fc-μTP-L309C, or s.c. injections of 2000 mg/kg of SCIg as indicated in the figure legends on days 1, 3, 5, 7, 9, and 11. HSA was used as a protein control.

5.3.4 Arthritis scoring

The clinical scores of the mice were monitored daily over the course of each experiment. The development of arthritis was assessed daily, and the severity of arthritis was scored for each paw on a 3-point scale, in which 0 = normal appearance, 1 = localized edema/ erythema over one surface of the paw, 2 = edema/ erythema involving more than one surface of the paw, 3 = marked edema/erythema involving the whole paw. The scores of all four paws were added for a composite score, with a maximum score of 12 per mouse.

5.3.5 Neutrophil histology

Dissected ankle joints from K/BxN mice that recieved 6 s.c. injections of 200 mg/kg of Fc-µTP-L309C were embedded in OCT, frozen in liquid nitrogen and mounted on a cryomicrotome support at -25°C. Mice treated with HSA and BALB/c mice were used as controls. Sagittal sections (6-8 µm thick) were cut and transferred to an adhesive coated slide. Slides were stored at -80°C until use and then acetone-fixed for 1 minute and air dried for 30 minutes. Sections were incubated with the primary antibody to mouse Ly-6G and Ly-6C (clone NIMP-R14, LifeSpan Biosciences, Seattle, WA) in 1% BSA/PBS overnight at 4°C. The slides were rinsed with PBS and incubated with the goat anti-rat IgG secondary antibody conjugated to HRP (Thermofisher Scientific) in 1% BSA/PBS for 1 hour at room temperature. The slides were rinsed with PBS, and the neutrophils were visualized by incubation with 0.015% H₂O₂/0.05% diaminobenzidene (DAB) (Thermofisher Scientific) for 10 minutes at room temperature. Sections were taken using the LeicaSP8/STED Confocal microscope and the images were analyzed using QuPath.

5.3.6 Neutrophil transwell migration assay

Bone marrow cells were collected into complete medium at 5×10^6 cells/ml and these cell suspensions (200 µl) were added to the top of a polycarbonate transwell filter with a 3 µm pore (MilliporeSigma, Burlington, MA) inserted into a 24-well plate (Costar, Acton, MA) containing 300 µl of the above buffer with or without chemoattractant. The chemoattractant used was recombinant mouse CXCL1 (Biolegend, San Diego, CA). The plates were incubated at 37 °C with or without 100 µg/ml of Fc-µTP-L309C added into the top chamber. After 1 h, the filter inserts were removed, and the cells from the upper and lower chambers were removed and retained separately. The cells recovered from each of the wells and were cytospun onto slides to be analysed by immunofluorescence. The neutrophils were counted using a primary antibody to mouse Ly-6G and Ly-6C and goat anti-rat IgG secondary antibody conjugated to AlexaFluor 555 (Thermofisher Scientific). Neutrophils were counted using ImageJ. The results are expressed as the mean \pm SD of the chemotactic index (CI) for triplicate wells. The CI represents the fold-change in the number of untreated cells that migrated in response to control medium.

5.3.7 TUNEL assay

Dissected ankle joints from K/BxN mice that recieved 6 s.c. injections of 200 mg/kg of Fc-µTP-L309C were embedded in OCT, frozen in liquid nitrogen and mounted on a cryomicrotome support at -25°C. Mice treated with HSA and BALB/c mice were used as controls. Sagittal sections (6-8 µm thick) were cut and transferred to an adhesive coated slide. Slides were stored at -80°C until use and then acetone-fixed for 1 minute and air dried for 30 minutes. To analyze neutrophil cell death in the ankle joint, neutrophils were stained with a primary antibody to mouse Ly-6G and Ly-6C and goat anti-rat IgG secondary antibody conjugated to AlexaFluor 555 Apoptotic cells were labeled using an In-Situ Cell Death Detection Kit (Sigma Aldrich) according to the manufacturer's instructions. Briefly, a TUNEL reaction mixture, which incorporates fluorescein dUTP at DNA strand breaks, was used to detect apoptotic neutrophils. Nuclei were counterstained with DAPI (Thermofisher Scientific). Representative images were taken using the LeicaSP8/STED Confocal microscope and the images were analyzed using QuPath.

5.3.8 Determination of cell death and apoptosis by flow cytometry

Bone marrow cells were collected into complete medium and neutrophils were enriched using the EasySep mouse neutrophil enrichment kit (Stemcell Technologies). Neutrophils were incubated at 37°C for 4 hours, 14 hours and 24 hours with medium or with 100 µg/ml of FcµTP-L309C. To analyze neutrophil cell death, neutrophils were stained with Ly6G conjugated to APC (clone 1A8, Biolegend). Dead cells and apoptotic cells were labelled using an AnnexinV/Dead Cell Apoptosis Kit (Thermofisher Scientific). Cells were incubated at 37°C for 4 hours, 14 hours and 24 hours with mouse recombinant TNF (R&D Sytstems) as a positive control and with mouse recombinant GMCSF (R&D Sytstems) as a negative control. Staining was analyzed by flow cytometry on a BD LSRFortessa (BD Biosciences), and the data was analyzed by using FlowJo software (Ashland, OR).

5.3.9 Neutrophil Respiratory Burst

For luminometry, bone marrow cells were collected into complete medium and neutrophils were enriched using the EasySep mouse neutrophil enrichment kit. Neutrophils were added to the wells of a microtiter plate containing 100 μ l of luminol solution (Sigma Aldrich), and medium or 1 μ g /ml diphenyleneiodonium chloride (DPI) (BioShop, Burlington, ON) (negative controls), 1 μ g/ml of mouse IgG and goat-anti-mouse IgG immobilized on the plate (iIIC) (Sigma Aldrich) (positive control), or 100 μ g/ml of Fc- μ TP-L309C were added. Chemiluminescence was recorded at 37°C for 30 minutes on a TECAN Infinite 200 PRO microplate reader (Tecan, Mannedorf, Switzerland). The area under the signal-to-time curve was calculated, and results were expressed as relative light units (RLU).

For flow cytometry, 100 μ g/ml of Fc- μ TP-L309C, 1 μ g/ml of mouse IgG and goat-anti-mouse IgG (Sigma Aldrich) or medium (negative control) were added to neutrophils enriched from the bone marrow of K/BxN mice for 10 minutes at 37 °C in a water bath. Respiratory burst activity was assessed using dihydrorhodamine-123 (Thermofisher Scientific). FITC staining was analyzed by flow cytometry on a BD LSRFortessa and the data were analyzed by using FlowJo software.

5.3.10 Binding of Fc-µTP-L309C to FcγRIII on Neutrophils

Neutrophils were enriched from the bone marrow of K/BxN mice using the EasySep mouse neutrophil enrichment kit. Neutrophils were treated with 100 μ g/ml of Fc- μ TP-L309C for 30 mins at 37°C and stained for Ly6G-APC, CD11b-FITC (Biolegend) and Fc γ RIII-PE (Thermofisher Scientific). Fc γ RIII staining was analyzed by flow cytometry on a BD LSRFortessa and the data were analyzed by using FlowJo software.

5.3.11 IL-1β ELISA

Neutrophils were enriched from the bone marrow of K/BxN mice using the EasySep mouse neutrophil enrichment kit. Neutrophils were preincubated with 100 μ g/ml of Fc- μ TP-L309C for 15 minutes at 37°C, followed by 1 μ g/ml of mouse IgG and goat-anti-mouse IgG for 16 hours at 37°C. IL-1 β levels were analyzed using IL-1 β ELISA kit (R&D Systems, Minneapolis, MN).

IL-1 β levels from the joints of K/BxN mice that were treated with 6 s.c. injections of 200 mg/kg of Fc- μ TP-L309C were analyzed using IL-1 β ELISA kit (R&D Systems, Minneapolis, MN). Mice treated with HSA were used as a control.

5.3.12 Joint washes

Joint washes were performed as previously described (Campbell et al., 2016). Malleoli and surrounding soft tissue (excluding fat) were removed from both rear limbs and placed in RPMI 1640 + 5% FCS on ice for 60 min. The medium was then removed, centrifuged, and the supernatant (joint wash) stored at -20°C until subsequent analysis.

5.3.13 Safranin-O stain

Mice were euthanized and the left rear paws were fixed in 10% neutral-buffered formalin, decalcified, and embedded in paraffin. Sagittal tissue sections were stained with H&E and Safranin O (HistoTox, Boulder CO). Cartilage depletion was identified by presence of diminished Safranin O staining of the matrix and was indicated with an arrow.

5.3.14 Statistical Analyses

Statistical tests were performed using GraphPad Prism 8 for Windows software. Analyses of differences between sample groups were performed using the tests indicated in the figure legends. Data shown are mean \pm standard deviation (SD), unless otherwise stated. P < 0.05 was considered statistically significant.

5.4 Results

5.4.1 Fc-µTP-L309C has no effect on neutrophil migration

K/BxN mice that were at high clinical scores of 9-12, were given 6 s.c. injections on days 1, 3, 5, 7, 9, and 11 of 200 mg/kg of Fc- μ TP-L309C, and HSA treated mice and BALB/c mice were used as controls. The ankle joints of these mice were dissected on day 12 and stained for neutrophils using immunofluorescence microscopy. The neutrophils were quantified using Qupath. There were significant number of neutrophils in the ankle joints of mice that were treated with HSA (Figure 5.1A and B). There were significantly less neutrophils in the ankle joints of mice that were treated with 6 s.c. injections of 200 mg/kg of Fc- μ TP-L309C (Figure 5.1A and B). There were no neutrophils in the ankle joints of BALB/c mice (Figure 5.1A and B).

Using the trans-well assay, we found that Fc-µTP-L309C did not inhibit CXCL1 promoted chemotaxis (Figure 5.1C).

5.4.2 The effects of Fc-µTP-L309C on neutrophil cell death are unclear

K/BxN mice that were at high clinical scores of 9-12, were given 6 s.c. injections on days 1, 3, 5, 7, 9, and 11 of 200 mg/kg of Fc- μ TP-L309C, and HSA treated mice and BALB/c mice were used as controls. The ankle joints of these mice were dissected on day 12 and stained for dead neutrophils using immunofluorescence microscopy. The dead neutrophils were quantified using Qupath. There were no dead neutrophils in the ankle joints of K/BxN mice treated with HSA or in the ankle joints of BALB/c mice (Figure 5.2 A and B). There were more dead neutrophils in the ankle joints of 200 mg/kg of Fc- μ TP-L309C however, the results were not significant. (Figure 5.2 A and B).

As a complementary assay we investigated the number of dead neutrophils after 4, 14, and 24 hours of exposure to medium, Fc- μ TP-L309C, TNF, or GMCSF using flow cytometry. Dead neutrophils were identified as cells that stained positive for Ly6G, AnnexinV and propidium

iodide. There was no significant difference in neutrophil death between neutrophils that were exposed to Fc- μ TP-L309C or medium for 4, 14, or 24 hours. (Figure 5.2 C).

5.4.3 The effects of Fc-µTP-L309C on respiratory burst in neutrophils is unclear

The production of ROS by mouse neutrophils was detected over 30 minutes and chemiluminescence was evaluated with a luminometer. Neutrophils treated with iIICs exhibited significant ROS production over time (Figure 5.3 A), as shown by an increase in RLU. Whereas, neutrophils treated with medium, DPI (a known inhibitor of NADPH oxidase), or Fc-µTP-L309C did not exhibit any ROS production over time (Figure 5.3 A).

However, when ROS production was assessed using dihydrorhodamine-123, which can be detected in the FITC channel when respiratory burst occurs, contradictory results were observed. Although the frequency of FITC⁺ cells was significantly higher in neutrophils treated with mIgG + GAM Abs in comparison to neutrophils treated with Fc- μ TP-L309C (Figure 5.3 B), the frequency of FITC⁺ cells was significantly higher in neutrophils treated with Fc- μ TP-L309C in comparison to neutrophils treated with medium, which did not exhibit respiratory burst (Figure 5.3 B).

5.4.4 Fc- μ TP-L309C prevents cartilage destruction in K/BxN mice by preventing IL-1 β release from neutrophils by binding to Fc γ RIII

Neutrophils from the bone marrow of K/BxN mice were treated *ex vivo* with 100 μ g/ml of Fc- μ TP-L309C to analyze Fc γ RIII staining. 62% of neutrophils treated with Fc- μ TP-L309C stained positive for Fc γ RIII (Figure 5.4 A).

Neutrophils from the bone marrow of K/BxN mice were treated *ex vivo* with Fc- μ TP-L309C followed by mouse IgG and goat-anti-mouse IgG and IL-1 β levels were measured by ELISA. Neutrophils that were pretreated with Fc- μ TP-L309C exhibited significantly less IL-1 β production after 16 hours in comparison to neutrophils just treated with mouse IgG and goat-anti-mouse IgG (Figure 5.4 B).

K/BxN mice that were at high clinical scores of 9-12, were given 6 s.c. injections on days 1, 3, 5, 7, 9, and 11 of 200 mg/kg of Fc- μ TP-L309C, and HSA treated mice and BALB/c mice were used as controls. The ankle joints of these mice were dissected on day 12 and the synovial fluid was collected for an ELISA to measure IL-1 β levels. IL-1 β levels were significantly lower in mice given 6 s.c. injections of 200 mg/kg of Fc- μ TP-L309C in comparison to mice treated with HSA (Figure 5.4 C).

Histological evaluation using a Safranin-O stain for cartilage showed that Fc-µTP-L309C prevented cartilage erosion in the ankle joints of mice given 6 s.c. injections of Fc-µTP-L309C in comparison to mice treated with HSA (Figure 5.4 D).

Α





Ly6G/Ly6C

В







Figure 5.1 Fc-µTP-L309C reduces neutrophils in the joints of K/BxN mice without chemotactic interference.

Representative images (**A**) of ankle sections from K/BxN mice that were given 6 s.c. injections of 200 mg/kg of Fc- μ TP-L309C. Mice treated with HSA and BALB/c mice were used as controls. Ankle sections were stained for anti-Ly6G/anti-Ly6C (neutrophils) in brown and the tissue was counterstained with Hematoxylin. (**B**) The number of neutrophils/area (mm²) is shown; error bars indicate the range of neutrophils/area (mean ± SD, n = 10). **** p <0.0001 compared with HSA, Kruskal–Wallis with Dunn test. (**C**) The cells that migrated to the bottom chamber in response to the chemotactic gradient were collected and enumerated by fluorescence microscopy. The results are expressed as the mean ± SD of the CI of triplicate cultures.





Ly-6G

dUTP

DAPI

В





Figure 5.2 The effects of Fc-µTP-L309C on neutrophil cell death.

Representative images (**A**) of ankle sections from K/BxN mice that were given 6 s.c. injections of 200 mg/kg of Fc- μ TP-L309C. Mice treated with HSA and BALB/c mice were used as controls. Ankle sections were stained for anti-Ly6G in red, dUTP in green and DAPI in blue. (**B**) The number of dead neutrophils/area (mm²) is shown; error bars indicate the range of neutrophils/area (mean ± SD, n = 10). (**C**) The number of dead neutrophils at 4, 14, and 24 hours is shown; error bars indicate the range of dead neutrophils (mean ± SD, n = 10).

ROS Production over Time 1250-Medium DPI 1000illC 750· RLU Fc-µTP-L309C 500 250· 0 10 30 0 20 Time (min)

В



Figure 5.3 The effects of Fc-µTP-309C on ROS production in neutrophils.

ROS production over time (**A**) expressed as RLU; error bars indicate the range of RLU (mean \pm SD, n = 6). **** p <0.0001 for Fc-µTP-L309C compared with ilIC, one-way ANOVA with Dunnet test. Frequency of FITC⁺ cells (**B**) as an indicator of respiratory burst; error bars indicate the range of frequency (mean \pm SD, n = 8). **** p <0.0001 for Fc-µTP-L309C compared with mIgG + GAM Abs, Kruskal–Wallis with Dunn test.



Α









1 indicates cartilage erosion

Figure 5.4 Fc-μTP-L309C prevents IC induced IL-1β release from neutrophils to prevent cartilage destruction in K/BxN mice.

Shown are the percentages (**A**) of neutrophils that stained positive for FcγRIII from the bone marrow of K/BxN mice that were treated *ex vivo* with 100 µg/ml of Fc-µTP-L309C or with HSA. Shown are the average percentages; error bars indicate range of percentages (mean \pm SD, n = 6). ** p <0.01, compared with HSA, Mann-Whitney test. Shown are the concentrations (**B**) of IL-1 β from neutrophils treated *ex vivo* with mIgG and GAM Abs or with Fc-µTP-L309C and mIgG and GAM Abs. Shown are the average average IL-1 β concentrations measured in pg/ml; error bars indicate the range of concentrations (mean \pm SD, n = 8). **** p < 0.0001, compared with Dunn test. Shown are the concentrations (**C**) of IL-1 β in the synovial fluid of K/BxN mice that were treated with 6 s.c. injections of 200 mg/kg of Fc-µTP-L309C and HSA was used as a protein control. Injections were done on days 1, 3, 5, 7, 9, and 11 and synovial fluid collection was done on day 12. Shown are the average IL-1 β concentrations measured in pg/ml; error bars indicate the range of concentrations (mean \pm SD, n = 8). **** p < 0.0001, compared with HSA, Kruskal–Wallis with Dunn test. The representative Safranin-O stained sections of ankle joints (**D**) of BALB/c mice, HSA-treated mice and, Fc-µTP-L309C-treated mice after 11 s.c. injections are shown here.

5.5 Discussion

We have previously shown that Fc- μ TP-L309C is effective at treating K/BxN arthritis (Lewis et al., 2019). Because neutrophils have been shown to be the critical mediators of arthritis in this model and are present in high numbers in the synovium of RA patients, where they can promote significant joint inflammation and destruction, we wanted to determine whether Fc- μ TP-L309C affects neutrophils in the joints of K/BxN mice (Pillinger and Abramson, 1995; Wipke and Allen, 2001). First, we used DAB staining to detect neutrophils in the joints of K/BxN mice. There were significantly less neutrophils in mice that were given 6 s.c. injections of 200 mg/kg of Fc- μ TP-L309C in comparison to mice treated with HSA (Figure 5.1 A and B). We hypothesized that Fc- μ TP-L309C could be affecting the neutrophils in K/BxN mice in two major ways such that there would be less neutrophils in the joints.

Since we have shown that $Fc-\mu TP-L309C$ has high avidity to $Fc\gamma Rs$, we decided to investigate whether it could inhibit neutrophil chemotaxis, using a transwell migration assay.

Fc γ Rs have been shown to maintain and enhance cytokine and chemokine responses that occur during the initiation phase of arthritis (Kaplan et al., 2002). We used CXCL1 as a potent chemokine in this assay since it has been shown to coordinate leukocyte trafficking to the joint in the endogenous K/BxN model (Jacobs et al., 2010). However, we found that Fc-µTP-L309C did not inhibit the CXCL1 promoted chemotaxis of neutrophils (Figure 5.1 C).

We also investigated the ability of Fc- μ TP-L309C to induce cell death in neutrophils as an alternative mechanism to the decreased number of neutrophils in the joints of K/BxN mice. IVIg has been shown to regulate the survival of neutrophils and to induce neutrophil apoptosis in Kawaski disease patients (Schneider et al., 2017; Tsujimoto et al., 2002). We used the TUNEL assay to show that there was more neutrophil cell death in the joints of mice that were given 6 s.c. injections of 200 mg/kg of Fc- μ TP-L309C in comparison to mice treated with HSA (Figure 5.2 A and B). However, when we used flow cytometry to confirm these results, we found that there was no difference in the number of dead neutrophils treated with Fc- μ TP-L309C for 4, 14, or 24 hours in comparison to neutrophils in medium for the same length of time (Figure 5.2 C). We hypothesize that these results were different because they were obtained *in vitro* with smaller

exposure times to Fc- μ TP-L309C, and with smaller doses of Fc- μ TP-L309C, and in the absence of the microenvironment of the joint. This suggests an indirect mechanism for apoptosis.

Although IVIg has been shown to induce apoptosis in neutrophils, this occurs through an F(ab)'2 mechanism through functional antibodies to Fas and Siglec-9 in IVIg (Casulli et al., 2011; Schneider et al., 2017; von Gunten et al., 2006). Seeing as though Fc- μ TP-L309C does not contain any F(ab)'2s, we decided to investigate whether Fc- μ TP-L309C induces respiratory burst in neutrophils as an alternative way of regulating cell survival. The engagement of Fc γ Rs on neutrophils with ICs can induce ROS production and furthermore, respiratory burst (García-García and Rosales, 2002; Jakus et al., 2008; van der Heijden et al., 2014). Upon activation of neutrophils to ICs, the NADPH oxidase is assembled at cellular and granular membranes and becomes activated (Sengelov et al., 1992). This activation results in extracellular ROS generation, which can be detected by dihydrorhodamine-123, or intracellular ROS generation, which can be detected by dipoptosis (Simon et al., 2000). Crosslinking Fc γ Rs on neutrophils with multimeric Fc proteins such as Fc- μ TP-L309C, could potentially have the same effect.

We used luminometry to determine whether there was extracellular ROS production from neutrophils that were treated *ex vivo* with Fc- μ TP-L309C. However, we found that there was significant extracellular ROS production from neutrophils treated with iIICs but that there was no extracellular ROS production from neutrophils treated with Fc- μ TP-L309C (Figure 5.3 A). Our results are confounding because we found that there was significant intracellular ROS production from neutrophils treated significant intracellular ROS production from neutrophils treated with Fc- μ TP-L309C (Figure 5.3 A). Our results are confounding because we found that there was significant intracellular ROS production from neutrophils treated with Fc- μ TP-L309C (Figure 5.3 B).

It has been shown that $Fc\gamma R$ engagement, specifically $Fc\gamma RIII$, by ICs in neutrophils in K/BxN mice mediates IL-1 β release (Corr and Crain, 2002; Ji et al., 2002; Monach et al., 2010; Sadik et al., 2012). IL-1 β is a crucial mediator of cartilage destruction in K/BxN mice (Joosten et al., 1999). We decided to investigate whether Fc- μ TP-L309C could prevent the release of IL-1 β from neutrophils. First, we found that Fc- μ TP-L309C binds to Fc γ RIII on neutrophils from K/BxN mice (Figure 5.4 A). Then we found that Fc- μ TP-L309C prevents the IC stimulated release of IL-1 β from neutrophils *ex vivo* and that these results are mimicked *in vivo* because

there was significantly less IL-1 β from the synovial fluid of K/BxN mice that received 6 s.c. injections of 200 mg/kg of Fc- μ TP-L309C in comparison to mice that received HSA (Figure 5.4 B and C). Our histological findings mimicked our clinical findings because the Safranin-O stain revealed that Fc- μ TP-L309C prevented cartilage erosion in the ankle joints of mice in comparison to mice treated with HSA (Figure 5.4 D).

When we investigated the functional effects of Fc- μ TP-L309C on neutrophils, we found that it prevented cartilage destruction in K/BxN mice, potentially through the inhibition of IL-1 β release by blocking Fc γ RIII. Although the effects of Fc- μ TP-L309C on neutrophil cell death and respiratory burst are unclear, it is evident that Fc- μ TP-L309C affects the function of neutrophils by blocking Fc γ Rs. Fc- μ TP-L309C could be a potential therapeutic candidate for autoimmune and inflammatory conditions whereby neutrophils are the predominant cell type involved in pathogenesis. Chapter 6

Discussion

6.1 Summary and Significance of Key Findings

The purpose of this thesis was to investigate the therapeutic utility of Fc-µTP-L309C as a treatment for various autoimmune diseases. High-dose IVIg/SCIg, are used to treat a variety of autoimmune diseases; however, there are challenges associated with product production, availability, access and efficacy. Additionally, the mechanism(s) of action of IVIg/SCIg is unknown. Fc-µTP-L309C serves as a potential replacement for IVIg/SCIg because it is composed of the Fc of IgG and the immunomodulatory properties of IVIg are suggested to be Fc dependant.

IVIg is a first line therapy for ITP (Bierling and Godeau, 2004; Bussel, 1989; Bussel 2006; Fehr et al., 1982; Imbach et al., 1989; Tarantino, 2006) and so we decided to compare the therapeutic efficacy of Fc-µTP-L309C and IVIg/SCIg in a mouse model of ITP. We observed that Fc-µTP-L309C was more effective at ameliorating ITP in comparison to IVIg/SCIg at 10-fold lower doses (Chapter 2). This result is significant because there is a pressing need for a replacement for IVIg in Canada. IVIg usage for ITP has a substantial burden on the Canadian health care budget and this will continue to increase in the upcoming years (Hsia et al., 2015). Additionally, there has been significant growth in the utilization of IVIg for various other clinical conditions and it is estimated that the supply may not be able to meet the demand (Hsia et al., 2015).

IVIg usage for ITP should be reduced for several reasons as well. IVIg is associated with numerous potential side effects such as, headache, nausea, flushing, fevers, chills, fatigue, and diarrhea (Provan et al., 2010). Less commonly, it may result in serious complications, including anaphylaxis, hemolysis, thrombosis, renal failure, and aseptic meningitis (Caress et al., 2010; Guo et al., 2018; Provan et al., 2010). The donor variation that exists in different IVIg preparations means that its efficacy can vary from person to person. Further, IVIg is a limited by a finite blood supply, which is widely utilized and can impact the quality of life of patients, including the need for travel to an infusion clinic, the need to sit for several hours during the infusion, and anxiety over possible reactions.

Other than the obvious benefits of efficacy, Fc-µTP-L309C would also potentially solve most of the problems associated with IVIg usage in ITP. First, we have not observed any adverse effects

on the *in vitro* exposure of Fc- μ TP-L309C to human cells (Spirig et al., 2018) and we did not observe any adverse effects when Fc- μ TP-L309C was administered subcutaneously to mice (Chapter 2). There is no donor variation in different Fc- μ TP-L309C preparations because it does not rely on human blood donations. This would alleviate some of the financial burden on the Canadian health care system and it would minimize lot to lot variations. Lastly, Fc- μ TP-L309C would only be formulated for s.c. administration, which would significantly increase the quality of life of patients because they could administer the drug from the comfort of their own home. Indeed, we have shown that the s.c. administration of Fc- μ TP-L309C has similar efficacy to IVIg (Chapter 2).

When we began to investigate the mechanism through which Fc- μ TP-L309C ameliorates ITP, we observed that Fc- μ TP-L309C internalizes and degrades mouse activating Fc γ Rs (Chapter 2). This is important because it potentially leaves Fc γ RIIb free to take up ICs with subsequent antigen presentation to T and B cells (Amigorena et al., 1992; Antoniou and Watts, 2002). This has implications for therapeutic utility of Fc- μ TP-L309C as diseases involving an *en masse* blockade of a phagocytic component such as ITP may find better utility than those requiring interruption of self-antigen presentation of ICs.

IVIg is currently under evaluation for many other diseases such as RA (Katz-Agranov et al., 2015). With the limited amount of studies performed on the therapeutic efficacy of IVIg in RA (Kanik et al., 1996; Maksymowych et al., 1996; Muscat et al., 1995; Prieur et al., 1990; Pyne et al., 2002; Tumiati et al., 1992; Vaitla and McDermott, 2010) we decided to examine whether IVIg/SCIg could ameliorate arthritis in the endogenous K/BxN mouse model of RA (Chapter 3). Although IVIg has been shown to be effective in the K/BxN serum transfer model of RA, no work has been done to investigate its effect in treating K/BxN mice endogenously (Anthony et al., 2011; Bruhns et al., 2003; Campbell et al., 2014; Fiebiger et al., 2015).

After we found that IVIg/SCIg was effective at ameliorating arthritis, we compared the therapeutic efficacy of IVIg/SCIg to that of Fc-µTP-L309C. We are the first to use Ig-based agents to treat the spontaneous generation of RA in K/BxN mice. We used a multiple dose and frequency of dosing approach to treat K/BxN mice endogenously and found that Fc-µTP-L309C showed greater efficacy at 10-fold lower doses than IVIg/SCIg.

These results are significant because we showed that K/BxN mice can be treated endogenously and this offers a superior alternative to using the K/BxN serum transfer model. Although the clinical manifestations of arthritis in the K/BxN serum transfer model are very similar to those experienced in humans with RA, the progression of arthritis is very different. The progression of arthritis in the K/BxN serum transfer model is very aggressive and it develops just a few days after the transfer of arthritic serum. The development of RA occurs over a longer period with a peak incidence between 30 and 50 years of age (Yazici and Paget, 2000). Although the development of arthritis in K/BxN mice does not take as long as the human disease, it is not as aggressive as the K/BxN serum transfer model and occurs 1-3 weeks after weaning.

The K/BxN serum transfer model only represents the effector phase of the disease and it occurs in the absence of the adaptive immune system. The K/BxN serum transfer model is solely mediated by auto-Abs that drive the inflammatory response by IC formation and deposition, complement activation as well as activation of $Fc\gamma Rs$ on innate immune cells such as neutrophils, macrophages and mast cells (Firestein, 2003). The arthritis in K/BxN mice involves both the initiation and the effector phases of the disease, which requires both T and B cells. This is more physiologically relevant to the human disease because it is driven by complex interactions between a range of different cell types from both the innate and the adaptive immune systems.

Although the K/BxN serum transfer model is very useful for studying the effector mechanisms involved in progression of RA, it does not allow for any investigation into the initiation of the disease or for the involvement of the adaptive immune system. Our results provide an alternative model to the K/BxN serum transfer model that is more physiologically relevant to the human disease and that is reliable for testing potential therapeutics.

After we showed that Fc- μ TP-L309C was effective at treating arthritis in the endogenous K/BxN model, we began to investigate its mechanism of action in this model. We found that Fc- μ TP-L309C had a significant effect on the humoral system by reducing Ab production by B cells (Chapter 4). It is unclear exactly how this is established but nevertheless it is an important finding.

Although the pathogenicity of auto-Abs in RA has not been fully elucidated, there is a strong association between auto-Abs, such as anti-ACPA Abs and RF, and inflammation and ultimately bone destruction (McInnes and Schett, 2011). When auto-Abs deposit in the joints, mast cells become activated via $Fc\gamma Rs$ and release pro-inflammatory cytokines and chemokines (Nimmerjahn, 2006). This leads to the recruitment of neutrophils and monocytes to propagate joint inflammation. The microenvironment of the joint favours the differentiation of monocytes into osteoclasts, which bind ICs via $Fc\gamma Rs$ to promote bone resorption (Seeling et al., 2013).

In addition to human studies, the strongest evidence for a critical role of the humoral immune system in RA comes from mouse models, such as the K/BxN serum transfer model. These mouse models have permitted studying the complex disease mechanisms involved in the molecular and cellular processes ultimately resulting in joint inflammation and bone destruction (Ji et al., 2002). In the K/BxN serum transfer model, the passive transfer of serum from K/BxN mice with arthritis into healthy animals was enough for induction of joint inflammation and bone destruction (Christensen et al., 2016).

In addition to RA there are many other auto-Ab mediated autoimmune diseases such as SLE, Sjogren's syndrome, Myasthenia Gravis, Pemphigus diseases, etc. Autoimmune diseases in general have been increasing worldwide and the cumulative prevalence of autoimmune diseases caused by auto-Abs is well over 2.5% (Bach, 2002; Eaton et al., 2007; Vento and Cainelli, 2016). Despite developing insights into the pathogenesis of auto-Ab-mediated autoimmune diseases, systemic immunosuppression with steroids is still the first line therapy. Consequently, patients suffer from severe adverse effects, treatment associated morbidities and an increased mortality (Joly et al., 2002). Thus, there is a high, and thus far, unmet medical need for development of novel treatments for patients suffering from autoantibody-mediated autoimmune diseases and potentially Fc-µTP-L309C could alleviate that void.

Not only did we find that $Fc-\mu TP-L309C$ affects B cells, but we also found that it prevents the IC mediated release of IL-1 β from neutrophils by blocking $Fc\gamma RIII$ (Chapter 5). This prevented cartilage destruction in K/BxN mice. Although this mechanism is specific to the K/BxN serum transfer model, the broader significance of this work highlights the fact that Fc- μ TP-L309C could be used to target $Fc\gamma Rs$ in inflammatory and autoimmune diseases.

Fc γ Rs play a major role in the induction and maintenance of inflammatory responses induced by ICs in autoimmune diseases. It was established that activating Fc γ Rs, especially Fc γ RIIIa in humans and Fc γ RIV in mice, are important in driving Ab-induced inflammation and are associated with the development or chronic inflammation in mouse models such as CIA, autoimmune hemolytic anaemia and IC glomerulonephritis (Baudino et al., 2008; Bergtold et al., 2006; Diaz de Stahl et al., 2002; Monach et al., 2004; Ravetch, 2010). Additionally, transgenic mice expressing human Fc γ RIIa, which is the most abundant and prevalent activating Fc γ R in humans, are highly susceptible to tissue damage by ICs, implicating Fc γ RIIa as a central mediator of inflammation in humans (Tan Sardjono et al., 2005; Tsuboi et al., 2011; Van de Velde et al., 2010). In these animal models, IC binding to Fc γ R activates inflammatory cells, resulting in mediator release and/or direct tissue damage (Ravetch, 2010).

Several studies have reported the importance of activating Fc γ Rs in patients with RA. In a subgroup of patients with RA who had anti-CII Abs, it was shown that *in vitro* stimulation of their monocytes with illCs induced the release of the pro-inflammatory cytokines TNF α , IL-1 β and IL-8. This response was inhibited by blocking Fc γ RIIa (Mullazehi et al., 2006). Furthermore, in patients who tested positive for RF, ICs from synovial fluid stimulated monocytes and macrophages to produce TNF α and this response was blocked by the binding of monoclonal Abs to Fc γ RIIa (Mullazehi et al., 2006). Thus, Fc γ Rs are potential targets for the treatment of inflammation involving pathogenic Abs and Fc- μ TP-L309C may be a therapeutic strategy that is considered.

6.2 Limitations

6.2.1 Limitations of the mouse model of ITP

One of the major limitations to testing therapeutics in the passive model of ITP is that this model only captures the innate response associated with platelet destruction. This model is not conducive to studying the initiation of platelet autoimmunity with regards to T cell involvement because there is no break in immune tolerance Importantly, it has also been shown that there is no inflammatory response in passive models of ITP (Leontyev et al., 2014; Semple, 2010). Nevertheless, this model has been instrumental in understanding the physiological process of $Fc\gamma R$ -mediated platelet destruction in ITP. It is important to note that because the repertoire of $Fc\gamma Rs$ expressed by mice and humans are distinct, in vitro assays of phagocytosis with human cells should be used to validate the results (McKenzie et al., 1999; Mechetina et al., 2002; Ravetch and Kinet, 1991).

6.2.2 Limitations of the K/BxN mouse models of RA

There are two major limitations to using the K/BxN models to study RA. First, the K/BxN models rely heavily on auto-Abs for its disease pathogenesis. In RA, approximately 20-30% of patients are seronegative, which means that this model cannot be used to assess potential therapeutics for these patients (Barra et al., 2014; Sokka et al., 2009). For patients who are seropositive, the auto-Abs that are known to contribute in pathogenesis, such as ACPA and RF, are not present in the K/BxN models (Firestein, 2005; Monach et al., 2004; Ytterberg et al., 2012). Anti-G6PI auto-Abs have an essential role in triggering and maintaining arthritis in the K/BxN models, their presence in humans with RA is controversial and their pathogenic and diagnostic relevance is currently not clear (Herve et al., 2003; Kassahn et al., 2002; Matsumoto et al., 2019; Matsumoto et al., 2003; Schaller et al., 2001; Schubert et al., 2002). Even though G6PI might not be an essential autoantigen in RA, the K/BxN models are useful tools to understand how auto-Abs, in general, drive the progression of arthritis by interacting with different downstream components of the innate immune system. Therefore, finding agents that
ameliorate the pathologic conditions in K/BxN mice may translate to human RA regardless of the causative nature of the disease.

TNF and IL-6 are important disease mediators in RA, shown by the success of TNF/IL-6 blockade in treating this disease (Nishimoto et al., 2000; Toussirot and Wendling, 2007). Although TNF has been shown to be a potent mediator in the K/BxN serum transfer model, IL-6 has been shown to be completely dispensable (Ji et al., 2002). The other major difference is that IL-1 β is absolutely required for arthritis development in the K/BxN serum transfer model whereas, neutralizing Abs to IL-1 β in RA patients have had no effect on their disease outcome (van den Berg, 2001). These differences in the inflammatory milieu of the mouse model and the human disease highlight the fact that there may be challenges in testing therapeutics for RA in the K/BxN models.

6.2.3 Limitations of Fc-µTP-L309C

There are two major limitations to using Fc- μ TP-L309C as a therapeutic for autoimmune diseases. First, the ability of Fc- μ TP-L309C to achieve effective Fc γ R blockade at low doses requires careful consideration with respect to potential safety concerns. Fc γ R crosslinking is known to initiate signalling cascades that lead to cytokine release, platelet activation/aggregation, and complement activation (Finco et al., 2014; Walker et al., 2010; Yun et al., 2016). Cytokine release and complement activation can lead to acute and systemic inflammation and platelet activation can lead to deep vein thrombosis, myocardial infarction and stroke. It is important to test the potential toxicity risks in in vitro assays with human cells because mice do not replicate all the relevant aspects of human Fc γ R biology. Human clinical trials can also address potential toxicity issues and they can determine what the optimal route of administration is.

The other major limitation of Fc- μ TP-L309C is that it is rapidly cleared from the serum of mice, which is reflected in its short serum half-life of approximately 3 hours (Spirig et al., 2018). This short circulating serum half-life is likely due to internalization and degradation of Fc γ Rs upon exposure of Fc- μ TP-L309C and due to its inability to efficiently interact with FcRn. These

factors pose challenges for the clinical development of Fc- μ TP-L309C because it has a short therapeutic window for drug testing.

6.3 Future Directions

This thesis has shown that Fc- μ TP-L309C is more effective than IVIg/SCIg at ameliorating both ITP and RA in mouse models. However, significant work still needs to be done to determine its mechanism of action. In Chapter 2 we showed that Fc- μ TP-L309C can internalize and degrade the activating Fc γ RIII. However, it would be useful to investigate whether Fc- μ TP-L309C has similar effects on the other activating and inhibitory Fc γ Rs. Fc γ RIIb would be a specific point of interest as it has previously been shown to be spared from internalization and degradation by a similar hexamer (Qureshi et al., 2017). It would also be interesting to investigate whether Fc γ R functions are disrupted for a prolonged period after Fc- μ TP-L309C exposure. Flow cytometry could be used to investigate Fc γ R expression patterns on cells after Fc- μ TP-L309C binding in conjunction with various assays that determine Fc γ R function such as, phagocytosis assays. These studies are important to understand whether Fc- μ TP-L309C could contribute to immune dysfunction in diseases involving the chronic generation of ICs.

It could also be of interest to determine whether the high binding avidity of Fc- μ TP-L309C coupled with the internalization of Fc γ Rs results in any cytokine/chemokine release from various cell types. This could be tested by collecting the culture supernatant from cells that have been exposed to Fc- μ TP-L309C and testing for various cytokines/chemokines using Luminex technology.

In Chapter 3 we showed that Fc- μ TP-L309C can treat K/BxN mice endogenously upon repeated administration of the drug. The reduced duration of therapeutic efficacy could be attributed to the short serum half-life of Fc- μ TP-L309C however, it could also be due to an immune response against the human protein upon repeat dosing in mice. Immunogenicity and pharmacokinetics studies should be done in nonhuman primates to determine the longevity of the therapeutic effect of Fc- μ TP-L309C. These nonhuman primate studies will also be important to study the safety and toxicity of Fc- μ TP-L309C because they are a better model for human Fc γ R biology.

In Chapters 4 and 5 we started to investigate the mechanism of action of Fc- μ TP-L309C in the K/BxN model. In Chapter 4 we showed that Fc- μ TP-L309C reduces Ab production by B cells in K/BxN mice, which we proposed to be through the increase in Tregs. However, we never showed that there was a direct effect of the upregulation of Tregs on Ab production. We could investigate this using Treg depletion studies with monoclonal Abs, or we could isolate Tregs from successfully treated K/BxN mice with Fc- μ TP-L309C and adoptively transfer these Tregs to K/BxN mice with severe arthritis to determine whether there is a positive effect on arthritis.

It was hypothesized in Chapter 4 that Fc- μ TP-L309C may serve as a Tregitope that could activate and expand Tregs to ameliorate arthritis. It would be worthwhile to investigate this hypothesis further by exposing DCs to Fc- μ TP-L309C, and then by co-culturing T cells with DCs to determine if there is any Treg expansion. If Tregs are expanded, a standard suppression assay can be used to assess T effector cell function. Furthermore, the modulation of cytokine responses by Tregs can be further investigated using ELISA or flow cytometry. Tregitopes may explain the mechanism by which IVIg exerts its tolerogenic effect. Where those effects are Tregmediated, Tregitopes might serve as an alternative therapy to IVIG for autoimmune conditions that may be safer and more effective.

In Chapter 5 we showed that Fc- μ TP-L309C reduces IL-1 β production by neutrophils, which prevents cartilage destruction in K/BxN mice. Although neutrophils are arguably the most important mediator of tissue destruction in K/BxN mice through their engagement of ICs with Fc γ Rs, there are also other cells that engage ICs through their Fc γ Rs. Mast cells are thought to be initiators of arthritis in the K/BxN model whereby they release IL-1 β and can degranulate when they are activated through Fc γ RIII by ICs (Nigrovic et al., 2007). They can also release tryptase/heparin complexes, which were shown to induce the expression of the neutrophil chemoattractants, CXCL1, CXCL5, and CXCL8, in cultured fibroblast-like synoviocytes (Shin et al., 2009). Macrophages have been shown to release MIF upon ligation of their Fc γ Rs with ICs (Paiva et al., 2009). MIF mediates cartilage destruction and can promote osteoclastogenesis. Lastly, ligation of Fc γ RIV on osteoclasts have been shown to promote osteoclastogenesis and enhance bone resorption in K/BxN mice (Seeling et al., 2013). It would be interesting to investigate whether Fc- μ TP-L309C could also limit the effects of Fc γ R engagement on these cell types in addition to neutrophils.

6.4 Conclusions

This thesis showed that Fc- μ TP-L309C is more effective than IVIg/SCIg at ameliorating ITP and RA in mouse models. Fc- μ TP-L309C serves as a potential replacement for IVIg for certain autoimmune diseases and it can be administered at lower doses and subcutaneously.

For the first time, it has been shown that endogenous, chronic arthritis in K/BxN mice can be ameliorated using both IVIg/SCIg and Fc-µTP-L309C. This endogenous K/BxN model is superior to the commonly used K/BxN serum transfer model because the endogenous model involves a break in tolerance whereas, the serum transfer model only involves the passive transfer of Abs. Not only does this work provide the opportunity for researchers to use an improved mouse model of RA, but it also encourages IVIg testing in RA, given the fact that there is limited research on this topic and that safe and effective therapies are needed to treat this disease.

The mechanism of action of Fc- μ TP-L309C is multifactorial and is likely different in different models of autoimmunity. This thesis highlighted the effects of Fc- μ TP-L309C on the innate immune system through its engagement of Fc γ Rs on monocytes/macrophages and neutrophils. It also highlighted the effects of Fc- μ TP-L309C on the adaptive immune system through its ability to expand Tregs and modulate Ab secretion from B cells. These mechanistic insights may help to expand our knowledge of how IVIg works in autoimmune diseases where its immunomodulatory effects are Fc dependant.

Fc-μTP-L309C is currently being investigated in mouse models of neuromyelitis optica and it is undergoing toxicity testing in *in vitro* human assays with the hope of entering human clinical trials soon (Tradtrantip et al., 2018).

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Rolf Spirig, Ian K. Campbell, Sandra Koernig, Chao-Guang Chen, Bonnie J. B. Lewis, Rebecca Butcher, Ineke Muir, Shirley Taylor, Jenny Chia, David Leong, Jason Simmonds, Pierre Scotney, Peter Schmidt, Louis Fabri, Andreas Hofmann, Monika Jordi, Martin O. Spycher, Susann Cattepoel, Jennifer Brasseit, Con Panousis, Tony Rowe, Donald R. Branch, Adriana Baz Morelli, Fabian Käsermann and Adrian W. Zuercher

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rIgG1 Fc Hexamer Inhibits Antibody-Mediated Autoimmune Disease via Effects on Complement and FcγRs

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Activation of Fc receptors and complement by immune complexes is a common important pathogenic trigger in many autoimmune diseases and so blockade of these innate immune pathways may be an attractive target for treatment of immune complex-mediated pathomechanisms. High-dose IVIG is used to treat autoimmune and inflammatory diseases, and several studies demonstrate that the therapeutic effects of IVIG can be recapitulated with the Fc portion. Further, recent data indicate that recombinant multimerized Fc molecules exhibit potent anti-inflammatory properties. In this study, we investigated the biochemical and biological properties of an rFc hexamer (termed Fc- μ TP-L309C) generated by fusion of the IgM μ -tailpiece to the C terminus of human IgG1 Fc. Fc- μ TP-L309C bound Fc γ Rs with high avidity and inhibited Fc γ R-mediated effector functions (Ab-dependent cell-mediated cyto-toxicity, phagocytosis, respiratory burst) in vitro. In addition, Fc- μ TP-L309C prevented full activation of the classical complement pathway by blocking C2 cleavage, avoiding generation of inflammatory downstream products (C5a or sC5b-9). In vivo, Fc- μ TP-L309C suppressed inflammatory arthritis in mice when given therapeutically at approximately a 10-fold lower dose than IVIG, which was associated with reduced inflammatory cytokine production and complement activation. Likewise, administration of Fc- μ TP-L309C restored platelet counts in a mouse model of immune thrombocytopenia. Our data demonstrate a potent anti-inflammatory effect of Fc- μ TP-L309C in vitro and in vivo, likely mediated by blockade of Fc γ Rs and its unique inhibition of complement activation. *The Journal of Immunology*, 2018, 200: 000–000.

ntibody-mediated autoimmune diseases can give rise to immune complexes that are not readily cleared from the circulation but instead become highly pathogenic by engaging Fc receptors, activating complement, and initiating inflammatory pathways (1, 2). Ideally, an effective therapeutic would target these key innate immune pathways.

Plasma-derived IgG that is administered either i.v. (as IVIG) or s. c. has been increasingly used at high dose for the treatment of patients with chronic or acute autoimmune and inflammatory diseases, such as immune thrombocytopenia (ITP), Guillain-Barré syndrome, Kawasaki disease, chronic inflammatory demyelinating polyneuropathy, myasthenia gravis, and several other rare diseases

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(3). In addition, IVIG is currently under evaluation for many other diseases such as rheumatoid arthritis (4).

Several mechanisms of action have been proposed for the antiinflammatory effect of high-dose IVIG (5, 6). Some of these are dependent on the $F(ab')_2$ portion and include neutralization of autoantibodies by anti-idiotypic Abs (7, 8) and binding/ neutralization of immune mediators, such as cytokines (9). Other protective mechanisms are mediated by the Fc domain and include blockade of Fc γ Rs (10), saturation of the neonatal Fc receptor (FcRn) to enhance autoantibody clearance (11), scavenging of complement protein fragments (12) or modulation of immune cell activity (regulatory T cells, B cells, or tolerogenic

D.R.B. designed and interpreted experiments, and revised the manuscript. A.B.M., F.K., and A.W.Z. designed and interpreted experiments, and wrote the manuscript.

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Abbreviations used in this article: ADCC, Ab-dependent cell-mediated cytotoxicity; A4F, asymmetric flow field-flow fractionation; AP, alternative pathway; CAbIA, collagen Ab-induced arthritis; CIA, collagen-induced arthritis; CII, type II collagen; CP, classical pathway; FcRn, neonatal Fc receptor; HAGG, heat-aggregated γ globulin; IRB-SRC, Interregional Blood Transfusion Service SRC; ITP, immune thrombocytopenia; LP, lectin pathway; MALS, multiangle light scattering; NHS, normal human serum; SEC, size exclusion chromatography; Tg, transgenic; WT, wild-type.

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FIGURE 1. Biochemical characterization of rFc multimers. (**A**) Schematic diagram of Fc- μ TP and Fc- μ TP-L309C hexamer structures. Light gray bars represent presumed disulphide bonds. (**B**) SDS-PAGE of Fc- μ TP (left) and Fc- μ TP-L309C (right) rFc multimers. Molecular mass markers in kDa are shown. (**C**) SEC and (**D**) A4F with MALS of Fc- μ TP (left panels) and Fc- μ TP-L309C (right panels). Chromatograms show the normalized UV (280 nm) signals, and the bold lines are the molecular mass (in kDa) of material eluted at the time indicated.

DCs), for example, by upregulation of inhibitory $Fc\gamma RIIb$ (CD32b) (13). A therapeutic role for the IgG Fc domain in autoimmune diseases has been suggested in experimental models of arthritis and ITP (13, 14). Furthermore, in a clinical study, plasma-derived monomeric Fc successfully alleviated acute ITP in children (15).

Recent studies have investigated the in vivo efficacy of rFc protein-based therapeutics. In particular, various approaches for controlled multimerization of Fc to form polyvalent molecules have been explored (16). Fusion of the human IgG2 hinge region to human IgG1 Fc or mouse IgG2a Fc led to expression of multimerized Fc fragments that bound $Fc\gamma R$ with high avidity (17). These molecules demonstrated therapeutic efficacy in animal models of arthritis and ITP (17), as well as in a model of inflammatory neuropathy (18) and in experimental autoimmune myasthenia gravis (19). Interestingly, in most models, efficacy was achieved with low doses of ~50 mg/kg body weight, compared with the standard dose of 1000-2000 mg/kg for IVIG in inflammatory indications. Using an alternative strategy, Ortiz et al. (20) studied Fc multimers of increasing valency and identified molecules that bound FcyRs with high avidity without triggering activating signals. A trivalent molecule termed Fc3Y showed protection in mouse models of ITP, arthritis, and epidermolysis bullosa acquisita (20). Finally, a hexameric Fc molecule showed increased binding to FcyRs and dendritic cell-specific intercellular adhesion molecule-3 grabbing nonintegrin, appeared to fully activate complement (C1q binding and C5b-9 deposition) (21), and effectively interfered with FcyR function (22). Ab hexamer structures are reported to have superior complement fixing properties; indeed, upon binding to cell-surface Ags, IgG monomers spontaneously arranged into ordered hexamer structures that recruited and activated C1 (23). None of the molecules described earlier have progressed into human trials, and their protective mechanisms of action have not been fully elucidated.

We developed a hexameric rFc multimer and compared its therapeutic efficacy and mechanism of action with that of IVIG and rFc monomer in autoantibody-driven disease models. The multimer demonstrated high-avidity binding to Fc γ Rs and inhibition of Fc γ R-mediated effector functions; furthermore, it was unique among this class of molecules in also inhibiting the full activation of complement. A rapid and pronounced therapeutic efficacy was observed in mouse models of inflammatory arthritis and ITP.

Materials and Methods

Generation of $Fc-\mu TP$ and $Fc-\mu TP-L309C$ expression constructs

Fc- μ TP was generated by fusing the 18-aa residues (PTLYNVSLVMSD-TAGTCY) of human IgM μ -tailpiece to the C terminus of the C region of human IgG1 Fc fragment (aa residues 216–447, EU numbering; Uni-ProtKB P01857). Fc- μ TP-L309C was generated by mutating the Leu residue at 309 (EU numbering) of Fc- μ TP to Cys.

The DNA fragments encoding $Fc-\mu TP$ and $Fc-\mu TP-L309C$ were synthesized and codon optimized for human cell expression by Thermo Fisher Scientific (Waltham, MA). The DNA fragments were cloned between ApaL1 and XbaI sites of pRhG4 mammalian expression vector with an InTag adaptor for positive selection as described previously (24). Miniprep plasmid DNA was purified using the QIAprep Spin Miniprep ki (Qiagen, Hilden, Germany), and the sequence was confirmed by DNA sequencing analysis. Restriction enzymes and T4 DNA ligases were purchased from New England BioLabs (Ipswich, MA).

Transient expression in Expi293 cells

Fc- μ TP and Fc- μ TP-L309C, as well as fragments of human Fc γ Rs CD16a, CD32a, CD32b/c and CD64, were produced by transient transfection using the Expi293 Expression System (Thermo Fisher Scientific) according to the manufacturer's instruction and as described recently (25, 26). rFc preparations were purified using standard Protein A affinity purification techniques. The recombinant products used in this study (Fc, Fc- μ TP, and Fc- μ TP-L309C) were shown to be endotoxin-free by the limulus amebocyte lysate test and based on their inability to stimulate NF- κ B activation in THP1 cells (data not shown).

Table I. Biochemical characterization of rFc multimers

rFc Molecule ^a	Technique	Monomer (%)	Dimer (%)	Trimer (%)	Hexamer (%)	Multimer (%)
Fc-µTP	SEC-MALS A4F-MALS	13 (73 kDa) 10 (60 kDa)		2 (168 kDa)	84 (355 kDa) 87 (305 kDa)	3 (491 kDa)
Fc-µTP-L309C	SEC-MALS A4F-MALS	2 (62 kDa)	4 (114 kDa)	4 (211 kDa)	84 (383 kDa) 83 (327 kDa)	8 (745 kDa) 15 (592 kDa)

^arFc multimers were analyzed as described in Fig. 1C and 1D. Data show the percentage of material that was identified as monomer, dimer, trimer, hexamer, and multimer (dodecamer). The corresponding molecular masses (in kDa) are indicated in brackets.

Size exclusion chromatography-multiangle light scattering

Size exclusion chromatography–multiangle light scattering (SEC-MALS) was performed using an Agilent 1200 series HPLC in series with a Wyatt DAWN Heleos II EOS MALS detector and Wyatt Optilab T-rEx refractive index detector. For analysis, 24 μ g of Fc- μ TP-L309C was injected onto a 4.6 \times 300 mm WTC-030N5 SEC column (Wyatt Technology, Santa Barbara, CA) at room temperature. Isocratic elution was performed with PBS as mobile phase and a flow rate of 0.2 ml/min. Data were analyzed with Astra 6 software (Wyatt Technology) using a refractive index increment value of 0.185 ml/g.

Asymmetrical flow field-flow fractionation-MALS

Samples were tested using a Postnova AF2000 asymmetrical flow field-flow fractionation (A4F) coupled with Wyatt HELEOS light scattering and

Optilab rEX refractive index detectors and a Shimadzu PDA detector. The concentration and size data were collected with Wyatt's Astra 5 software and analyzed using Astra 6.1. The A4F channel was equipped with a regenerated cellulose membrane with a molecular mass cutoff of 10 kDa and a 350- μ m spacer.

Binding to $Fc\gamma Rs$ and FcRn by Biacore and Octet analyses

Binding to $Fc\gamma Rs$ was carried out using a Biacore T200 biosensor (GE Healthcare, Waukesha, WI). Recombinantly expressed $Fc\gamma R$ components CD16a, CD32a, CD32b/c, and CD64 were captured via their His tags on a nitrilotriacetic acid sensor chip, precharged with Ni²⁺, and the surface washed with 3 mM EDTA. Fc- μ TP-L309C was injected over captured receptors for 3 min at 100 nM, and dissociation was monitored for a further 30 min. After each injection, the surface was regenerated with a 60-s injection of 350 mM EDTA and 30-s injection of 50 mM NaOH. Sensorgrams were double

FIGURE 2. Binding of Fc- μ TP-L309C to Fc receptor. (**A**) Biacore analysis of binding of Fc- μ TP-L309C to Fc γ R components CD16a, CD32a, CD32b/c, and CD64. Affinity of monomeric Fc to CD16 was too low to generate a signal. (**B**) Binding of Fc- μ TP-L309C and IVIG to primary human monocytes and granulocytes. Data shown as mean + SEM, n = 3 individual experiments using cells from three different donors.



A

referenced by subtraction of signals from a blank injection, and a reference cell with no protein was captured. Running buffer throughout was 10 mM HEPES, 150 mM NaCl (pH 7.3). The analysis temperature was 37° C using a flow rate of 30 μ l/min.

Binding to FcRn at pH 6 (Sino Biological, Peking, China) was analyzed in a 96-well format on an Octet QKe device (FortéBio, Menlo Park, CA). The assays were visualized with the Octet Software 7.0.1.1 (FortéBio).

FACS evaluation of Fc-µTP-L309C binding to human granulocytes and monocytes

Cells were isolated from buffy coats obtained from healthy blood donors (Interregional Blood Transfusion Service SRC [IRB-SRC], Bern, Switzerland) by density gradient centrifugation over Ficoll-Paque (GE Healthcare). Granulocytes were isolated from the lowest layer with additional hypotonic lysis of the remaining erythrocytes. Monocytes were isolated from PBMCs using a CD14 microbeads kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were incubated with Fc-µTP-L309C or IVIG (CSL Behring AG, Bern, Switzerland) for 45 min at 4°C, washed four times with PBS containing 0.1% BSA (Sigma) and 0.01% NaN₃ (Sigma), and stained with a FTTC-labeled goat polyclonal F(ab')₂ Ab-fragment against human IgG (ab98534; Abcam) for 45 min at 4°C. Stained cells were washed a further four times and then analyzed with a BD FACSCanto II flow cytometer (BD Biosciences AG, Allschwil, Switzerland), and the data were evaluated using FlowJo.

Effect of Fc constructs on fluid-phase complement activation in human serum and whole blood

For the assay, normal human serum (NHS) was diluted 1:5 with GVB^{2+} buffer (Complement Tech) and incubated for 1 h at 37°C. The reaction was stopped by adding EDTA. Activation of human complement in serum was analyzed by the generation of C4a and C5a by ELISA (Quidel, San Diego, CA).

Analysis of complement activation in human whole blood was based on anticoagulation with recombinant hirudin (Sarstedt, Numbrecht, Germany). Whole blood was diluted 1:5 in GVB²⁺ buffer and incubated as described earlier for NHS. Complement activation was analyzed by the generation of C4a and sC5b-9 by ELISA (Quidel).

Binding of Fc constructs to Clq

Binding of rFc constructs to C1q was analyzed by ELISA (Inova Diagnostics, San Diego, CA). Wells were precoated with human C1q, and samples were added to allow binding. After washing of the wells to remove all unbound protein, purified peroxidase-labeled goat anti-human IgG conjugate was added. Unbound protein was removed by a further wash step and bound conjugate visualized with 3,3',5,5' tetramethylbenzidine substrate.

Effect of Fc constructs on complement deposition

The effect of Fc constructs on the function of the classical pathway (CP), lectin pathway (LP), and alternative pathway (AP) was examined in the Wieslab Complement System Screen (Euro-Diagnostica, Malmø, Sweden), which is an enzyme immunoassay for the specific detection of the three pathways with deposition of C5b-9 (detected with an anti-C9^{necopitope} mAb) as a common read-out. Samples were preincubated with 20% NHS diluted with GVB²⁺ buffer as described earlier. After 1-h incubation at 37° C, the samples were transferred to the ELISA plates. Further dilutions were made according to the instructions, that is, 1:101 for the CP and LP and 1:18 for the AP. The complement inhibitor Futhan (FUT-125; nafamostat mesilate; BD Biosciences) was used as a positive control. C1q-depleted serum was purchased from Quidel and purified C1q from Quidel and Complement Tech.

HUVECs were cultured according to the manufacturer's description (Lonza, Visp, Switzerland). For analysis of C3b, C1q, and C4b deposition, HUVECs were opsonized with an anti-CD105 (Endoglin) mAb (MEM-226; ab60902; Abcam) before incubation with 20% NHS diluted in GVB²⁺(1:5) for 30–60 min at 37°C. C3b deposition was detected using an FITCconjugated anti-C3c polyclonal Ab (F0201; Dako), anti-C1q polyclonal Ab (F0254; Dako), and anti-C4b polyclonal Ab (F0169; Dako) and quantified by FACS.

Analysis of cleavage of C2 by Western blot

Samples containing equal amounts of human serum (1 μ l each) were separated on a 4–12% NuPage Bis-Tris SDS-PAGE gel (Invitrogen) and transferred onto a polyvinylidene difluoride membrane (Invitrogen). Purified plasma-derived C2 was used as an additional control (Complement Tech). Equal loading was controlled by Coomassie staining of an identical



FIGURE 3. Fc- μ TP-L309C and the FcRn. (**A**) Binding to FcRn. Octet analysis of binding of Fc and Fc- μ TP-L309C (each at 25 μ g/ml) to FcRn at pH 6. (**B**) Pharmacokinetics in FcRn-Tg mice. IVIG and Fc- μ TP-L309C measured in the blood of FcRn-Tg mice following a single i.v. injection (all doses were 100 mg/kg) at time 0. Data show the means \pm range (n = 2); where absent, error bars are smaller than the symbol size.

gel (GelCode Blue; Thermo Scientific). After blocking the membrane overnight at 4°C with Superblock (Thermo Scientific), we detected C2 using mouse anti-C2 Ab (269716; MAB1936; 1:1000 in Superblock; R&D Systems) with HRP-conjugated goat anti-mouse IgG as the secondary Ab (P0447; 1:1000 in Superblock; Dako). Wash steps were performed with PBS/0.05% Tween 20. Finally, the membrane was developed with a chemiluminescence detection kit (SuperSignal West Pico; Thermo Scientific).

Respiratory burst in purified human granulocytes and monocytes

Human granulocytes were purified from buffy coats (IRB-SRC Bern) by dextran sedimentation (molecular mass 450–650 kDa) followed by Ficoll gradient centrifugation and hypotonic lysis of residual erythrocytes. A total of 50 μ l of granulocytes (2 × 10⁷/ml in HBSS containing 10 mmol/l HEPES, 1 mmol/l CaCl₂, 1 mmol/l MgCl₂, 1 mg/ml BSA) was added to the wells of a microtiter plate containing 100 μ l of luminol solution (0.01 mmol/l PBS; Sigma), and the test articles were added. Chemiluminescence was recorded at 37°C during 90 min, the area under the signal-to-time curve was calculated, and results were expressed as relative light units. Rabbit RBC (Charles River), previously treated with human IgG (CSL Behring) and therefore decorated with human anti-rabbit IgG, were used as positive control.

For inhibition experiments, granulocytes were preincubated with inhibitors (Fc monomer or Fc- μ TP-L309C) for 15 min at 37°C, followed by the luminol solution and IgG-treated rabbit RBCs.



FIGURE 4. Therapeutic effect of Fc- μ TP-L309C in acute Ab-mediated arthritis. (**A**) Protocol for evaluating therapeutic efficacy in CAbIA. (**B**) The clinical response to therapeutic administration of Fc- μ TP-L309C (200 mg/kg) or IVIG (2000 mg/kg) i.p. at day 6 (arrow). PBS served as control. Kinetics of response (left) and mean clinical scores over days 7–14 (right) are shown. (**C**) CD45⁺ cells recovered from knee joints of mice at day 8 of disease. (**D**) Histopathology of arthritic joints. Representative H&E-stained sections of tarsal joints from arthritic mice at day 8. The joint of a naive nonarthritic mouse is also shown. Original magnification ×40. (**E**) Histological analysis of joints. Data show the mean (\pm SEM) histological scores of joints at days 8 and 14. All data are means \pm SEM, pooled from two experiments. (**F**) Complement components C3 and C5a in arthritic mouse joint washes taken at day 8, determined by ELISA. Nonarthritic naive mouse joint washes were also included. Data show the complement component concentrations (means \pm SEM), which have been normalized as a percentage of the PBS control. *p < 0.05, **p < 0.01, ***p < 0.001 compared with PBS control, (**B** and **E**) Kruskal–Wallis with Dunn test, (**C** and **F**) one-way ANOVA with Dunnett test.

Monocytes were purified from peripheral blood by direct adherence to the wells of the microtiter plates; chemiluminescence was measured as described earlier for granulocytes.

Ab-dependent cell-mediated cytotoxicity assay

PBMCs were isolated from buffy coats obtained from healthy volunteers with blood group O (IRB-SRC Bern) by Ficoll gradient centrifugation. Subsequently, PBMCs were depleted of monocytes by adherence on polystyrene. As target cells, purified human Rh(D)⁺ RBCs of blood group O volunteers (IRB-SRC Bern) were used. The RBCs were papain treated and labeled with 10 μ mol/l CFSE (Molecular Probes) at 1.25 \times 10⁶ cells/and then opsonized with anti-D (Rhophylac; CSL Behring).

Lymphocytes $(2.2 \times 10^7/\text{ml})$ were preincubated with inhibitors (IVIG or FcµTP-L309C) for 30 min at 37°C; then 100 µl aliquots of the mixtures were added in triplicates to wells of microtiter plates previously incubated with 50 µl of target cells and 50 µl of anti-D (Rhophylac; 6 ng/ml) for 1 h at 37°C. After an overnight incubation at 37°C and 5% CO₂, microtiter plates were centrifuged and washed once with 0.9% NaCl before lysis of the sediment with 1% Triton X-100. Aliquots of the lysates were pipetted into the wells of a microtiter plate and fluorescence was determined (excitation 480 nm/emission 535 nm). Ab-dependent cell-mediated cytotoxicity (ADCC) was calculated using control samples (labeled RBCs without incubation with anti-D) and 100% lysis samples (RBCs treated with Triton X-100).

Phagocytosis assay with THP1 cells

THP1 cells were preincubated with IVIG or Fc- μ TP-L309C for 45 min on ice, followed by the incubation with IgG-coated FITC-labeled latex beads (Polysciences, Warrington, PA) for 3 h in the presence of IVIG or Fc- μ TP-L309C at 37°C. Afterwards, uptake of beads was analyzed by FACS. As control, Fc receptors were blocked using human Fc receptor binding inhibitor (Fc Block; eBioscience).

Analysis of calcium mobilization in human leukocytes

The effect on Ca²⁺ flux or mobilization of calcium was evaluated in a FACS assay using purified human leukocytes. Cells were incubated with the calcium indicator dye Cal-520 (AAT Bioquest) for 60 min at 37°C, washed, and resuspended in 5 ml of HBSS containing 1 mg/ml BSA/HEPES. Samples were added to FACS tubes, and recording was started. After equilibration, Fc- μ TP-L309C or heat-aggregated γ globulin (HAGG;



FIGURE 5. Therapeutic effect of Fc- μ TP-L309C in chronic autoimmune arthritis. (**A**) Protocol for evaluating therapeutic efficacy in CIA. (**B**) Clinical response to therapeutic administration of Fc- μ TP-L309C (200 mg/kg) or IVIG (2000 mg/kg) at the days indicated (arrows). PBS served as control. Kinetics of response (left) and mean clinical scores over days 2–28 (right) are shown (n = 6-8 mice). (**C**) Histopathology of arthritic joints. Representative H&E-stained sections of ankle joints from arthritic mice at day 28 of disease. Joints from IVIG-treated mice (data not shown) appeared similar to the Fc- μ TP-L309C group. Original magnification ×40. (**D**) Histological analysis of joints. Data show the mean (± SEM) histological scores of joints (n = 12-16) at day 28 of disease. *p < 0.05, **p < 0.01 compared with PBS control, Kruskal–Wallis with Dunn test.

control) were added and fluorescence recorded at room temperature for the indicated times. For analysis, cellular subsets were distinguished using forward and side scatter. For inhibition experiments, cells were equilibrated for 1 min before adding rFc multimers; after a 3 min fluorescence recording, activator (HAGG) was added and fluorescence was recorded for another 3 min. Total recording time was 7 min per sample.

Pharmacokinetics of Fc constructs

Human FcRn-transgenic (Tg) mice were injected i.v. or s.c. with IVIG or Fc- μ TP-L309C (100 mg/kg). In rats, the doses were 250 mg/kg IVIG and 25 mg/kg Fc- μ TP and Fc- μ TP-L309C. Serum concentrations were measured with an anti-human IgG1 ELISA (Cayman).

Collagen Ab-induced arthritis

Male BALB/c mice, aged >7 wk, were purchased from the Animal Resources Centre (Canning Vale, WA, Australia) and housed at the University of Melbourne Bio21 Institute animal facility for experimentation. Mouse anti–type II collagen (anti-CII) 5 clone mAb mixture kit was purchased from Chondrex (Redmond, WA). On day 0, mice were injected i.p. with 2 mg of mAb mixture followed by 50 µg of LPS i.p. on day 3. Mice were monitored for up to 14 d for clinical signs of arthritis. A clinical score was assigned by investigators who were blinded to the treatment groups as follows: 0, normal; 0.5, swelling confined to digits; 1, mild paw swelling; 2, marked paw swelling; 3, severe paw swelling and/or ankylosis. Mice that showed signs of arthritis at day 6 (i.e., clinical score ≥ 1) were given a single i.p. or s.c. injection of rFc multimer, IVIG, or PBS.

Collagen-induced arthritis

Male DBA/1J mice (aged 8–12 wk), obtained from Jackson Laboratories (Bar Harbor, ME) and bred in-house (Bio21 Animal Facility, University of Melbourne), were injected intradermally at the base of the tail with 0.1 ml of emulsion containing equal volumes of chick CII (2 mg/ml in 10 mM acetic acid; Sigma) and CFA (containing 5 mg/ml *Mycobacterium tuberculosis* H37RA; Becton-Dickinson), as described previously (27). The injection was repeated after 3 wk. Mice were assessed daily and recruited on the first day of clinical disease (as detailed earlier) into one of three treatment groups (PBS, 200 mg/kg Fc-µTP-L309C, or 2000 mg/kg IVIG). Mice were given multiple s. c. injections with reagents as detailed in the *Results*.

Histology of arthritic joints

Mice were killed and the left rear paws were fixed in 10% neutral-buffered formalin, decalcified, and embedded in paraffin. Sagittal tissue sections were stained with H&E and scored blinded to the treatment groups. Ankle joints were scored for exudate (presence of inflammatory cells within the joint space), synovitis (degree of synovial membrane thickening and inflammatory cell infiltration), and tissue destruction (cartilage and bone erosion and invasion), each out of 5 (0 = normal, 1 = minimum, 2 = mild, 3 = moderate, 4 = marked, and 5 = severe), and these were tallied for a total score out of 15.

Joint washes and cell digestions

Joint washes and cell digestions were performed as previously described (28). Patellas and surrounding soft tissue (excluding fat) were removed from both rear limbs and placed in RPMI 1640 + 5% FCS on ice for 60 min. The medium was then removed, centrifuged, and the supernatant (joint wash) stored at -30° C until subsequent analysis. The washed patellas and cell pellets were combined for each individual mouse and digested for 30 min at 37°C in a shaking incubator (140 cycles/min) with 1 mg/ml collagenase (CLS-1, 250 U/mg; Worthington Biochemical, Lakewood, NJ) and 0.1 mg/ml DNase I (type IV from bovine pancreas, 2100 kU/mg; Sigma). The digests were strained (70- μ m cutoff), washed, and resuspended in PBS + 2% FCS for cell counts and FACS.

FACS analysis of mouse blood and joint digests

Single-cell suspensions of peripheral blood and joint digests were resuspended in PBS containing 2% (v/v) FCS. Cells were stained with the

following anti-mouse mAbs: Ly6C (HK1.4; eBioscience), Ly6G (1A8; BioLegend), CD11b (M1/70; eBioscience), CD16/32 (93; eBioscience), CD64 (X54-5/7.1.1; BD Pharmingen, Mississauga, ON, Canada), and CD45 (30-F11; BD Pharmingen). Fixed cells were acquired on a BD Fortessa and analyzed with FlowJo software.

Protein array of cytokines/chemokines and complement

Joint washes and sera were evaluated for cytokine, chemokine, and complement levels using Luminex-Multiplex (Millipore) and C5a (R&D Systems) and C3 (Geneway) ELISAs.

Murine model of ITP

A passive Ab dose-escalation mouse model of ITP was used (29, 30). In brief, BALB/cJ mice (Jackson Laboratories) were given an i.p. injection of 68 $\mu g/kg$ anti-platelet mAb (rat anti-mouse glycoprotein IIb; CD41; clone MWReg30; BD Pharmingen) each of 2 d followed by a daily increase in dose of 34 $\mu g/kg$ platelet Ab for the duration of the experiment. Fc- μ TP-L309C or IVIG was given i.p. on day 2 when platelet nadir was attained. Platelet counts were followed daily using FACS (29).

Statistics

Statistical tests were performed using GraphPad Prism 6.0 for Mac OSX or Windows software. Analyses of differences between sample groups were performed using the tests indicated in the text. Data shown are means \pm SEM, unless otherwise stated. A *p* value < 0.05 was considered statistically significant.

Study approval

The CSL/Zoetis Animal Ethics Committees and the University Toronto Health Network Animal Research Committee approved all procedures and protocols.

Results

Development of multimerized Fc molecules by fusing IgG1 Fc with IgM μ -tailpiece

Recombinant human IgG1 Fc was multimerized by fusing the 18-aa IgM μ -tailpiece to the C terminus of either a wild-type (WT) human IgG1 Fc (Fc- μ TP) or a variant with a point mutation at position 309 (Fc- μ TP-L309C) (31). This resulted in the formation of Fc hexamers, with the point mutation of leucine 309 to cysteine (Fc- μ TP-L309C) providing a more stable structure than the WT (Fc- μ TP), because of the formation of covalent bonds between Fc molecules (Fig. 1A).

Nonreducing SDS-PAGE of the Protein A-purified rFc multimers showed a laddering pattern for each preparation, corresponding to the expected m.w. for monomer, dimer, trimer, tetramer, pentamer, and hexamers of the Fc fragment. Fc- μ TP-L309C, but not Fc- μ TP, had a predominant band at the expected m.w. for a hexamer, which is consistent with a more stable structure under the disruptive electrophoresis buffer conditions (Fig. 1B). Higher-order structures, most likely dimers of the hexameric molecule, were also evident for Fc- μ TP-L309C.

To examine the multimerization of the Protein A–purified rFc multimers under nondisruptive conditions, we performed SEC and A4F followed by MALS (Fig. 1C, 1D, respectively). Similar distribution patterns with a predominant hexamer peak of ~85% were observed for each of the rFc multimers with both procedures (Table I). The remaining material was mostly lower order (monomer) for Fc- μ TP or higher order species (dimers of the hexameric molecule) for Fc- μ TP-L309C. Because of its more stable structure, subsequent studies were performed using the Fc- μ TP-L309C molecule.

Fc- μ TP-L309C binds to Fc γ Rs and primary human myeloid cells

Fc-mediated effects are initiated through the binding of the Fc portion to specific receptors on the surface of leukocytes. To explore whether Fc- μ TP-L309C could engage Fc γ Rs, we first examined its qualitative binding to the specific Fc γ Rs, CD16a (Fc γ RIIIa), CD32a (Fc γ RIIa), CD32b/c (Fc γ RIIb/c), and CD64



FIGURE 6. Therapeutic effect of rFc multimers in experimental ITP. Dose escalation of anti-platelet Ab (MWReg30) maintains platelet nadir over time. Treatment with a single i.p. dose of Fc- μ TP-L309C at day 2 results in alleviation of ITP comparable with IVIG. Shown are the mean platelet values in the blood; data show the mean (± SEM) of platelet counts (Fc- μ TP-L309C, n = 5; IVIG and PBS, n = 3).

(Fc γ RI), by surface plasmon resonance. Fc- μ TP-L309C bound to all four receptors and displayed slower apparent off-rates compared with Fc monomer, consistent with an avidity effect either through its binding to multiple immobilized Fc γ R molecules or rebinding effects (Fig. 2A).

Next, the binding of Fc- μ TP-L309C to primary human myeloid cells was evaluated by FACS using fluorescently labeled anti-Fc Abs for detection. Fc- μ TP-L309C bound to primary human monocytes and granulocytes (Fig. 2B) with higher levels of binding to granulocytes as compared with IVIG.

Fc-µTP-L309C binds to FcRn

The ability of Fc- μ TP-L309C to bind FcRn was examined by Octet analysis using immobilized human FcRn at pH 6. Fc- μ TP-L309C bound to FcRn with a slower apparent off-rate compared with rFc monomer (Fig. 3A), suggesting an avidity effect.

Pharmacokinetics of Fc-µTP-L309C

The pharmacokinetics of Fc- μ TP-L309C was compared with that of IVIG after a single i.v. injection into human FcRn-Tg mice (Fig. 3B). Despite the ability to bind to FcRn with higher avidity (Fig. 3A), Fc- μ TP-L309C displayed a more rapid clearance from serum than IVIG, reflected in a shorter serum $t_{1/2}$ (3.1 h). Similar results were obtained in rats ($t_{1/2}$ of 2.5–3.0 h; data not shown).

$Fc-\mu TP$ -L309C provides therapeutic benefit in acute inflammatory Ab-induced arthritis

Fc- μ TP-L309C was examined for therapeutic efficacy in the collagen Ab–induced arthritis (CAbIA) model in mice (14, 28). Disease development in the CAbIA model is dependent on both Fc γ R engagement and activation of the complement system (32, 33) triggered particularly by the CP (34). Disease was induced in WT BALB/c mice by i.p. injection of a mixture of anti-CII mAbs followed by i.p. injection of LPS 3 d later (Fig. 4A). Mice with clinical signs of disease at day 6 were randomized and given a single i.p. injection of Fc- μ TP-L309C or IVIG, and disease was clinically evaluated up to day 14; untreated mice (PBS only)



FIGURE 7. Effects of Fc- μ TP-L309C on human complement in vitro. (**A**) Inhibition of classical complement pathway by Fc- μ TP-L309C. Wieslab ELISA Complement system kit was used. Data show the percentage of C5b-9 formation normalized to NHS values (mean \pm SEM, n = 3). HAGG and Futhan served as controls. (**B**) Fc and Fc- μ TP-L309C binding to C1q, determined by ELISA (mean \pm SD, n = 2). (**C**) Effect of Fc- μ TP-L309C on the generation of C4a (left) and sC5b-9 (right) in human whole blood. HAGG served as a positive control (mean \pm SEM, n = 4). ****p < 0.0001, one-way ANOVA with Dunnett test, compared with nonactivated. (**D**) Effect of Fc- μ TP-L309C on C2 cleavage in the presence and absence of HAGG, demonstrated by SDS-PAGE and Western blot for C2. The position of C2 is indicated by the arrow; molecular weight markers are shown at left in kiloDaltons. Representative Western blot from three independent experiments. (**E**) Inhibition by Fc- μ TP-L309C (1 mg/ml) of sC5b-9 generated in response to HAGG (1 mg/ml) in human whole blood (mean \pm SEM, n = 4). ****p < 0.0001, one-way ANOVA with Dunnett test, compared with HAGG. (**F**) Dose-dependent inhibition of C1q, C4b, and C3b deposition on HUVECs by Fc- μ TP-L309C, but not Fc monomer (mean \pm SEM, n = 4).

served as control. Fc- μ TP-L309C (200 mg/kg) reduced the clinical signs of disease more rapidly than IVIG (2000 mg/kg), with effects being evident within 24 h and sustained until day 14 (Fig. 4B). With 50 mg/kg a statistically nonsignificant trend to reduced clinical scores was observed (data not shown). Similarly, rapid and persistent effects were achieved via the s.c. route (I.K. Campbell, S. Koernig, and A.W. Zuercher, unpublished observations).

The reduced disease was reflected in the lower numbers of infiltrating (CD45⁺) leukocytes recovered from knee joints of mice treated with Fc- μ TP-L309C at day 8 of disease (Fig. 4C). Histopathology of the arthritic joints confirmed the clinical assessment; reduced inflammatory cell infiltrate, synovitis, and cartilage and bone destruction were evident in the Fc- μ TP-L309C-treated mice as early as 48 h after treatment (day 8 in Fig. 4D, 4E).

To further examine the effects of $Fc-\mu TP-L309C$ on the local inflammatory response, we evaluated cytokine/chemokine levels

in joint tissue washes at day 8 using protein arrays. Compared with the PBS control, Fc- μ TP-L309C reduced the levels of the proinflammatory cytokines IL-6, LIF, and G-CSF, as well as the chemokines IP-10 (CXCL10), MCP-1 (CCL2), KC (CXCL1), RANTES (CCL5), and MIP-2 (CXCL2) (Supplemental Fig. 1). The multimer did not reduce the levels of IL-9, MIG (CXCL9), or eotaxin (CCL11). In contrast, IVIG had minimal impact on the cytokine/chemokine levels at day 8, with a significant reduction observed only for MIP-2. This could reflect the slower kinetics of the IVIG response (Fig. 4B), which we previously reported in this model (14).

$Fc-\mu TP$ -L309C reduces complement activation in arthritic mouse joints

To determine whether Fc-µTP-L309C could alleviate disease through an effect on the complement system, we treated arthritic



FIGURE 8. Effect of Fc- μ TP-L309C on Fc receptor expression and function. (**A**) Induction of calcium flux in human granulocytes (n = 4 experiments) and monocytes (n = 2 experiments). HAGG served as positive control; one representative experiment is shown. (**B**) Fc- μ TP-L309C fails to activate the respiratory burst in human granulocytes (n = 4 experiments) and monocytes (n = 2 experiments); one representative experiment is shown. (**C**) Calcium flux in human granulocytes induced by HAGG is inhibited by Fc- μ TP-L309C; one representative of four experiments is shown. (**B**) AGG is inhibited by Fc- μ TP-L309C; one representative of four experiments is shown. (**B**) AGG is inhibited by Fc- μ TP-L309C; one representative of four experiments is shown. (**B**) AGG is inhibited by Fc- μ TP-L309C; one representative of four experiments is shown. (**B**) AGG is inhibited by Fc- μ TP-L309C; one representative of four experiments is shown. (**B**) AGG is inhibited by Fc- μ TP-L309C; one representative of four experiments is shown. (**B**) AGG is inhibited by Fc- μ TP-L309C; one representative of four experiments is shown. (**B**) AGG is and **C**) Mean \pm SD of duplicates are shown. (**D**) Fc- μ TP-L309C inhibits the activation of the respiratory burst in response to IgG-coated rabbit RBCs in human granulocytes. (**B** and **D**) Left and right columns are 1.5 and 0.4 mg/ml doses, respectively. (**E**) Inhibition of ADCC of anti-D-treated O⁺ human RBCs by Fc- μ TP-L309C (mean \pm SEM, n = 3). (**F**) Inhibition of phagocytosis of IgG-coated FITC-labeled latex beads by THP1 cells. Fc Block was used as control (mean \pm SEM, n = 3).

mice at day 6 with PBS, IVIG, or Fc- μ TP-L309C, and C3 and C5a levels were determined in joint washes by ELISA at day 8. Both complement components were elevated in the joint washes of arthritic mice compared with naive mice (Fig. 4F), whereas Fc- μ TP-L309C reduced these levels. In contrast, IVIG treatment only slightly reduced C3, and C5a levels were unaffected.

$Fc-\mu TP$ -L309C provides therapeutic benefit in the chronic inflammatory collagen-induced arthritis model

Having shown the therapeutic benefit of a single injection of FcµTP-L309C in the treatment of acute inflammatory arthritis, we next determined its effect in collagen-induced arthritis (CIA), a model of chronic inflammatory autoimmune disease. In CIA, mice generate a continuous supply of anti-CII autoantibodies following immunization with CII, resulting in chronic disease, which enables the evaluation of a prolonged treatment regimen with multiple drug injections. DBA/1 mice were immunized and boosted with chick CII in CFA (27) and treated on the first day of clinical disease with s.c. injection of Fc-µTP-L309C (200 mg/kg), IVIG (2000 mg/kg), or PBS. The mice were monitored for 28 d, and treatment was repeated as outlined in Fig. 5A. As observed in the CAbIA model, Fc-µTP-L309C (at 200 mg/kg) rapidly reduced disease (within 24 h), whereas IVIG (at 2000 mg/kg) was also effective, but the response was relatively delayed (Fig. 5B). Repeated Fc-µTP-L309C injections were necessary to sustain the therapeutic response as disease returned when treatment was ceased for 8 d (see Fig. 5B, days 15-23). Histological evaluation confirmed the clinical findings, with both Fc-µTP-L309C and IVIG significantly reducing disease (inflammatory cell infiltrate and tissue destruction) (Fig. 5C, 5D).

$Fc-\mu TP$ -L309C provides the rapeutic benefit in a murine ITP model

The therapeutic effect of Fc- μ TP-L309C was tested in a murine ITP model (Fig. 6). Mice were treated with a single dose of Fc- μ TP-L309C (200 mg/kg) when the platelet nadir was attained 2 d after initiating Ab-mediated depletion. Treatment with Fc- μ TP-L309C resulted in increased platelet numbers, which were significantly higher at days 3 and 4 (p < 0.01 and p < 0.0001, respectively, two-way ANOVA with Tukey test) than for mice treated with a 10-fold larger dose of IVIG (2000 mg/kg) (Fig. 6).

$Fc-\mu TP$ -L309C inhibits complete classical complement pathway activation and is C1q dependent

Because an attenuated complement response was observed in arthritic mice (Fig. 4F), we investigated whether Fc- μ TP-L309C interfered with complement activation. Using the Wieslab ELISA Complement system screen kits, we found that Fc- μ TP-L309C inhibited the full activation of the CP and LP complement pathways but had no effect on the AP (Fig. 7A, Supplemental Fig. 2A). Furthermore, we have evaluated the effect of Fc- μ TP-L309C on the CP without preincubation of rFc multimer with serum. As shown in Supplemental Fig. 3A, Fc- μ TP-L309C still prevented C5b-9 formation with similar potency. These findings were confirmed in hemolytic CP and AP complement assays using sheep and rabbit RBCs, respectively (data not shown).



- Opsonization of cells with C3b

- Deposition of the terminal complex C5b-9 and cell lysis

FIGURE 9. Effects of rFc constructs on human complement. Inhibition of the classical complement pathway by Fc- μ TP-L309C. High-avidity binding of C1q and subsequent depletion of C4, but no downstream activation of the complement cascade. Consequently, Fc- μ TP-L309C inhibits generation of anaphylatoxins C3a and C5a, opsonization by C3b, and deposition of the terminal membrane attack complex C5b-9.

Next, we examined in detail the effect of Fc-µTP-L309C on the CP. We observed incomplete activation of the CP, evidenced by strong binding to C1q (Fig. 7B) and cleavage of C4 (Fig. 7C, Supplemental Fig. 2B), but no generation of sC5b-9 (Fig. 7E) and C5a (Supplemental Fig. 2B). Interestingly, it appeared that activation of the CP stalled at the assembly of the C3 convertase because there was no evidence for cleavage of C2 (Fig. 7D). As the next step, we investigated whether the inhibitory effect of FcµTP-L309C on the LP is C1q dependent. As shown in Supplemental Fig. 4A, no cleavage of C4 was observed when Fc-µTP-L309C was incubated in C1q-depleted serum. Furthermore, no inhibition of the LP was observed when Fc-µTP-L309C was incubated in C1q-depleted serum, whereas the inhibitory effect was restored when C1q-depleted serum was reconstituted with purified C1q (Supplemental Fig. 4B). As a control, C1qdepleted serum was tested in the CP and, as expected, the CP was inactive, but activity was restored when reconstituted with purified C1q (Supplemental Fig. 4B). Surprisingly, we observed a reduced activity in C1q-depleted serum reconstituted with purified C1q in the LP. Purified C1q from two different distributors were tested with similar results.

The effect of Fc- μ TP-L309C on activation of the CP mediated by HAGG was also investigated (Fig. 7D, 7E). As observed earlier (Fig. 7C), preincubation of human serum or whole blood with Fc- μ TP-L309C led to the generation of C4a, which was not further affected by subsequent addition of HAGG (data not shown). However, the Fc- μ TP-L309C fully inhibited further downstream activation of the CP, as shown by the inability of HAGG to induce cleavage of C2 (Fig. 7D) or promote the formation of sC5b-9 (Fig. 7E) and C5a (data not shown). Next, we investigated whether Fc- μ TP-L309C still inhibited HAGG-induced sC5b-9 formation when given simultaneously and in less diluted whole blood (90% whole blood instead of the 20% whole blood shown in Fig. 7D). As shown in Supplemental Fig. 3B, Fc- μ TP-L309C significantly prevented sC5b-9 formation even under these assay conditions.

$Fc-\mu TP$ -L309C inhibits complement deposition on human endothelial cells in vitro

We investigated whether Fc-µTP-L309C prevented complement deposition on cells in a more physiological system using a complement fragment deposition assay with HUVECs (see *Materials and Methods*). Incubation of opsonized HUVECs with serum containing Fc- μ TP-L309C resulted in a dose-dependent inhibition of C1q, C4b, and C3b deposition (Fig. 7F), whereas monomeric Fc or IVIG did not have an effect at the same doses (data not shown for IVIG). As the next step, we investigated whether Fc- μ TP-L309C could still prevent complement deposition without preincubation or even postactivation. As shown in Supplemental Fig. 3C, Fc- μ TP-L309C still inhibited C3b deposition when given simultaneously or even up to 15 min postactivation.

$Fc-\mu TP$ -L309C inhibits $Fc\gamma R$ -mediated functions in vitro

Next we investigated whether the anti-inflammatory effect of FcµTP-L309C, as observed in the arthritis models, might be caused by modulation of $Fc\gamma R$ function. Human in vitro systems were used for this purpose. Exposure to Fc-µTP-L309C did not lead to calcium flux or respiratory burst in primary human granulocytes (Fig. 8A, 8B). In contrast, Fc-µTP-L309C induced calcium flux in monocytes (Fig. 8A), but no respiratory burst (Fig. 8B). Importantly, preincubation of granulocytes with Fc-µTP-L309C inhibited subsequent calcium flux induced by HAGG (Fig. 8C) and respiratory burst induced by IgG-coated RBCs (Fig. 8D). In more complex and functional in vitro systems, Fc-µTP-L309C inhibited ADCC (Fig. 8E) and reduced FcyR-mediated phagocytosis (Fig. 8F). Taken together, our data suggest that, in addition to effects on the complement pathways (summarized in Fig. 9), blockade of FcyRs is another possible anti-inflammatory effector mechanism of Fc-µTP-L309C.

Discussion

In this study, we describe the functional characterization of a hexameric IgG1-Fc molecule, termed Fc- μ TP-L309C, that was designed to therapeutically block effector pathways in immune complex-mediated diseases. Indeed, Fc- μ TP-L309C effectively and rapidly suppressed established acute (Fig. 4B) and chronic arthritis (Fig. 5B), as well as ITP (Fig. 6), in mice. Importantly, these effects were achieved with 10-fold lower doses than IVIG and were reproduced using s.c. administration in the arthritis models. The main mechanisms responsible for these effects are likely to be inhibition of Fc γ R-mediated effector functions of pathological autoantibodies and inhibition of the CP. Blockade of

FcRn could be an additional mechanism potentially contributing to the therapeutic effects.

The concept of generating IgG1 hexamers by C-terminal fusion with the IgM µ-tailpiece was originally described by Smith et al. (35) using full-length IgG. Hexameric IgG1 Fc molecules were described more recently as a potential carrier for vaccine Ags (36) and subsequently as a therapeutic candidate (21). Initially, in these studies an additional mutation of the Fc sequence at position 310 was proposed (36). This additional mutation appeared to destabilize the hexameric structure (36), and it abolished binding to FcRn (21). Therefore, in their later work the authors focused on a variant with the L309C mutation only (21). This molecule was analyzed structurally, for its binding to Fc receptors and other receptors, as well as for complement activation when bound to solid phase. No analyses of the effect on complement activation under more physiological conditions were performed; likewise, the molecule was not studied in animal models of inflammatory disease (21). More recently, an IgG1/IgG4 hybrid hexameric Fc molecule has also been described (37).

In our experiments, we showed that introducing the L309C mutation stabilized the hexameric structure, demonstrated by the higher proportion of hexameric species in the L309C mutant compared with the WT sequence by SDS-PAGE (Fig. 1B). Interestingly, both SEC-MALS and A4F analyses indicated the presence of ~85% hexamers in both the Fc- μ TP and Fc- μ TP-L309C, suggesting that a large proportion of the hexamers in Fc- μ TP were associated noncovalently (Table I). For this reason, subsequent studies were performed exclusively with the more stable Fc- μ TP-L309C molecule.

As expected, hexamerization of IgG-Fc led to a dramatic increase in binding avidity to FcyRs (Fig. 2A). This finding is in line with recent reports on other multimeric Fc molecules such as the Stradomer (17), hexameric Fc (21, 22), and Fc3Y (20). Ortiz et al. (20) established that structures with more than three Fc fragments led to Syk and ERK phosphorylation; furthermore, a trimeric lead molecule (Fc3Y) did not activate but, on the contrary, potently inhibited immune complex-mediated effector cell activation. Indeed, the activation of innate cells through FcyR would not be a desirable property of an Fc multimer in the treatment of autoimmune diseases. Therefore, we investigated in detail the effect of Fc-µTP-L309C on granulocytes and monocytes. In vitro, exposure of monocytes to Fc-µTP-L309C under serum-free conditions led to calcium-flux, an effect that was not seen in granulocytes (Fig. 8A). Importantly, despite this apparent activation of monocytes, no signs of unwanted downstream cellular effector functions were detected, including the absence of respiratory burst in neutrophils and monocytes (Fig. 8B) and platelet activation. Importantly, similar to the Stradomer and Fc3Y, Fc-µTP-L309C potently inhibited disease-relevant Fc-mediated effector functions, such as ADCC and phagocytosis and the CP (see later).

The FcRn has an important role in protecting IgG from degradation and therefore contributing to the extended $t_{1/2}$ of serum IgG, including pathogenic Abs. Enhanced degradation of autoantibodies and alleviation of autoimmune symptoms have been demonstrated after treatment of animals with molecules competing for binding of IgG to FcRn (38), and such molecules are currently in clinical development (39). Fc- μ TP-L309C bound to FcRn with high avidity (Fig. 3A), and so it may block recycling of IgG through FcRn, thereby increasing autoantibody degradation. Overall, the importance of FcRn blockade as an effector mechanism of Fc- μ TP-L309C will need to be further investigated, and studies are currently under way to examine the ability of Fc- μ TP-L309C to use FcRn-mediated recycling pathways.

The potential impact of the short vascular $t_{1/2}$ of Fc- μ TP-L309C will need to be studied in more detail. Fc- μ TP-L309C

showed profound and sustained efficacy in the CAbIA (Fig. 4B) and ITP mouse models (Fig. 6). The short $t_{1/2}$ of Fc-µTP-L309C should not have a great impact in these models because they both rely on passively administered autoantibodies against CII and platelets, respectively, for disease induction and, therefore, have a short therapeutic window for drug testing. For this reason, we further tested Fc-µTP-L309C in the chronic CIA model, in which mice were immunized with CII to produce anti-CII autoantibodies. In this model, the effect of FcµTP-L309C seemed less sustained (Fig. 5B). The reduced duration of therapeutic efficacy might be attributed to the short $t_{1/2}$ of Fc-µTP-L309C or the development of an immune response against the human protein upon repeat dosing. Efficacy studies are currently ongoing using a mouse surrogate rFc multimer to minimize the potential effect of anti-drug Abs. Ultimately, immunogenicity and pharmacokinetics studies in nonhuman primates and humans will be required to determine the longevity of the therapeutic effect of Fc-µTP-L309C.

The protective effects of Fc-µTP-L309C appeared to be mediated by a "collaboration" of various mechanisms of action. First, in the CAbIA model, Fc-µTP-L309C inhibited activation of complement, evidenced by decreased levels of C3 and C5a at the inflammatory site (the joint) (Fig. 4F). Because the ELISA used does not discriminate between C3, C3b/c, or C3a, Fc-µTP-L309C might have prevented leakage of uncleaved C3 from the periphery into the joints or decreased generation of C3a by reduced cleavage of local C3 present. In vitro analyses confirmed inhibition of the activation of the CP and LP (Fig. 7A, Supplemental Fig. 2A). Further in-depth in vitro analyses on the interference of Fc-µTP-L309C with complement activation showed partial activation of the CP up to cleavage of C4 (Fig. 7C), but a failure to cleave C2 (Fig. 7D). Thus, no inflammatory downstream products such as C5a (Supplemental Fig. 2B), sC5b-9 (Fig. 7E), or cell-bound C3b (Supplemental Fig. 3C) or C5b-9 (Fig. 7A) were generated (see Fig. 9 for overview). This functional feature is surprising and appears to be unique to Fc-µTP-L309C compared with similar molecules. For example, Ortiz et al. (20) reported no significant impact of the trimeric Fc molecule on complement activation. Studies with Hexa-Fc (21) bound to a solid phase indicated full complement activation; therefore, the inhibitory activity of Fc-µTP-L309C in solution was unexpected. A similar complement inhibitory activity was most recently reported by Zhou et al. (40), demonstrating that GL-2045 bound C1q, leading to generation of C4a, but limited levels of C3a and no C5a. In a second publication, the same group reported novel versions of the Stradomer with reduced binding to FcyRs but intact capability of inhibiting full complement activation (41). Second, FcyR-mediated effector functions relevant to pathological autoantibodies for mediating disease were effectively inhibited by Fc-µTP-L309C, including inhibition of respiratory burst (Fig. 8D), phagocytosis (Fig. 8F), and ADCC (Fig. 8E). These findings are in line with previous data obtained with similar molecules (17, 20). Third, as mentioned earlier, blockade of FcRn may be an additional protective mechanism.

In summary, we describe a unique rFc hexamer with therapeutic efficacy in multiple models of immune complex-mediated autoimmunity when administered at 10-fold lower doses than IVIG. The multimer mimics IVIG Fc-mediated immunomodulatory effects, in particular blockade of $Fc\gamma R$ and inhibition of the CP, and may block Ab recycling through FcRn. Fc- μ TP-L309C may be a promising candidate as an alternative to IVIG for the treatment of autoimmune disorders and diseases.

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Disclosures

All authors except B.J.B.L. and D.R.B. are employees of CSL Ltd./CSL Behring AG. The other authors have no financial conflicts of interest.

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GM-CSF and IL-4 are not involved in IVIG-mediated amelioration of ITP in mice: a role for IL-11 cannot be ruled out

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Summary

Previously, we have reported that interleukin (IL)-4, granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-11, but not IL-33, are up-regulated in two strains of mice with immune thrombocytopenia (ITP) that are responsive to intravenous immunoglobulin (IVIg) treatment. Previously, IL-4 was ruled out in the mechanism of IVIg; however, other publications have suggested this cytokine as a major player in the mechanism of IVIg action. Thus, we sought to further investigate a role for IL-4 and, in addition, GM-CSF and IL-11 in the mechanism of action of IVIg using a murine model of ITP. A passive platelet antibody model was used to generate ITP in IL-4 receptor knock-out (IL-4R^{-/-}), IL-11 receptor knock-out (IL-11Ra^{-/-}) and GM-CSF knock-out $(Csf2^{-/-})$ mice. We also used a neutralizing antibody to IL-11 and recombinant human IL-11 (rhIL-11) in addition to depleting basophils in vivo to study the effect of IVIg to ameliorate ITP. Our results showed that basophils, IL-4 and GM-CSF were unimportant in both ITP induction and its amelioration by IVIg. The role of IL-11 in these processes was less clear. Even though IL-11R $\alpha^{-/-}$ mice with ITP responded to IVIg similarly to wildtype (WT) mice, treatment of ITP WT mice with rhIL-11 instead of IVIg showed an increase in platelet numbers and WT mice administered anti-IL-11 showed a significant reduction in the ability of IVIg to ameliorate the ITP. Our findings indicate that neither IL-4, basophils or GM-CSF have roles in IVIg amelioration of ITP; however, a role for IL-11 requires further study.

Keywords: GM-CSF, IL-4, IL-11, ITP, IVIg

Introduction

Intravenous immunoglobulin (IVIg) has been used for many years as an antibody replacement therapy for patients with primary immunodeficiencies [1,2] and, since the early 1980s, as a therapy for several autoimmune diseases and inflammatory conditions [3–8]. High-dose IVIg has been used to treat immune thrombocytopenia (ITP) for more than 30 years; however, the mechanism of IVIg action in ITP and in other autoimmune/inflammatory diseases is currently unresolved [9–15].

Several mechanisms of action for the immunomodulatory and anti-inflammatory effects of IVIg have been proposed; however, there remains controversy regarding this subject. Previously proposed mechanisms include FcR blockade [15], anti-idiotypic antibodies [16], inhibition of complement [17], increased regulatory T cell involvement [18,19], up-regulation of the inhibitory Fcy receptor (Fc γ RIIb) on macrophages 20, modulation of peripheral tolerizing dendritic cells (DCs) [21,22] and saturation of the neonatal FcR (FcRn) to enhance autoantibody clearance [23,24]. Most of these have been challenged or even refuted by subsequent publications [25-31].

Currently, one model for the mechanism of action of IVIg continues to be discussed, despite the numerous publications that refute this model [32-39]. In this model, the Fc γ sialylated fraction of IgG is responsible for the antiinflammatory properties of IVIg [32,35,37]. The model theorizes that a minor population (~10%) of total IgG sialylated in the Fc γ domain engages the CD209 receptor (DC-SIGN) on regulatory macrophages/dendritic cells, which causes their release of IL-33 [35-39]. IL-33 is a Th2-polarizing cytokine that further causes basophils to release IL-4 at sites of inflammation. IL-4 acts on macrophages to upregulate the expression of Fc γ RIIb, which acts further to inhibit the activating Fc γ receptors and dampen the autoimmune response [36]. Indeed, this model proposes that the subsequent increase in expression of $Fc\gamma RIIb$ on effector cells raises the threshold of activation for inflammatory effector cells allowing for a reduction in inflammation 20,36.

Numerous scientists in the field have not been able to support this model of IVIg action [25-29]. A previous study where the investigators used interleukin (IL)-4 and common gamma chain-deficient mice indicated that IL-4 was not involved in the mechanism of action of IVIg to ameliorate ITP [40]. However, other studies maintain that IL-4 is a major cytokine, produced by activated basophils, in the mechanism of IVIg action [32-39]; thus, whether or not IL-4 plays any role in the mechanism of IVIg action in ITP remains an open question.

Few studies have focused on the cytokines produced in mouse models of ITP with and without treatment with IVIg. A recent report that looked at cytokine/chemokine induction by IVIg, using a multiplex cytokine assay and two different strains of mice with ITP that respond to IVIg, has shown that IL-33 is produced following IVIg treatment only in BALB/cJ mice, but not in C57BL/6J mice [41]. This supports other studies which have shown that IL-33 is not involved in the mechanism of IVIg action [42]. However, high amounts of IL-4 were produced in both mouse strains upon IVIg induction. Despite shown previously that both IL-4 deficient and common cytokine receptor gamma chain-deficient mice respond to IVIg [40], other published data still support a role for IL-4 and basophils that produce the IL-4 in the mechanism of IVIg action [32-39]. Thus, a role for IL-4 and basophils requires further study. Interestingly, in addition to IL-4, both IL-11 and granulocyte-macrophage colony-stimulating factor (GM-CSF) were produced significantly following IVIg treatment in both mouse strains, with IL-11 reaching nanogram levels [41].

The work reported herein investigates whether any of the cytokines induced by IVIg in both mouse strains could be playing a role in the mechanism of action of IVIg in ITP. Using a murine model of ITP, we confirm a previous report that IL-4 (or basophils) are not involved in the IVIg mechanism in the amelioration of ITP. We show further that GM-CSF is also unnecessary. In contrast, a role for IL-11 cannot be ruled out.

Materials and methods

Mice and reagents

Wild-type (WT) BALB/cJ and C57BL/6J females were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) at 6–8 weeks of age. IL-4 receptor knock-out (IL-4R^{-/-}), GM-CSF knock-out ($Csf2^{-/-}$) and IL-11 receptor α -chain knock-out (IL-11R $\alpha^{-/-}$) breeding pairs were purchased from The Jackson Laboratory and bred in our animal facility. Genotyping confirmed the knock-outs (data not shown). Mice were kept under a natural light-dark cycle, maintained at 22 ± 4°C, and fed with standard diet and water ad libitum. The use of animals was consistent with the requirements of the Canadian Council on Animal Care (CCAC) and the specific animal use protocol (AUP 829.35) was reviewed and approved by the University Health Network (UHN) Animal Research Committee in Toronto. Typically, three to six mice were used in each experimental and control group of animals. Specific numbers used for each experiment are given in the corresponding figure legends. To justify the minimum number of animals used for each experiment, we calculated the group size (n) required at 80% power and significance level (two-tailed, alpha = 0.05) to observe an effect of expected size using the t-statistic and non-centrality parameter [for comparing two means, effect size (E/S) = $k \times \delta$, where *t*-value at degrees of freedom (d.f.) = n_{total} -2, where $n_{\text{total}} = n_1 + n_2$, δ is the non-centrality parameter, and $k = (1/n_1 + 1/n_2)^{1/2}$. For example, we used three animals per group aiming to resolve an effect size of > 3.0standard deviations and five animals per group where we aimed to resolve an effect size as small as 2.0 standard deviations. In some control (baseline) groups, to minimize the number of animals used, where standard deviation was expected to be smaller based on previous observations, a smaller n = 3 group size was used to achieve the desired statistical power of 80%.

The rat monoclonal anti-mouse glycoprotein IIb (CD41; clone MWReg30, rat IgG1 λ) antibody used to induce ITP was purchased from BD PharMingen (Mississauga, ON, Canada). The IVIg preparation Gamunex 10% used to treat ITP was purchased from Talecris Biotherapeutics (Research Triangle Park, NC, USA). Privigen 10% IVIg was from CSL Behring (Bern, Switzerland). The Armenian hamster monoclonal anti-mouse Fc epsilon receptor I alpha (FceR1) (MAR-1; hamster IgG) antibody used to deplete basophils *in vivo* was purchased from eBioscience (San Diego, CA, USA). The rhIL-11 protein used to treat ITP was purchase from R&D Systems, Inc. (Minneapolis, MN, USA). The rat monoclonal anti-mouse IL-11 (clone 188520, rat IgG2a) antibody used to neutralize mouse IL-11and the goat immunoglobulin (Ig)G were purchased from R&D Systems, Inc.

ITP mouse model

A dose-escalation mouse model of passive platelet (PLT) antibody (MWReg30)-induced ITP was used, as described previously 43. Briefly, mice were injected with 68 μ g/kg of MWReg30 on days 0 and 1, followed by increasing doses of an additional 34 μ g/kg on each subsequent day. It has been well established that, under these conditions, PLT nadir is achieved on day 2 and maintained until day 5

41,43,44. Platelets in whole blood samples were quantified daily using a calibrated flow cytometer [fluorescence activated cell sorter (FACS)Calibur; Becton Dickinson, Franklin Lakes, NJ, USA] as described previously [43]. For treatment of ITP, IVIg was given intraperitoneally (i.p.) 2h after PLT antibody administration on day 2 of the experiment at 1–2 g/kg for BALB/cJ or IL-4R^{-/-} mice or at 2–2.5g/kg for C57BL/6J or IL-11Ra^{-/-} or IL-11Ra^{+/+} or Csf2^{-/-} mice, as optimized previously [43,44]. To control for the protein content of IVIg, we used human serum albumin (HSA; Canadian Blood Services, Toronto, ON, Canada) at the same g/kg dose used for IVIg.

In-vivo depletion of basophils in BALB/cJ mice

Basophils were depleted by injection of 5 μ g of anti-FceR1 (MAR-1) twice daily i.p. for 3 consecutive days starting 4 days before ITP induction, as optimized previously [28,45]. Controls received the same dose of the isotype control (Armenian hamster IgG antibody). Basophil populations were analysed by flow cytometry, using a FACSCalibur, by staining monocytes/granulocytes in the peripheral blood with anti-CD49b-phycoerythrin (PE) (5 μ g/ml) and CD123-allophycocyanin (APC) (5 μ g/ml) antibodies (eBioscience).

Treatment of ITP with recombinant human IL-11

For treatment of ITP, rhIL-11 was given i.p. 2h after PLT antibody administration on day 2 of the experiment at $10-12 \mu g/mouse$ for BALB/cJ mice. This was compared to treatment with IVIg given to BALB/cJ mice in a separate treatment group with similar conditions at 1g/kg.

Neutralizing antibody to mouse IL-11

To neutralize IL-11 and for treatment of ITP, both antimouse IL-11 antibody and IVIg were given with a 30-min interval i.p. 2h after PLT antibody administration on day 2 of the experiment at 100 μ g/mouse (5mg/kg) and 1g/kg, respectively, using BALB/cJ mice. To control for the added IgG from the neutralizing antibody, when given with the IVIg, goat IgG was injected at 5 mg/kg along with the IVIg as a non-neutralizing control.

Flow cytometric analysis

To quantify basophil numbers in peripheral blood, singlecell suspensions of red blood cell (RBC)-depleted peripheral blood were resuspended in phosphate-buffered saline (PBS) containing 1% (w/v) bovine serum albumin (BSA). Cells ($\sim 1 \times 10^6$) were stained with APC-conjugated anti-CD123 (5B11; eBioscience) and PE-conjugated anti-CD49b monoclonal antibody (mAb) (DX5; BD Pharmingen). Propidium iodide (1 µg/ml; Sigma-Aldrich, St Louis, MO, USA) was used to exclude dead cells, and live cells were analysed on a BD LSR II (BD Biosciences) using FlowJo software (Tree Star, Inc., Ashland, OR, USA).

Statistical analysis

All statistical analyses were performed using GraphPad Prism version 6.0 (GraphPad Inc., San Diego, CA). Significant differences between experimental groups was determined using a two-tailed Student's t-test and P-values of less than 0.05 were regarded as significant. All experiments were designed to have at least 80% power (beta of 0.2) to detect the desired effect size. The group comparisons for statistical analysis were as follows: (1) comparison of mice with ITP treated with IVIg and isotype control compared to mice with ITP treated with IVIg and MAR-1; (2) comparison of IL-4R^{-/-} mice with ITP treated with IVIg compared to WT mice with ITP treated with IVIg; (3) comparison of $Csf2^{-/-}$ mice with ITP treated with IVIg compared to WT mice with ITP treated with IVIg; (4) comparison of IL-11R $\alpha^{-/-}$ mice with ITP treated with IVIg compared to WT mice with ITP treated with IVIg; (5) comparison of mice with ITP treated with rhIL-11 compared to untreated mice with ITP; and (6) comparison of mice with ITP treated with IVIg compared to ITP mice treated with IVIg and anti-IL-11.

Results

Basophils and IL-4 are not required for IVIg amelioration of ITP

We had previously quantified cytokine serum levels in BALB/cJ (and C57BL/6J) mice following IVIg treatment in a murine model of ITP and found IL-4, among others, to be elevated significantly 41. Although a previous report using IL-4 and common cytokine gamma chain knock-out mice 40 suggested that IL-4 does not play a significant role in the mechanism of IVIg amelioration of ITP, other groups have claimed that IL-4 produced by basophils is a main player in the IVIg mechanism [32-39]. Thus, based on our cytokine results showing increased IL-4 following IVIg treatment, we investigated the role of IL-4 and basophils in the ability of IVIg to ameliorate ITP. To investigate a role for IL-4, we approached this in two ways: (i) we used an IL-4 receptor knock-out mouse instead of an IL-4 knock-out mouse, as in previous work [40], and (ii) we examined a role for basophils in our ITP mouse model. Given that previous reports showed that basophils play no role in the mechanism of IVIg action in mouse arthritis models [28], we expected to find that basophils were not involved in the mechanism of IVIg action in our murine mouse models of ITP; however, this has never been examined.

First, we showed that BALB/cJ mice were depleted successfully of basophils *in vivo* using a MAR-1 antibody (Fig. 1a). Next, we induced ITP in these mice that were essentially devoid of basophils and examined whether they responded differently to IVIg treatment than WT BALB/cJ mice. We found that



Fig. 1. Basophils and interleukin (IL)-4 are not required for intravenous immunoglobulin (IVIg) amelioration of immune thrombocytopenia (ITP) in BALB/cJ mice. ITP was induced by daily injections of MWReg30 at an escalating dose, as described previously 43,44. (a) Depletion of basophils, as measured in the peripheral blood of mice treated with either an Armenian hamster monoclonal Fc epsilon RI antibody (MAR-1) antibody to deplete basophils or with a corresponding isotype control antibody. (b) On day 2, when platelet nadir was reached (data not shown), mice were treated with IVIg (1·0g/kg) or received an injection of phosphate-buffered saline (PBS). Blood samples were collected at day 4 from the saphenous vein and platelets were counted using flow cytometry. The results shown represent the mean ± standard error of the mean (s.e.m.), n = 3. *P ≤ 0·05. (c) IL-4R^{-/-} knock-out BALB/cJ mice respond to IVIg (2·0g/kg) treatment, similarly to BALB/cJ treated with IVIg (2·0g/kg) compared with the untreated group. Control treatment was human serum albumin (HSA). The results shown represent the mean ± s.e.m., n = 3. Similar results were obtained in four additional experiments.

neither ITP induction nor its amelioration by IVIg depends on basophils, as these mice responded similarly to IVIg treatment, as did WT BALB/cJ mice (Fig. 1b). In addition to basophils being disposable in the mechanism of IVIg action, we showed that IL-4 was not required. We achieved this by inducing ITP in IL-4 receptor knock-out (IL-4R^{-/-}) BALB/cJ mice and examining whether these mice responded differently to IVIg treatment than WT BALB/cJ mice. We found that neither ITP induction nor its amelioration by IVIg depends upon IL-4, as the IL- $4R^{-/-}$ mice responded similarly to IVIg treatment as the WT BALB/cJ mice (Fig. 1c).

GM-CSF is not required for IVIg amelioration of ITP

In addition to showing that IL-4 was elevated in BALB/cJ and C57BL/6J mice following IVIg treatment in a murine model of ITP, we also showed that GM-CSF was elevated

Fig. 2. Granulocyte–macrophage colony-stimulated (GM-CSF) is not required for intravenous immunoglobulin (IVIg) amelioration of immune thrombocytopenia (ITP) in C57BL/6J mice. *Csf2*^{-/-} knock-out C57BL/6J mice respond to IVIg (1·0g/kg) treatment compared with control group treated with human serum albumin (HSA). The results shown represent the mean \pm standard error of the mean (s.e.m.), n = 5-6.



[40]. Because there has been little attention given to this cytokine in the mechanism of action of IVIg in ITP, we were curious as to whether this cytokine played a role. We induced ITP in Csf2^{-/-} C57BL/6J GM-CSF knock-out mice and examined whether they responded differently to IVIg treatment in comparison to C57BL/6J WT mice. In accordance with our previous results with basophils and IL-4, we found that neither ITP induction nor its amelioration by IVIg depends upon GM-CSF, as the Csf2^{-/-} C57BL/6J knock-out mice responded similarly to IVIg treatment as the C57BL/6J WT mice (Fig. 2).

IL-11 may be involved in IVIg amelioration of ITP

We have shown previously that IL-11 is greatly elevated in BALB/cJ and C57BL/6J mice following IVIg treatment of ITP [41,46,47]. To begin to investigate a possible role for IL-11 in the mechanism of action of IVIg, we first examined the ability of IVIg to ameliorate ITP in IL-11R $\alpha^{-/-}$ knock-out mice. Using these mice, we found that neither ITP induction nor its amelioration by IVIg depends upon the IL-11 receptor, as the IL-11Ra^{-/-} C57BL/6J knock-out mice responded similarly to IVIg treatment as the C57BL/6J WT littermate mice (Fig. 3a). However, it was a surprise to us, given that IL-11 plays an important role in megakaryopoiesis/thrombopoiesis [48-51], that these knock-out mice had such high platelet numbers (Fig. 3a). As this may have been due to some redundancy in this cytokine family 52, we looked further at whether rhIL-11 could replace IVIg to ameliorate ITP. We found that platelet numbers increased following rhIL-11 treatment alone, and that this was statistically significant $(P \le 0.0081)$ (Fig. 3b). Finally, we tested IVIg in combination with neutralizing antibody to IL-11. We showed that C57BL/6J mice with ITP administered anti-IL-11 in parallel with IVIg only achieved an approximately 50% increase in platelet counts compared to WT mice given IVIg alone. This 50% reduction in the IVIg response in the presence of neutralizing anti-IL-11 was statistically significant ($P \le 0.0001$) (Fig. 3c).

Discussion

We have reported previously that the serum levels of IL-4, GM-CSF and IL-11 are elevated significantly in both BALB/cJ and C57BL/6J mice with ITP following IVIg treatment 41, and as IVIg could ameliorate ITP in both these mouse models we asked whether these cytokines may play a role in the mechanism of action of IVIg. We conclude that neither ITP induction nor its amelioration by IVIg depends upon IL-4 (or basophils) or GM-CSF (Figs. 1 and 2). Results for IL-11, however, did not rule out a possible role for this cytokine conclusively in the mechanism of IVIg amelioration of experimental ITP.

It has been reported previously that the mechanism of IVIg action in ITP does not require IL-4 when using IL-4 or a common cytokine gamma chain knock-out mouse 40. This contrasts with previous reports that indicate IL-4 as a critical cytokine in the mechanism of IVIg action and that this cytokine is produced by basophils [35,36]. In our previous work [41], we observed significantly elevated production of IL-4 in two mouse strains that respond to IVIg treatment; thus, we sought to resolve the controversy of a role for IL-4 by using an IL-4R knock-out mouse and to evaluate the role of basophils in our ITP mouse model. Not unexpectedly, our results with basophil depletion indicated clearly that basophils do not play a role in the mechanism of action of IVIg in ITP (Fig. 1b). These results mirror previous studies in two different arthritis mouse models, where basophil depletion had no effect on the ability of IVIg to ameliorate the arthritis [28]. Our results with IL-4R knock-out mice indicate clearly that this cytokine also does not play a role in the mechanism of action of IVIg (Fig. 1c), which confirms previous work [40] and is contrary to other investigators' proposed model [34-36].

To our knowledge, we are the first group to investigate a possible role of GM-CSF in the mechanism of action of IVIg. We looked at GM-CSF based on our previous work that showed that GM-CSF was elevated significantly in both BALB/cJ and C57BL/6J mice given IVIg [40]. Using GM-CSF knock-out mice we did not see any differences



Fig. 3. Role for interleukin (IL)-11 in intravenous immunoglobulin (IVIg) amelioration of immune thrombocytopenia (ITP) appears to be equivocal. (a) IL-11R $\alpha^{-/-}$ knock-out and wild-type littermate mice were given ITP and then administered IVIg (2.5g/kg) treatment on day 2. Control treatment was human serum albumin (HSA). The results shown represent the mean (s.e.m.), n = 3-5. Similar results were obtained in one additional experiment. (b) C57BL/6J mice respond to IVIg (2.5g/kg) treatment and rhIL-11 (11–12 $\mu g/kg)$ -treated C57BL/6J mice compared to control mice treated with human serum albumin (HSA). The results shown represent the mean \pm s.e.m., n = 4-8. **P ≤ 0.0081 . (c) C57BL/6J mice respond to IVIg (2.5g/kg) treatment compared with anti-IL-11 (100 µg/kg)-treated C57BL/6J mice and control mice treated with human serum albumin (HSA). The results shown represent the mean \pm s.e.m., n = 5. *** $P \le 0.0001$.

of ability of IVIg to ameliorate ITP in either the knockout or WT mice, thus ruling out GM-CSF as having a role in the mechanism of action of IVIg (Fig. 2).

We became interested in a possible role for IL-11 in the mechanism of action of IVIg when we observed that mice with or without ITP produce very high amounts of IL-11 when administered IVIg [41,46,47]. Recently, as a small part of another study, we have looked at IL-11 production in 60 patients receiving 2 g/kg of IVIg for various conditions. In this cohort, we have seen that 43 of 60 (72%) patients showed an increase in IL-11 24 hours after IVIg treatment, six of 60 (10%) patients had a higher expression of IL-11 prior to IVIg treatment and 11 of 60 (18%) showed no difference in IL-11 pre- or post-IVIg treatment. Thus, production of IL-11 appears to be a common feature of both murines and humans when given immunomodulatory doses of IVIg. This has piqued our interest in a possible role for IL-11 in the mechanism of action of IVIg [47].

IL-11 is a pleotropic cytokine belonging to the IL-6 family of cytokines, which includes IL-6 and leukaemia inhibitory factor (LIF), among others [53]. IL-11 has been suggested to play an important role in immunity; however, its role is poorly defined [48]. IL-11 has been described as an inflammatory molecule and an anti-inflammatory molecule [48]. Importantly, IL-11 seems to play a critical role in thrombopoiesis, as it is required for optimal mega-karyopoiesis along with thrombopoietin [49–51,54]. Indeed,

previous work has shown that IVIg treatment in a mouse model of ITP resulted in an increase in young, newly produced, reticulated platelets, and this was proposed as a possible mechanism of IVIg action to ameliorate ITP in this mouse model [43]. It is probable that this increase in platelet production resulted from the induction of IL-11 upon IVIg treatment [47]. Increased thrombopoiesis has been shown to occur in ITP patients receiving IVIg therapy [55,56]. In our current work, it appears that a role for IL-11 is plausible, because we show that platelet counts decreased by ~50% when a neutralizing anti-IL-11 was used in conjunction with IVIg (Fig. 3c). Furthermore, there was a significant increase in platelet numbers (50% increase) when using rhIL-11 in place of IVIg (Fig. 3b). These two findings suggest that IL-11 induced by IVIg treatment plays some role in the mechanism of action of IVIg to ameliorate ITP.

A significant caveat to the conclusion that IL-11 is important for the mechanism of IVIg amelioration of ITP is our finding that mice deficient in IL-11 receptor, IL-11R $\alpha^{-/-}$, did not show any differences in amelioration between knock-out and WT littermates (Fig. 3a). Given the role for IL-11 in megakaryopoiesis/thrombopoiesis [49-51,54], we were surprised to see that these mice had normal to higher platelet counts than WT littermates (Fig. 3a). We fully expected these mice to have lower platelet numbers unless there may be redundancy within this cytokine family. Indeed, redundancy in the IL-11R $\alpha^{-/-}$ mouse has been speculated previously to explain a lack of an overt haematological phenotype in these animals [52]. It is intriguing to speculate that IL-6 and/or LIF have replaced the role of IL-11 in this knock-out mouse. It has been proposed that both IL-6 and LIF can be thrombopoietic cytokines [57]. This potential redundancy could explain why IVIg was still capable of ameliorating the platelet deficit in this knock-out mouse, by inducing another thrombopoietic cytokine such as LIF.

In summary, our results confirm that IL-4 or basophils are not involved in the mechanism of action of IVIg in the amelioration of ITP, nor is GM-CSF involved. However, a role for IL-11 cannot be ruled out with certainty, and further studies are necessary to determine if IL-11 may be involved in the mechanism of effect of IVIg.

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Disclosure

The authors assert that there are no conflicts of interest

Author contributions

D. R. B., D. L. and A. N. designed the research; B. J. B. L., M. B., D. L. and A. N. performed experiments; B. J. B. L., D. L., A. N. and D. R. B. analysed and interpreted data; B. J. B. L. and D. R. B. wrote the manuscript.

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