Intravenous immunoglobulin skews macrophages to an anti-inflammatory, IL-10 producing activation state

by

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Abstract

Macrophages initiate the immune response and contribute to the inflammation that characterizes many diseases. Macrophages play an equally important role in turning off the inflammatory response, by producing the anti-inflammatory cytokine IL-10. Intravenous immunoglobulin (IVIg) is a drug made up of pooled polyclonal IgGs, which is used to treat immune-mediated diseases. However, its mechanism of action is not completely understood. We found that IVIg induced high production of IL-10 and low production of pro-inflammatory cytokines by murine bone marrow-derived macrophages (BMDMs) treated with lipopolysaccharide (LPS), an inflammatory stimulus. MAPKs, Erk5, Erk1/2, and p38, were activated by co-stimulation with IVIg and LPS and their activation was required for IL-10 production. *In vivo*, murine peritoneal macrophages also produced high levels of IL-10 and low levels of IL-12/23p40 when treated with IVIg + LPS.

Inflammatory bowel disease (IBD) is characterized by chronic inflammation of the intestine. IVIg-treated macrophages or IVIg treatment ameliorated intestinal inflammation in mice during dextran sulfate sodium (DSS)-induced colitis. Moreover, IVIg-induced macrophage IL-10 production was required for IVIg-mediated protection.

In human monocytes, IVIg also increased IL-10 production and reduced pro-inflammatory cytokine production in response to LPS. IVIg-induced IL-10 production required the FcγRI and FcγRIIB as well as activation of MAPKs, ERK1/2 and p38. An FcγRIIA gene variant predisposes people to develop immune-mediated diseases, such as IBD, and has been linked to a failure to respond to antibody therapy. The FcγRIIA disease risk variant changes this receptor from a low to a high affinity receptor. My results demonstrated that IVIg-induced anti-

inflammatory responses were compromised in monocytes from people with the FcγRIIA risk variant.

Together, these results describe a novel mechanism of action for IVIg, the induction of anti-inflammatory, IL-10 producing macrophages. IVIg may provide an effective therapeutic option to treat people with IBD. However, induction of this anti-inflammatory activation state may be impaired in monocytes from people with the disease-associated FcγRIIA gene variant. In summary, understanding IVIg's mechanism of action may inform new applications, prompt development of new therapeutic strategies for immune-mediated diseases, and identify individuals for whom IVIg will be most effective.

Lay Summary

My research involves studying how a drug called IVIg (intravenous immunoglobulin) works. IVIg is made of human antibodies and very little is known about how it works, even though it is used to treat a variety of diseases. Macrophages, which are a type of white blood cell, are important for protecting us against infections, but are also important in stopping inflammation in the body. I have found that IVIg can switch macrophages to become anti-inflammatory. Inflammatory bowel disease (IBD) is a disease that causes inflammation in the intestine leading to symptoms such as abdominal pain and diarrhea. Current treatments are not effective for almost 40% of people with IBD. Using mice, I have found that IVIg makes macrophages anti-inflammatory and could be a good therapy to treat IBD. I have also found that people with certain genetics are better at switching cells to become anti-inflammatory with IVIg.

Preface

Animal studies were approved by the University of British Columbia according to guidelines provided by the Canadian Council on Animal Care (protocol numbers A13-0014, A13-0054, A17-0061, and A17-0076). All experimental procedures using human samples were performed in accordance with ethical guidelines and approved by the University of British Columbia research ethics board (H13-03524 and H14-00622). All subjects provided informed, written consent for blood collection for immune cell isolation and functional assays, DNA isolation, and genotyping.

Chapter 1. Figure 1.2 was reproduced with permission of Nature Publishing Group: Saraiva M. and O'Garra A. The regulation of IL-10 production in immune cells. *Nat Rev Immunol.* 2010; 10(3):170-181. Figure 1.3 was reproduced with permission of Nature Publishing Group: Nimmerjahn F. and Ravetch J. Fcγ receptors as regulators of immune responses. *Nat Rev Immunol.* 2008; 8(1):34-47. Figure 1.4 was reproduced according to the terms of the Creative Commons Attribution License (CC BY): Fuller J. et al. New roles for Fc receptors in neurodegeneration-the impact on immunotherapy for Alzheimer's disease. *Front Neurosci.* 2014; 8(235). Figure 1.5 was reproduced with permission of The Rockefeller University Press: Nimmerjahn F. and Ravetch J. The antiinflammatory activity of IgG: the intravenous IgG paradox. *J Exp Med.* 2007; 204(1):11-15. Figure 1.6 was reproduced with permission of Nature Publishing Group: Neurath M. Cytokines in inflammatory bowel disease. *Nat Rev Immunol.* 2014; 14(5):329-342.

A version of Chapter 2 has been published and was used with permission of the Journal of Leukocyte Biology. Kozicky, L., Zhao, Z., Menzies, S., Fidanza, M., Reid, G., Wilhelmsen, K., Hellman, J., Hotte, N., Madsen, K., and Sly, L. Intravenous immunoglobulin skews macrophages to an anti-inflammatory, IL-10 producing activation state. *J Leukoc Biol.* 2015; 98(6):983-984. I performed all experiments and data analysis described herein, with the exception of the following contributions from additional authors: Zheng Zhao helped with ELISAs presented in Figure 2.4. Susan Menzies helped with derivation of macrophages from bone marrow used to generate data presented in Figure 2.3. Mario Fidanza and Gregor Reid helped with quantitative PCR experiments and data analysis. Kevin Wilhelmsen and Judith Hellman provided technical guidance on Erk5, used to generate data presented in Figure 2.4. Naomi Hotte and Karen Madsen isolated and shipped bones from $Il10^{+/+}$ and $Il10^{-/-}$ mice used to generate data in Figure 2.7. Dr. Laura Sly and I conceived of the experiments and wrote the manuscript for the published paper.

A version of Chapter 3 has been submitted for publication. I performed all experiments and data analysis described herein, with the exception of the following contributions from additional authors: Susan Menzies helped perform tail vein injections for data presented in Figure 3.1 and Figure 3.2, helped with tissue collection used for data presented in Figure 3.5, and scored for histological damage and cell numbers presented in all Figures. Naomi Hotte and Karen Madsen isolated and shipped bones from $Il10^{+/+}$ and $Il10^{-/-}$ mice used to generate data in Figure 3.1 and Figure 3.2. Dr. Laura Sly and I conceived of the experiments and wrote the manuscript for submission.

A version of Chapter 4 has been published and used with permission of Frontiers in Immunology. Lisa K. Kozicky, Susan C. Menzies, Zheng Yu Zhao, Tariq Vira, Kiera Harnden, Kwestan Safari, Kate L. Del Bel, Stuart E. Turvey and Laura M. Sly. IVIg and LPS Co-stimulation Induces IL-10 Production by Human Monocytes, Which Is Compromised by an FcγRIIA Disease-Associated Gene Variant. *Front. Immunol.* 2018; 9:2676. I performed all experiments and data analysis described herein, with the exception of the following contributions from additional authors: Susan Menzies collected blood samples from participants. Zheng Zhao helped with isolation of human monocytes, stimulations, and ELISAs presented in Figure 4.5. Tariq Vira helped with isolation of human monocytes, stimulations, and ELISAs presented in Figure 4.3 and Figure 4.7. Kiera Harnden helped with isolation of human monocytes, stimulations, and ELISAs presented in Figure 4.2A. Kwestan Safari helped with isolation of human monocytes, stimulations, and ELISAs presented in Figure 4.6. Kate Del Bel and Stuart Turvey helped with genotyping of the FcγRIIA gene variants included in data presented in Figures 4.5, 4.6, and 4.7. Dr. Laura Sly and I conceived of the experiments and wrote the manuscript for the paper.

Table of Contents

List of Tables

List of Figures

List of Symbols and Abbreviations

α	Alpha
β	Beta
δ	Delta
γ	Gamma
μg	Microgram
μm	Micrometer
μM	Micromolar
°C	Degrees Celsius
3'UTR	3' untranslated region
5-ASA	5-aminosalicylic acid
AIEC	Adherent invasive *E. coli*
ADCC	Antibody-dependent cell-mediated cytotoxicity
ADCP	Antibody-dependent cell-mediated phagocytosis
AMP	Anti-microbial peptide
ANOVA	Analysis of variance
AP-1	Activator protein 1
APC	Antigen presenting cell
ArgI	Arginase I
BIX	BIX02189 inhibitor
BMDM	Bone marrow-derived macrophage
BIR	BIRB-796 inhibitor
BLT mice	Bone marrow liver thymic mice

BTK	Bruton's tyrosine kinase
C/EBPβ	CCAAT enhancer binding proteins β
C/EBPδ	CCAAT enhancer binding proteins δ
CD	Crohn’s disease
CIDP	Chronic inflammatory demyelinating polyneuropathy
CLL	Chronic lymphocytic leukemia
CLR	C-type lectin receptor
c-Maf	c-musculoaponeurotic fibrosarcoma oncogene
CREB	Phosphorylated cyclic AMP element binding protein
DAIs	Disease Activity Index score
DAMP	Danger-associated molecular pattern
DAPI	4′,6-diamidino-2-phenylindole
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DMSO	Dimethyl sulphoxide
DSS	Dextran sodium sulphate
DUSP1	Dual specificity protein 1
EDTA	Ethylenediaminetetraacetic acid
Erk	Extracellular signal-regulated kinase (mice)
ERK	Extracellular signal-regulated kinase (human)
ELISA	Enzyme-linked immunosorbent assay
Emr1	F4/80 gene name (mice)
Fab	Fragment antigen-binding

FBS	Fetal bovine serum
Fc	Fragment crystallizable
Fcγ R	Fcγ receptor
FcRn	Neonatal Fc receptor
FDA	Food and Drug Administration
Foxp3	Forkhead box protein 3
g	Gram
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GBS	Guillain-Barré syndrome
GFP	Green fluorescent protein
GPI	Glycosylphosphatidinositol
GWAS	Genome wide association study
H&E	Hematoxylin and eosin
HIV	Human immunodeficiency virus
hr	Hour
HSV	Herpes simplex virus
IBD	Inflammatory bowel disease
Ic	Immune complex
IDO	Immune-regulatory protein indoleamine-2,3-dioxygenase
IFNγ	Interferon γ
IgG	Immunoglobulin G
IL	Interleukin
IL-1R2	IL-1 receptor 2

IL-1Ra	IL-1 receptor antagonist
IL-10R	IL-10 receptor
ILC	Innate lymphoid cell
IMDM	Iscove's modified Dulbecco's medium
iNOS	Inducible nitric oxide synthase
IRF1	Interferon regulatory factor 1
ITAM	Immunoreceptor tyrosine activation-based motif
ITIM	Immunoreceptor tyrosine inhibitory-based motif
ITP	Idiopathic thrombocytopenic purpura
IVIg	Intravenous immunoglobulin
Jak	Janus kinase
KD	Kawasaki disease
kg	Kilogram
L	Liter
LPS	Lipopolysaccharide
Mϕ	Macrophage
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MCSF	Macrophage colony-stimulating factor
MDP	Muramyl dipeptide
mg	Milligram
MHC II	Major histocompatibility complex II
min	Minute

miRNA	MicroRNA
mL	Milliliter
MMN	Multifocal motor neuropathy
MMP	Matrix metalloproteases
Mreg	Regulatory macrophage
MS	Multiple sclerosis
MSK	Mitogen and stress-activated protein kinase
Muc19	Mucin 19
MyD88	Myeloid differentiation primary response protein 88
NFκB	Nuclear factor kappa B
ng	Nanogram
NK cell	Natural killer cell
NLR	NOD-like receptor
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain containing protein
nM	Nanomolar
ns siRNA	Non-silencing siRNA
NSAID	Non-steroidal anti-inflammatory drug
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PBX1	Pre-B-cell leukemia transcription factor 1
PD	PD98059 inhibitor

PGE_2	Prostaglandin E2
PI3K	Phosphatidylinositol 3-kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PIP_3	Phosphatidylinositol 3,4,5-trisphosphate
PLCγ	Phospholipase C γ
PREP1	PBX-regulating protein 1
PRR	Pattern recognition receptor
RA	Rheumatoid arthritis
RAG1	Recombination-activating gene 1
rhIL-10	Recombinant human IL-10
RLR	RIG-I like receptor
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
SB	SB203580 inhibitor
SCH	SCH772984 inhibitor
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Siglec-9	Sialic acid-binding Ig-like lectin-9
SIGN-R1	Specific ICAM3 grabbing nonintegrin-related 1
siRNA	Small interfering RNA
SHP-1	SH2-domain containing inositol phosphate 5′phosphatase-1
SHIP	SH2 domain-containing inositolpolyphosphate 5′-phosphatase
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphisms

SOCS3	Suppressor of cytokine signalling 3
SOS	Son of sevenless
Sp1	Specificity protein 1
SRBC	Sheep red blood cell
STAT	Signal transducer and activator of transcription
SYK	Spleen tyrosine kinase
TGFβ	Transforming growth factor β
Th cell	T helper cell
TIR	Toll/IL-1 receptor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TPL2	Tumor progression locus 2
Tr1 cell	Type 1 regulatory cell
Treg	Regulatory T cell
TRIF	TIR domain-containing adaptor-inducing interferon β
TRAF3	Tumor necrosis factor receptor-associated factor 3
TSLP	Thymic stromal lymphopoietin
Tyk2	Non-receptor tyrosine protein kinase 2
UC	Ulcerative colitis
UnRx	Untreated
WISP-1	WNT1 inducible signalling pathway-1
XLA	X-linked agammaglobulinemia
XMD	XMD8-92 inhibitor

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Chapter 1: Introduction

1.1 Macrophages

Macrophages are heterogeneous innate immune cells, which have important roles in the immune response[1]. Macrophages play essential parts in all stages of the immune response, including promoting an inflammatory response, limiting the inflammatory response to prevent damage to the host, and participating in wound healing and repair[2]. Macrophages play pleiotropic roles in the murine and human immune response including phagocytosis of pathogens and cellular debris, presentation of antigens, production of reactive oxygen species (ROS), secretion of pro- and anti-inflammatory cytokines, secretion of chemokines to recruit immune cells, and production of extracellular matrix and growth factors during tissue remodeling[3].

Murine and human macrophages express cell surface receptors, which allow them to perform required functions in response to cues in their local environment. They express pattern recognition receptors (PRRs) to detect conserved microbial associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs), including toll-like receptors (TLRs), C-type lectin receptors (CLRs), nucleotide-binding oligomerization domain containing protein (NOD)-like receptors (NLRs), and RIG-I like receptors (RLRs). They express a variety of other receptors, including cytokine and chemokine receptors, Fc receptors, complement receptors, adhesion molecules, and hormone receptors[4].

Macrophages in mice and humans can be tissue resident cells or can be differentiated from monocytes recruited from the blood. Tissue resident macrophages are seeded during the embryonic stage of development by yolk sac or fetal liver precursors[5, 6]. These embryonic seeded macrophages can self-renew and are long lived[5]. In some tissue, such as the intestine, mammary gland, or heart, Ly6C^{hi} monocytes in mice derived from bone marrow precursors are continuously recruited to become tissue resident macrophages, which are short lived and non-renewing[7]. Tissue resident macrophages in mice and humans have specialized functions *in vivo*, depending upon their niche. For example, microglia in the brain are involved in synaptic pruning during development, osteoclasts in the bone are involved in bone and joint remodeling, and Kupffer cells in the liver are involved in detoxification and iron recycling[8]. During an inflammatory response or tissue injury, Ly6C^{hi} monocytes in mice and CD14^{+} monocytes in humans are recruited from the blood to the site of infection or injury and can differentiate into macrophages with various effector functions[7].

1.1.1 Macrophage activation

Murine and human macrophages are complex cells that have a diverse range of phenotypes. Macrophages are "plastic" cells; they have the ability to respond to local environmental cues and readily change their phenotype in order to mount an appropriate response[9]. Thus, macrophages are heterogeneous cells, which have a diverse range of distinct, but potentially overlapping, activation states[1].

Macrophage plasticity makes them difficult to study *in vivo,* but there are three well-studied activation states that are modeled *in vitro* in mice and humans by treating macrophages with exogenous stimuli[3] (Figure 1.1). Macrophage activation states are defined in the context of exogenous stimuli that are used to "re-program" them. Macrophages, formerly referred to as M1 macrophages, primed with interferon γ (IFNγ) and activated with TLR agonists, such as Gram-negative bacterial lipopolysaccharide (LPS), now defined as M(IFNγ + LPS), have an inflammatory phenotype, which is important in host defense against pathogens[10]. In order to limit tissue damage, the inflammatory response needs to be turned off[11]. Macrophages, formerly known as regulatory macrophages or Mregs, which can be primed with immune complexes (Ic) and LPS, now defined as M(Ic + LPS), have a potent anti-inflammatory phenotype[11]. Macrophages formerly known as M2 macrophages, primed with interleukin (IL)-4 or IL-13, now defined as M(IL-4) or M(IL-13), have a wound healing and tissue remodeling phenotype, in order to repair tissue damage from the inflammatory response[12].

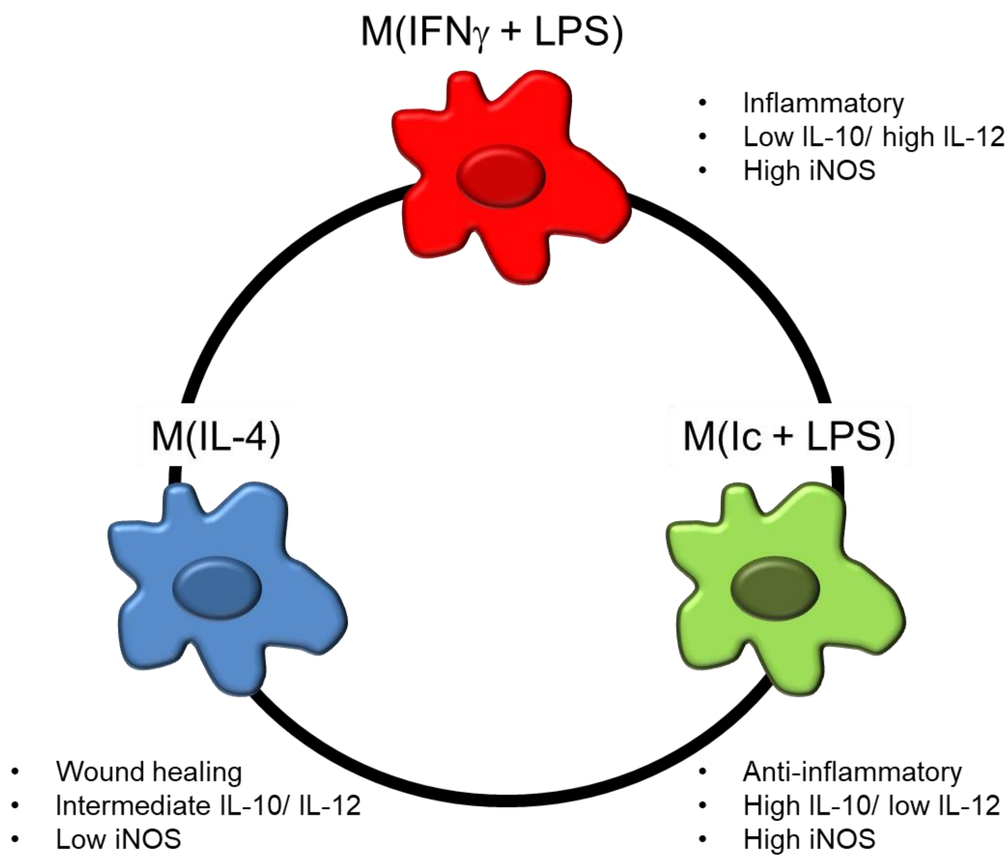

Figure 1.1 Macrophage activation states. Macrophages change their phenotype based on signals in the microenvironment and lie on a continuous spectrum of activation states. Activation states that are well defined *in vitro* are described.

1.1.1.1 M(IFNγ + LPS)

IFNγ primes murine and human macrophages to have higher inflammatory responses when stimulated with TLR ligands, such as LPS[13]. IFNγ can be produced by both innate and adaptive immune cells in mice and humans; it can be transiently produced from natural killer (NK) cells or produced from T helper (Th) 1 cells[1]. M(IFNγ + LPS) in mice and humans secrete high amounts of pro-inflammatory cytokines, such as IL-12, IL-23, IL-6, and tumor necrosis factor (TNF), which can cause pathology if production is not tightly controlled[10]. IL-12 potently drives $CD4^+$ T cells into Th1 cells and induces IFNγ production, whereas IL-23 enhances the expansion of Th17 cells[14]. IL-6 causes survival and proliferation of T cells, and drives the differentiation of $CD4^+$ T cells towards Th2 and Th17 phenotypes[15]. TNF causes inflammation, recruitment of immune cells, and tissue destruction[16]. Murine and human M(IFNγ + LPS) have higher antigen presentation on major histocompatibility complex II (MHC II) and initiate Th1-type responses[10, 17]. In mice, M(IFNγ + LPS) have higher production of bactericidal ROS and nitric oxide (NO), which is produced by the mouse, but not human, enzyme inducible nitric oxide synthase (iNOS)[18, 19].

Inflammatory macrophages can have either protective or pathogenic roles in disease. IFNγ-activated macrophages are protective during *F. tularensis* infection in the lungs of mice, with high production of IL-12 and recruitment of neutrophils[20]. Inflammatory macrophages have been implicated in the pathogenesis of many inflammatory and autoimmune diseases, such as inflammatory bowel disease (IBD), rheumatoid arthritis (RA), and multiple sclerosis (MS), wherein they produce high amounts of pro-inflammatory cytokines, such as IL-23, which contributes to disease pathology[21-24].

1.1.1.2 M(Ic + LPS)

Murine and human macrophages can have potent anti-inflammatory properties that turn off the immune response to prevent damage to host tissues after an inflammatory response. A defining characteristic of this macrophage activation state is the production of high amounts of the anti-inflammatory cytokine, IL-10, in response to a normally inflammatory stimulus[11]. IL-10 limits immune responses, which limits tissue damage and restores tissue homeostasis[25]. In addition to high levels of IL-10 production, some anti-inflammatory macrophages produce lower levels of pro-inflammatory cytokines, such as IL-12[26].

Two stimuli are required for the "re-programming" of macrophages to an anti-inflammatory activation state. The first does not induce cytokine production by itself, for example Ic or IL-10[11]. The second stimuli is normally inflammatory, such as a TLR agonist, which results in the modulation of cytokine production[11]. This activation state more commonly arises during the late stages of the adaptive immune response, although some innate immune stimuli can induce this activation state as well[1].

The best characterized example of this activation state are murine macrophages activated with Ic and LPS, M(Ic + LPS). LPS normally induces low levels of IL-10 and high levels of IL-12 in murine macrophages, whereas co-treatment with Ic results in a conversion of macrophages to an anti-inflammatory state, with high production of IL-10 and very low production of IL-12[27]. Murine M(Ic + LPS) do not have lower IL-6 and TNF production compared to macrophages activated with LPS alone[10, 32]. In mice, this population of macrophages retains antigen presentation capabilities and the production of iNOS[11]. Human M(Ic + LPS) also have high IL-10 production, but do not have reduced IL-12 production[28-30]. Unlike murine M(Ic + LPS), human M(Ic + LPS) have reduced IL-6 production[29, 30]. Similar to mice, human M(Ic + LPS) do not have lower TNF production[29, 30].

Other stimuli induce anti-inflammatory macrophages *in vitro*. In murine macrophages, IL-10 production is higher when cells are stimulated with apoptotic cells, prostaglandin E2 (PGE_2), glucocorticoids, or IL-10[31-35]. Although IL-10 production has not been reported, human and murine macrophages activated with serum, macrophage colony-stimulating factor (MCSF), and IFNγ, M(MCSF + IFNγ), have anti-inflammatory properties, including the suppression of T cell proliferation and the production of the immune-regulatory protein indoleamine-2,3-dioxygenase (IDO)[36, 37]. In human macrophages, the anti-TNFα antibody, infliximab, also induces IL-10 production and reduces IL-1β production in response to LPS[35].

The molecular mechanism for conversion of macrophages to a high IL-10 producing activation state has been elucidated for murine M(Ic + LPS). Ligation of Ic to the Fcγ receptor I (FcγRI) in M(Ic + LPS) results in high activation of the mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase 1 and 2 (Erk1/2), which leads to phosphorylation of serine 10 on histone 3, opening up the *Il10* promoter[27]. Activation of p38 in M(Ic + LPS) drives specificity protein 1 (Sp1)- and signal transducer and activator of transcription 3 (STAT3)-mediated transcription of IL-10 from the accessible *Il10* promoter [38, 39]. Erk activation has been proposed as a defining mechanistic switch to induce this IL-10 producing activation state in murine macrophages[1].

Anti-inflammatory macrophages can have either protective or pathogenic roles in disease. Anti-inflammatory macrophages can be protective *in vivo*, high IL-10 and low IL-12 producing microglia that are activated through FcγRI and TLR4 are protective in a murine model of breached blood brain barrier inflammation[40]. However, anti-inflammatory macrophage activation can increase susceptibility to infection, as is the case for *Leishmania*, which has developed a virulence mechanism co-opting this macrophage activation state. *Leishmania* can enter murine macrophages through Fcγ receptors, causing Erk activation and IL-10 production, resulting in blunted intracellular killing, survival, and spread of the infection[41].

1.1.1.3 M(IL-4)

Wound healing and tissue restitution occurs in mice and humans to return the tissue to homeostasis after the immune response has been suppressed by IL-10. Wound healing macrophages contribute to the production of extracellular matrix and remodeling of tissues[1]. IL-4 or IL-13 are stimuli that induce this activation state in mice and humans *in vitro* and *in vivo*[1, 10]. Murine and human basophils and mast cells are important sources of IL-4 during infection or injury and Th2 cells produce both IL-4 and IL-13[42-45].

Wound healing macrophages have unique characteristics from other macrophages. Markers for this activation state in mice, but not in humans, include arginase I (ArgI), Ym1, and FIZZI[10, 46]. ArgI converts arginine to precursors, which are used for extracellular matrix production, Ym1 binds to extracellular matrix proteins, and FIZZ1 promotes deposition of extracellular matrix[19, 47]. In mice, M(IL-4) do not present antigens to T cells and are less efficient at intracellular killing[10]. They are less efficient because they produce less reactive oxygen and nitrogen species, as ArgI competes for common substrates of iNOS[11, 48, 49]. In response to inflammatory stimuli, murine M(IL-4) or M(IL-13) have lower production of IL-12 and higher production of IL-10 compared to murine M(IFNγ + LPS)[27, 50]. However, they have higher IL-12 production and lower IL-10 compared to murine M(Ic + LPS)[27, 50]. Markers for wound healing macrophages in humans include CD206, which is a mannose receptor, and the IL-1 receptor 2 (IL-1R2)[51].

Wound healing macrophages can also have either protective or pathogenic roles in disease. They can prevent type 2 diabetes in mice, by preserving glucose tolerance, insulin sensitivity, and protecting adipocytes from inflammation[3, 52]. This same activation state can also cause pathogenic fibrosis in murine models of lung inflammation[53, 54].

M(IL-4) are distinct from M(Ic + LPS) in mice. M(Ic + LPS) are not involved in the production of extracellular matrix and do not express enzymes such as ArgI, Ym1, or FIZZ1[1, 2]. There is evidence in mice that anti-inflammatory, high IL-10 producing macrophages are activated prior to the wound healing response. IL-10 receptor signalling activates STAT3, which up-regulates expression of the IL-4 receptor α on macrophages that makes them more sensitive to activation to a wound healing phenotype by IL-4 or IL-13[55, 56].

1.2 IL-10

IL-10 is a non-redundant, anti-inflammatory cytokine, which has multiple roles in the suppression of adaptive and innate immune responses in both mice and humans. IL-10 was first described as a factor that inhibits cytokine production from murine Th1 cells in 1989[57, 58]. IL-10 limits adaptive immunity in mice and humans by preventing antigen presentation and $CD4^+$ T cell activation by preventing expression of MHC II and CD80 and CD86 co-stimulatory molecules[59, 60]. IL-10 can also suppress innate immune responses in mice and humans. In 1991, IL-10 was identified as a factor that inhibits cytokine production from activated human macropahges[61]. IL-10 suppresses macrophage production of pro-inflammatory IL-1β, IL-12, IL-6, IL-8, and TNF and production of inflammatory chemokines and matrix metalloproteases

(MMPs)[59, 61]. IL-10 acts on macrophages in an autocrine or paracrine manner to inhibit inflammatory mediators and antigen presentation[60, 62]. IL-10 limits dendritic cell (DC) responses by limiting their maturation and differentiation[61, 63]. IL-10 blocks the effects of IFNγ on epithelial cells and blocks the proliferation and remodeling of extracellular matrix proteins in fibroblasts[64-66]. IL-10 can also promote, rather than suppress, adaptive and innate immune responses in mice and humans. IL-10 can enhance B cell activation, B cell survival, and $CD8^+$ T cell activation[67, 68]. It can also promote mast cell, granulocyte, and keratinocyte differentiation and growth, and NK cell proliferation and production of IFNγ[59]. IL-10 can stimulate the production of other anti-inflammatory mediators in innate immune cells. IL-10 can induce IL-1 receptor antagonist (IL-1Ra), which inhibits IL-1β signalling, in LPS-stimulated human monocytes and neutrophils[61, 69].

Numerous immune and a few non-immune cell types produce IL-10 in both mice and humans. Innate immune cells, which produce IL-10 include monocytes, macrophages, myeloid DCs, NK cells, eosinophils, neutrophils, and mast cells[25]. Adaptive immune cells, which produce IL-10 include B cells and T cells; Th1 cells, Th2 cells, regulatory T cells (Tregs), Type 1 regulatory (Tr1) cells, Th17 cells, and $CD8^+$ T cells[70]. Epithelial cells, keratinocytes, and even some viruses and tumor cells are non-immune cell producers of IL-10[61, 71, 72]. Cells in mice and humans can produce different amounts of IL-10. For example, in response to TLR ligands, macrophages produce high amounts of IL-10, myeloid DCs are able to produce intermediate amounts of IL-10, whereas plasmacytoid DCs do not produce IL-10[73]. The amount of IL-10 produced by a particular cell type is dependent on the type and strength of the IL-10-inducing stimulus[61].

1.2.1 IL-10 signalling

The IL-10 receptor is expressed in mice and humans by most hematopoietic cells, epithelial cells, and some fibroblasts[74]. The IL-10 receptor has common structural features in mice and humans[75]. The IL-10 receptor (IL-10R) is a hetero-tetramer comprised of two subunits of the IL-10Rα (or IL-10R1) and two subunits of the IL-10Rβ (or IL-10R2), and binds homo-dimeric IL-10[62, 76]. The IL-10Rα is responsible for binding IL-10, whereas the IL-10Rβ is responsible for initiating signalling[61]. The IL-10Rα uniquely binds IL-10, whereas the IL-10Rβ also shares signalling of other IL-10 receptor superfamily cytokines, IL-22 and IL-28 in mice and humans, IL-26 in mice, and IL-29 in humans[77].

IL-10 activates Janus kinase/STAT (Jak/STAT) signalling in mice and humans, and STAT3 is the main transcription factor that is activated in response to IL-10 in immune cells of both species[76, 78]. Upon receptor ligation, Jak1 is recruited to the IL-10Rα, while the non-receptor tyrosine protein kinase 2 (Tyk2) is recruited to the IL-10Rβ chain. Jak1 and Tyk2 phosphorylate the cytoplasmic tails of the receptor subunits[76]. STAT3 is recruited to the phosphorylated receptor, homo-dimerizes, and translocates to the nucleus, where it binds to the promoters of various IL-10-responsive genes, including the suppressor of cytokine signalling 3 (SOCS 3) and IL-10 itself[61].

Antigen presenting cells (APCs), specifically macrophages and DCs, are the cells that IL-10 impacts the most in mice and humans[59, 61]. The mechanisms by which IL-10 suppresses myeloid cell responses remain only partially described. IL-10 signalling inhibits MAPK and nuclear factor kappa B (NFκB) activation in mice and humans, which may contribute to the inhibition of pro-inflammatory cytokine production and antigen presentation by macrophages and DCs[79].

1.2.2 IL-10 production by macrophages

Murine and human macrophages produce IL-10 and pro-inflammatory cytokines in response to PAMPs, through conserved PRRs, including TLRs (Figure 1.2) and CLRs, such as dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) and Dectin-1[80]. Ic, PGE_2, and IL-10 can promote enhanced IL-10 production in response to TLR ligands, in mice and humans[1, 29].

TLR ligation induces IL-10 production through downstream signalling pathways, common to mice and humans[80]. Toll/IL-1 receptor (TIR) domain-containing adaptor molecules, such as myeloid differentiation primary response protein 88 (MyD88) and TIR domain-containing adaptor-inducing interferon β (TRIF) initiate different signalling pathways[62]. Through MyD88, MAPKs and NFκB are activated, and through TRIF signalling, tumor necrosis factor receptor-associated factor 3 (TRAF3) is activated, promoting IL-10 production[81, 82]. MyD88-mediated pathways induce MAPKs; Erk1 (ERK1 in humans) and Erk2 (ERK2 in humans), and p38, through the activation of tumor progression locus 2 (TPL2)[80]. The Erk (ERK) and p38 pathways co-operate to induce IL-10 production in LPS or CpG-stimulated macrophages, and mitogen and stress-activated protein kinase 1 and 2 (MSK1 and MSK2) are activated downstream of both Erk (ERK) and p38[80]. NFκB induces IL-10 through Erk (ERK)-dependent and -independent pathways[83]. Type I IFNs, which are induced by the TRIF-dependent pathway also help to induce maximal production of IL-10 in LPS-stimulated macrophages[84-86]. Larger amounts of IL-10 are produced when MyD88 and TRIF pathways are both activated in macrophages[80, 84, 85]. MAPKs activate several transcription factors, which bind to the murine *Il10* promoter, including phosphorylated cyclic AMP element binding protein (CREB), STAT1, STAT3, Sp1, interferon regulatory factor 1 (IRF1), activator protein 1 (AP-1), CCAAT enhancer binding proteins β and δ (C/EBPβ and C/EBPδ), c-musculoaponeurotic fibrosarcoma oncogene (c-Maf), pre-B-cell leukemia transcription factor 1 (PBX1), PBX-regulating protein 1 (PREP1), and NFκB[61, 87]. In human macrophages, transcription factors that bind to the *IL10* promoter and promote IL-10 production are similar, including STAT3, Sp1, Sp3, IRF1, C/EBPβ, PBX1, and PREP1[25, 80, 86].

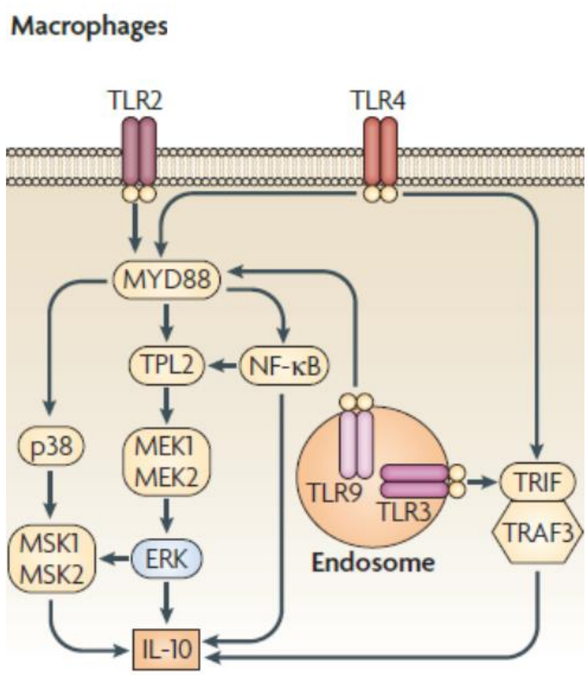

Figure 1.2 IL-10 production by macrophages. IL-10 is produced in response to TLR activation through MyD88- and TRIF-dependent pathways. Through MyD88, MAPK and NFκB activation promotes IL-10 production, whereas TRIF activation promotes IL-10 production through TRAF3. Reproduced with permission from Nature Publishing Group: Saraiva M and O'Garra A, Nature Reviews Immunology 2010[80].

IL-10 production by macrophages can be positively or negatively regulated. IL-10 amplifies its own production in human macrophages, through STAT3-dependent signalling[88]. Post-transcriptionally, IL-10 production regulates itself. In humans and mice, IL-10 promotes *IL10* (or *Il10* in mice) mRNA degradation, whereas other signals, such as adenosine receptor signalling in mice, can increase *Il10* mRNA half-life and production[80]. *Il10* expression is controlled epigenetically in mice; remodeling of chromatin at the *Il10* promoter initially leads to *Il10* transcription, and further histone acetylation or phosphorylation results in higher production of IL-10 by macrophages[38, 89]. Conversely, IFNγ negatively regulates IL-10 production by macrophages, by preventing CREB and AP-1 binding to the *Il10* promoter in mice[90]. IL-10 also negatively regulates its own production in murine macrophages, by inducing dual specificity protein 1 (DUSP1), which negatively regulates p38 activation[91]. MicroRNAs (miRNAs) can also regulate IL-10 production, miR-106a in humans binds to the 3' untranslated region (3'UTR) of *IL10* mRNA and causes its degradation[92].

1.2.3 IL-10 in disease

IL-10 serves a critical role in promoting the balance between inflammation and its resolution, to prevent damage to the host. IL-10 production by murine mast cells prevents leukocyte infiltration, inflammation, and tissue damage in allergic contact dermatitis and during ultraviolet B exposure to the skin[93]. IL-10 limits symptoms of multiple sclerosis and stroke in humans, by preventing pro-inflammatory cytokine production and by limiting infiltration of T cells into the brain[94]. IL-10 production is essential in mice during infections such as *Toxoplasma gondii*, *Mycobacterium* spp., and herpes simplex virus (HSV), in order to prevent tissue damage that results from a strong inflammatory response to fight infection[95]. In contrast, excessive or inappropriate production of IL-10 can lead to improper control of a pathogen that results in persistent or fatal infections in mice, such as with *Plasmodium* spp. or *Leishmania* spp.[95]. IL-10 deficiency in mice and humans leads to the development of inflammatory diseases, such as IBD, which will be discussed in section 1.5.10.

1.3 Fcγ receptors

Fcγ receptors are extracellular receptors that are in both mice and humans. They bind antibodies via their fragment crystallizable (Fc) portions, which are constant portions of antibodies that are comprised of two identical heavy chain proteins of immunoglobulin G (IgG) antibodies[96]. Genes for the Fcγ receptors are located in close proximity on chromosome 1, in mice and humans[97]. Apart from the human FcγRIIIB, which is glycosylphosphatidinositol (GPI)-anchored, murine and human Fcγ receptors are type I transmembrane glycoproteins (Figure 1.3)[98].

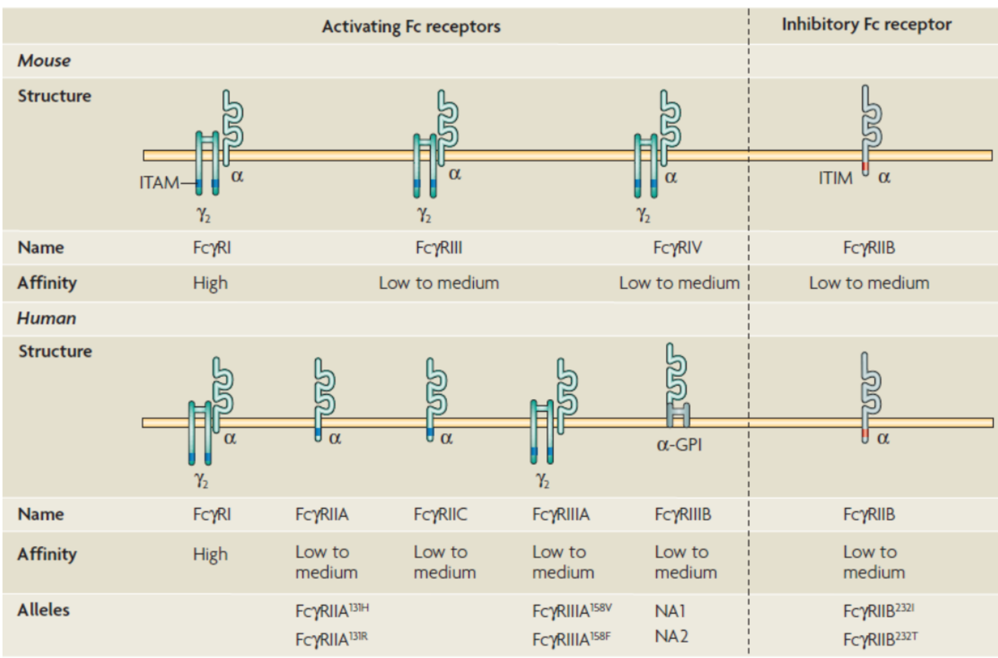

Figure 1.3 Fcγ receptors in mice and humans. Fcγ receptors in mice and humans can be categorized based on activating or inhibitory intracellular signalling motifs and by antibody affinity. Fcγ receptors contain intracellular ITAM motifs that activate signalling pathways. The FcγRIIB, which is the only receptor that contains an intracellular ITIM motif, inhibits cellular activation. FcγRI has the highest affinity for IgG antibodies, whereas the rest of the receptors range from low to medium affinity. Humans have gene variants for the FcγRIIA and FcγRIIIA that confers differential binding affinity for IgGs. The FcγRIIB has a gene variant, which prevents signalling downstream of its ITIM motif. Reproduced with permission from Nature Publishing Group: Nimmerjahn F and Ravetch J, Nature Reviews Immunology 2008[98].

Fcγ receptors can be categorized as activating or inhibitory receptors based on their intracellular signalling domains. The FcγRI (CD64), FcγRIII (CD16), and FcγRIV (CD16.2) in mice and FcγRI, FcγRIIA (CD32A), FcγRIIC (CD32C), FcγRIIIA (CD16A), and FcγRIIIB (CD16B) in humans, contain immunoreceptor tyrosine activation-based motifs (ITAMs)[99]. Activating receptors contain α chains, which bind the ligand extracellularly and, except for the FcγRIIA and FcγRIIC in humans, a γ chain dimer that contains the ITAMs and transduces the signal intracellularly[98]. ITAM signalling leads to activation of signalling cascades[100]. There is only one inhibitory receptor, the FcγRIIB (CD32B) that is common to mice and humans[98]. The FcγRIIB has a single ligand binding α chain, which contains an immunoreceptor tyrosine inhibitory-based motif (ITIM)[101]. ITIM signalling leads to inhibition of signalling cascades[100].

Fcγ receptors can also be classified based on their affinity for IgG. Affinities in mice and humans range from high for the FcγRI to medium or low affinity for the remaining receptors[102]. In mice, the FcγRI has the highest affinity for IgG1, IgG2, and IgG3[102]. In humans, the FcγRI has highest affinity for IgG1, IgG3, and IgG4[102]. In humans the FcγRI does not bind IgG2, whereas mice do not express the IgG4 isotype[102]. The remaining receptors in mice and humans, the FcγRIIA/B/C, FcγRIIIA/B, and FcγRIV range from medium to low affinity for IgGs, with 100-1000-fold lower binding affinity than the FcγRI[98].

Murine and human Fcγ receptors can bind IgGs as monomers, multimers, Ic, or on opsonized particles or cells[103]. All Fcγ receptors bind monomeric IgGs in mice and humans[104, 105]. It is widely thought that the receptors, except for the FcγRI, exclusively bind to aggregated or complexed IgGs, but evidence does not support this idea[106]. It was once thought that the FcγRI was saturated with IgGs in mice and humans *in vivo,* making it unavailable to bind and mount responses to additional IgGs, but evidence now suggests that this is not true[102, 107].

IgG binding affinities can change based on Fcγ receptor polymorphisms or post-translational modifications to antibodies. There are Fcγ receptor gene variants in mice and humans that confer differing affinities for IgGs[108]. IgG binding affinities can also change in mice and humans based on IgG Fc glycosylation status or changes to critical amino acids in areas responsible for binding to the Fcγ receptor[109].

In mice, Fcγ receptors are found on hematopoietic cells including B cells, T cells, neutrophils, NK cells, basophils, eosinophils, mast cells, DCs, monocytes, and macrophages[98, 110]. B cells in mice only express the FcγRIIB, which inhibits B cell receptor signalling[98]. Murine memory $CD8^+$ T cells also express the FcγRIIB[101]. Neutrophils express the FcγRIIB and FcγRIII in mice[111]. Murine NK cells express the FcγRIIB and FcγRIII[106]. The FcγRIII plays an important role in antibody-dependent cell-mediated cytotoxicity (ADCC) in neutrophils and NK cells[112]. Basophils, eosinophils, mast cells, and DCs in mice have expression of activating receptors and the inhibitory receptor[113]. Monocytes and macrophages express all Fcγ receptors in mice[98].

In humans, Fcγ receptors are found on hematopoietic cells, as with mice[98, 110]. Human B cells also only express the FcγRIIB[98]. Human memory $CD8^+$ T cells express the FcγRIIB, whereas human activated $CD4^+$ T cells express the FcγRIIIA[110, 114]. In humans, neutrophils express the FcγRIIA and FcγRIIIB, but not the FcγRIIB, [111]. Human NK cells express the FcγRIIB and FcγRIIIA[112, 115, 116]. As with mice, the FcγRIIIA and FcγRIIIB in humans play an important role in ADCC in neutrophils and NK cells[112]. Platelets express the FcγRIIA exclusively in humans, whereas murine platelets do not express Fcγreceptors[117].

Basophils, eosinophils, mast cells, and DCs have expression of activating receptors and the inhibitory receptor in humans, as with mice[113]. Monocytes and macrophages in humans express all Fcγ receptors, with the exception of the FcγRIIIB[98].

1.3.1 Fcγ receptor signalling in macrophages

ITAM-mediated signalling downstream of Fcγ receptor ligation causes activation of signalling cascades that are common between mice and humans (Figure 1.4)[99, 105]. ITAMs present on activating receptors become rapidly and transiently tyrosine phosphorylated by Src family kinases when the receptors are cross-linked[118, 119]. This creates SH2 docking sites for spleen tyrosine kinase (SYK)-family kinases that are recruited and downstream targets, such as son of sevenless (SOS) and Phosphatidylinositol 3-kinase (PI3K) are activated[98, 120]. Bruton's tyrosine kinase (BTK) and phospholipase C γ (PLCγ) can dock at the membrane once PI3K is activated and converts phosphatidylinositol 4,5-bisphosphate (PIP_2) to phosphatidylinositol 3,4,5-trisphosphate (PIP_3)[98, 121]. PLCγ activation leads to higher release of calcium from the endoplasmic reticulum and downstream signalling events, such as MAPK activation[122, 123]. SOS activation downstream of Syk also activates the RAS-RAF-MAPK pathway[98, 124]. Signalling events then lead to cytokine release, the oxidative burst, ADCC, and phagocytosis in murine and human macrophages[98].

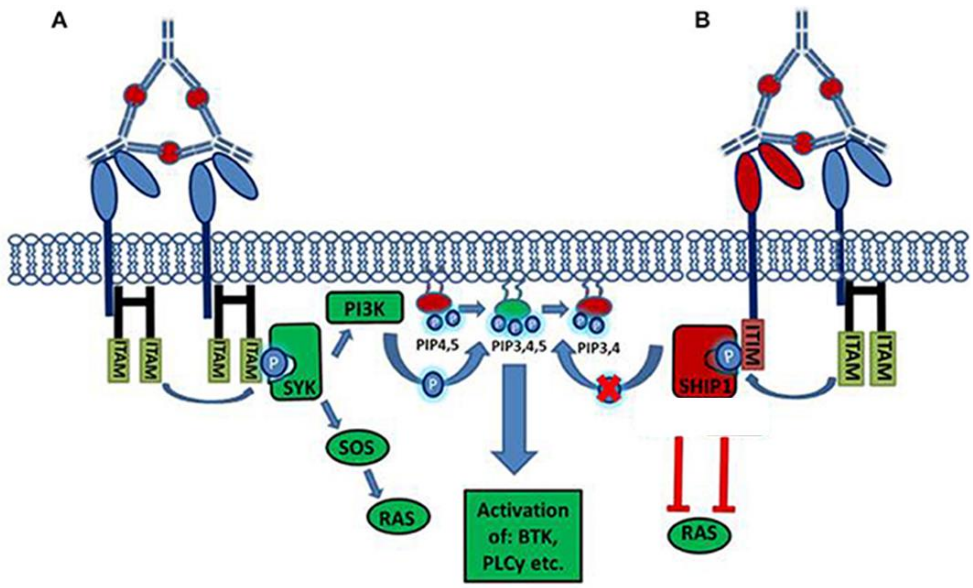

Figure 1.4 Fcγ receptor signaling. Fcγ receptor cross-linking can result in (A) activation or (B) inhibition of signalling pathways. (A) Fcγ receptor cross-linking with receptors that contain ITAM motifs results in phosphorylation of ITAMs, which recruits Syk. PI3K is activated resulting in activation of BTK and PLCγ. SOS and RAS are activated by separate pathways, and both pathways converge to activate MAPK. (B) Cross-linking ITIM motif-containing FcγRIIB causes phosphorylation of the ITIMs by activating receptors, which recruits SH2 domain-containing inositol phosphatases, such as SHIP. SHIP limits the PI3K and RAS signalling pathways. Reproduced according to the terms of the Creative Commons Attribution License (CC BY) ©Fuller, Stavenhagen and Teeling Frontiers in Neuroscience 2014[125].

ITIMs within the cytoplasmic domain of the FcγRIIB in mice and humans cause inhibition of signalling cascades, from the BCR, activating Fcγ receptors, or TLRs[99, 126]. Engagement of the FcγRIIB causes phosphorylation of the ITIM by Lyn or other Src-family kinases that are activated from co-aggregated receptors, and results in the recruitment of the phosphatases SHP-1 (SH2-domain containing inositol phosphate 5′phosphatase 1) and SHIP (SH2 domain-containing inositolpolyphosphate 5′-phosphatase)[102, 127, 128]. SHP-1 and SHIP inhibit signalling pathways that promote activation, such as the PI3K pathway, by dephosphorylating intermediates, which prevents the recruitment of kinases BTK and PLCγ to the cell membrane[98].

Inhibitory signaling can also occur downstream of ITAM-containing Fcγ receptors in mice and humans and is referred to as ITAMi signalling. ITAMi-meditated signalling can occur through the activating receptor FcγRIIA in humans and the FcγRIII in mice and humans, in a similar manner as ITIM-mediated inhibition of activating signals[129, 130]. ITAMi inhibition of signalling cascades occurs in a similar manner in mice and humans. Low engagement of activating receptors with monomeric IgGs results in low phosphorylation of ITAMs[127, 129, 131]. This causes a transient recruitment of Syk followed by a stable recruitment of SHP-1[127, 129, 131]. SHP-1 then co-localizes with activating receptors in an "inhibisome" cluster, leading to inhibition of activating signalling cascades[132, 133].

1.3.2 Fcγ receptor function in macrophages

Fcγ receptors can trigger a variety of processes in murine and human macrophages, depending on the type of antibody stimulus. In addition to participating in antigen processing and presentation, Fcγ receptors can modulate macrophage activation states and cytokine production, killing of pathogens, or can induce antibody-dependent cell-mediated phagocytosis (ADCP)[134].

As mentioned in the previous section, macrophage activation and cytokine production are affected by Fcγ receptor signalling. Ic and LPS co-stimulation of murine bone marrow-derived macrophages (BMDMs) induces higher production of IL-10 and lower production of IL-12, compared to LPS stimulation[1, 135]. Human macrophages also have higher production of IL-10, but not lower production of IL-12, when co-stimulated with Ic and LPS[29]. The density of IgG coating on Ic can determine the level of activation in murine macrophages. High densities of IgGs on Ics cause receptor cross-linking, which results in high LPS-induced IL-10 production and low IL-12 production[39]. The balance of activating (ITAM-mediated) signalling and inhibitory (ITIM-mediated) signalling can also determine the threshold for a response. Macrophages from *Fcgr2b*$^{-/-}$ mice, which are deficient in ITIM-mediated signaling, are more sensitive to inflammatory activation than wild type mice and are more sensitive to Ic-induced inflammatory conditions, such as arthritis[136].

Opsonization of pathogenic microorganisms with IgG antibodies leads to internalization and better killing of the pathogen. Activating Fcγ receptors in mice and humans direct the opsonized pathogen towards lysosomes, where intracellular replication is limited, and the pathogen is efficiently killed[137, 138]. When the Fcγ receptor common γ chain, which is present in activating receptors, is absent, mice have a higher susceptibility to intracellular infections due to reduced lysosomal killing[137]. The FcγRIIB can limit inflammatory responses to opsonized and internalized pathogens. When the FcγRIIB is absent in mice, there is higher production of inflammatory cytokines and *Mycobacterium tuberculosis* killing by macrophages[139].

Fcγ receptors can cause ADCP in murine and human macrophages[137, 138]. The FcγRIIIA in human monocytes and the FcγRIII in murine macrophages can bind opsonized infected cells or cancer cells, which causes efficient internalization and killing of these cells along with TNF production[102, 140-142]. ADCP is enhanced in human macrophages by activation with TLR agonists, DAMPs, or cytokines such as IFNγ[140].

1.3.3 Human Fcγ receptor IIA gene variant (rs1801274)

There are gene variants described for Fcγ receptors that result in altered function of these receptors (Figure 1.3)[112]. The FcγRIIA (rs1801274) single nucleotide polymorphism (SNP) occurs in the region of *FCGR2A* that encodes the amino acids that confer binding affinity for IgGs, which changes the receptor from a relatively low affinity to high affinity[143]. The low affinity gene variant for the FcγRIIA-R131, or CC genotype, has an arginine at amino acid position 131 that confers a lower binding affinity for IgG1 and IgG3[144-147]. The FcγRIIA-H131, or TT genotype, has a histidine substituted at amino acid 131, which confers binding for IgG2 that is not present in the CC genotype and confers a higher binding affinity for IgG1 and IgG3[144-147]. The frequency of the TT or CC genotype is between 25-35% depending on the ethnicity. In a European population, the genotype frequencies are 28.3% CC, 45.1% CT, and 26.6% TT (dbSNP)[148].

The TT gene variant (H131) has been associated with a higher risk of inflammatory and autoimmune diseases. Ulcerative colitis (UC) and Kawasaki disease (KD) are more common in people with the TT gene variant than the CC variant[149-151]. People with the TT gene variant are more likely to develop Guillain-Barré syndrome (GBS), celiac disease, or type 1 diabetes[152, 153].

While people with the TT gene variant have a higher risk of inflammatory and autoimmune diseases, they have a lower risk of getting infectious diseases. People with the TT gene variant are less likely to get *S. pneumoniae* infections compared to people with the CC gene variant[154]. The rates of sepsis and severe sepsis, from community acquired pneumonia due to *H. influenzae* infection, are also lower in people with the TT gene variant[155, 156]. Finally, the risk of severe meningococcal disease is lower in people with the TT gene variant[157].

The TT gene variant is also associated with a higher risk of failure to respond to therapy with the anti-TNFα antibodies, infliximab or adalimumab, in people with RA[144, 158]. Lower response rates were seen in people with the TT genotype at 30 weeks after starting treatment, with 60% of people with the CC genotype responding to treatment compared to 33.3% of people with the TT genotype[144].

Although few, *in vitro* experiments suggest that cells from people with the TT, high affinity gene variant have higher inflammatory responses. Peripheral blood mononuclear cells (PBMCs) from people with the TT genotype have higher IL-1β production compared to the CC genotype in response to IgG2 *in vitro*[159]. Neutrophils from people with periodontitis, who have the TT genotype are hyper-reactive; they have higher levels of phagocytosis and degranulation in response to serum opsonized bacteria compared to neutrophils from people with periodontitis, who have the CC genotype[160].

1.4 Intravenous immunoglobulin (IVIg)

IVIg is a drug made up of polyclonal IgG antibodies, pooled from the plasma of over 1000 healthy blood donors. More than a dozen IVIg preparations are approved by the U.S. Food and Drug Administration (FDA)[161]. As IVIg is a blood product, healthy donors are screened for potential infectious diseases and blood borne pathogens are inactivated and removed[162]. The method of preparation differs between IVIg brands, but can include cold ethanol fractionation, ultrafiltration, and/or chromatography[163]. Although relative distributions of antibody subtypes differ between IVIg preparations, IgG1 is the predominant isotype present making up 60% of the antibodies[164]. IgG2, IgG3, and IgG4 comprise the majority of the remaining 40% of the antibodies with trace amounts of IgM and IgA present in IVIg[164]. Small amounts of IgG dimers are present, with the addition of stabilizing agents, such as glycine or sorbitol, to prevent aggregation[163].

IVIg is given as an intravenous infusion, although use of subcutaneous preparations is increasing[165]. Side effects of intravenous infusion are often mild and self-limiting; these include hemolysis, headache, fever, chills, lower back pain, nausea, shortness of breath, and tightness in the chest[164].

Although IVIg treatment is efficacious, it is expensive. A single dose of IVIg for a 70 kg adult can cost from $2000 - $8000 Canadian dollars, as of 2007[166]. Since it is an expensive drug that is used at high doses, alternative synthetic products are being developed. Studies to determine its mechanism of action are important to inform the development of these products.

1.4.1 Uses of IVIg

IVIg is used therapeutically for two very different purposes, as an antibody supplement in people who have deficiencies, and as an anti-inflammatory therapy for immune-mediated diseases[167]. 75% of IVIg's use is for autoimmune and inflammatory diseases[161]. IVIg is increasingly used to treat diseases that it is not yet approved for[161].

Antibodies that are missing in a person can be passively administered to prevent or treat infection, because of the diverse antibody repertoire in IVIg. It is given at relatively lower doses for this purpose, at 400 mg/kg of body weight[167]. As an antibody replacement therapy, the FDA-approved indications include primary immunodeficiency diseases such as X-linked agammaglobulinemia (XLA), chronic lymphocytic leukemia (CLL), pediatric human immunodeficiency virus (HIV) infection, and to prevent infections after allogeneic bone marrow transplant[161]. IVIg is also used as an off-label treatment, for uses that are not approved by the FDA. Examples of off-label uses as an antibody supplement are cytomegalovirus infection in people undergoing bone marrow transplantation, necrotizing fasciitis, recurrent infections with unknown origin, and infections in geriatric people with low antibodies[165].

IVIg is used for inflammatory and autoimmune diseases with a diverse range of pathologies. It is given at higher doses for this purpose, at 1-2 g/kg of body weight[167]. The FDA has approved the use of IVIg for KD, idiopathic thrombocytopenic purpura (ITP), chronic inflammatory demyelinating polyneuropathy (CIDP), multifocal motor neuropathy (MMN), and kidney transplantation involving a recipient with a high antibody titer or an ABO-incompatible donor[161]. Off-label uses include neuromuscular disorders, such as GBS and relapsing and remitting multiple sclerosis, hematologic disorders, such as autoimmune hemolytic anemia and graft-versus-host disease, and for dermatological disorders, such as pemphigus vulgaris and Stevens-Johnson syndrome[161]. Other un-approved uses include treatment of severe rheumatoid arthritis, systemic lupus erythematosus (SLE), dermatomyositis, macrophage activation syndrome, inflammatory bowel disease, and autoimmune hemophelia[165, 168]. Off-label uses account for 50-60% of IVIg sales[149]. Large-scale, controlled studies are needed to determine whether IVIg use is warranted in an increasing number of immune-mediated diseases.

1.4.2 Proposed mechanism(s) of action of IVIg

As a source of passive immunity, IVIg provides a diverse repertoire of IgG antibody fragment antigen-binding (Fab) specificities, which are able to bind to a wide variety of pathogens and protect people from infections[169]. However, its mechanism of action in treating inflammatory and autoimmune diseases remains undefined. A unifying mechanism does not exist that explains how IVIg works at high doses to limit immune responses. IgG antibodies have diverse functions in the immune system, and mechanisms are proposed, which involve either the Fab portion or the Fc portion of the antibody. Fc-mediated mechanisms are shown in (Figure 1.5).

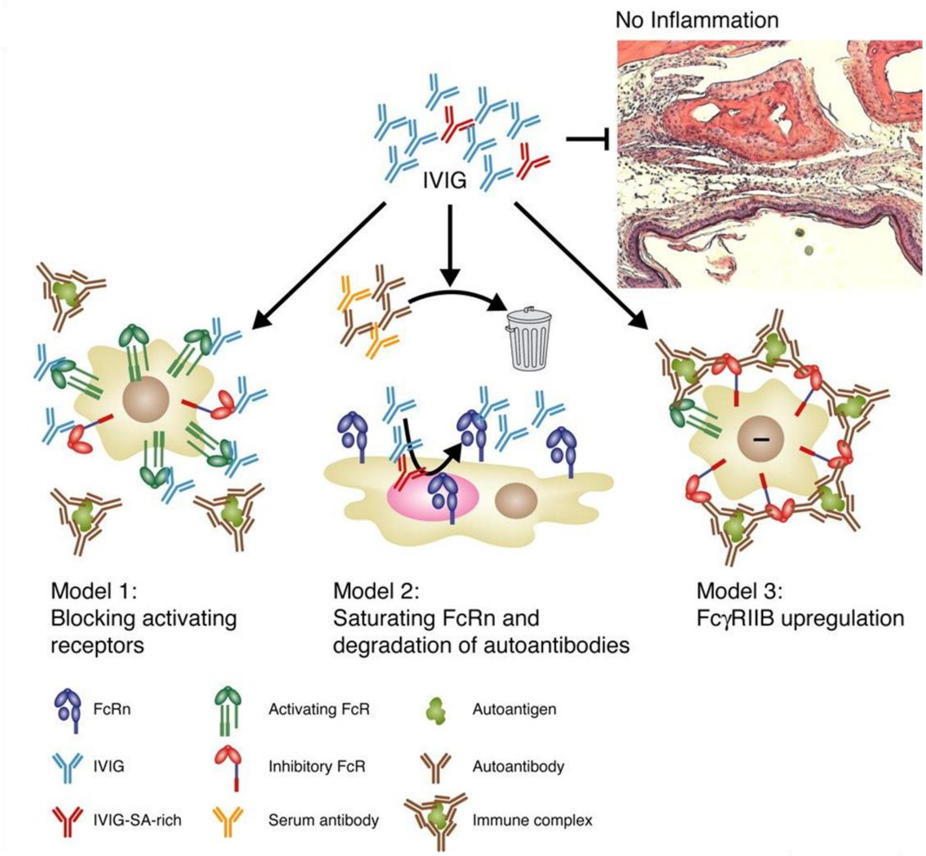

Figure 1.5 Proposed mechanism(s) of action of IVIg. Model 1: IVIg binds to activating Fcγ receptors, blocking autoantibody Ic-mediated immune activation. Model 2: IVIg binds to FcRn, causing saturation, which prevents recycling of autoantibodies that prevents activation of immune responses. Model 3: Sialic acid-linked IgGs in IVIg cause up-regulation of the inhibitory FcγRIIB, causing inhibition of autoantibody Ic-mediated immune activation. Reproduced with permission from The Rockefeller University Press: Nimmerjahn F and Ravetch J, Journal of Experimental Medicine 2007[170].

Fab-mediated binding of immunomodulatory proteins has been proposed as a mechanism of action of IVIg. IVIg contains small amounts of antibodies to inflammatory cytokines, chemokines, and receptors like Fas, sialic acid-binding Ig-like lectin-9 (Siglec-9), and CD5[161]. Fab portions have been proposed to bind to these inflammatory mediators, which could switch an immune response from inflammatory to anti-inflammatory[171]. However, little evidence exists for this theory and the importance of the Fc region in immunomodulation by IVIg has been demonstrated[161, 172].

Blocking activating Fcγ receptors by high doses of IgGs is another mechanism of action that has been proposed, with little direct evidence. In the untreated state, Fcγ receptors are available for autoantibody binding, which promote inflammation by inducing pro-inflammatory cytokine production in response to disease-associated TLR agonists[173]. A large increase in serum IgG concentration from IVIg treatment could cause monomeric IgGs to saturate and block Fcγ receptors, which would prevent autoantibody-induced pathology, as monomeric IgGs do not promote an inflammatory immune response[106, 170]. However, it has not been demonstrated in mice or in humans that saturation of Fcγ receptors with monomeric IgGs can prevent functioning with IgG multimers or complexes[106, 112]. The high dose requirements required for IVIg's effectiveness is explained by this theory, nonetheless. A similar theory has been proposed recently, by which IVIg induces ITAMi signaling through the FcγRIIA or FcγRIII, which reduces LPS-induced pro-inflammatory cytokine production by human macrophages *in vitro*[129, 130]. IVIg-induced ITAMi signaling through the FcγRIIA also ameliorates disease symptoms in a murine collagen-induced arthritis model, which provides further evidence that IVIg can prevent auto-antibody-induced pathology by binding to Fcγ receptors[129].

IVIg has also been proposed to work by saturating the neonatal Fc receptor (FcRn), preventing pathogenic autoantibody re-cycling, in autoimmune diseases. The murine and human FcRn is present on vascular endothelial cells, epithelial cells of the intestine, liver, and lung, as well as immune cells[174]. The FcRn re-cycles IgGs in mice and humans, by preventing their degradation by the lysosome and returning them to the serum[174]. In a murine model of ITP, IVIg promoted the clearance of pathogenic platelet IgG antibodies, through saturation of the FcRn[175]. FcRn saturation in mice prevents pathogenic autoantibody recycling and they are degraded more readily. However, the FcRn was not required for IVIg's effect in another murine model transgenic murine model of ITP suggesting that other mechanism(s) of action contribute to IVIg's efficacy in that model[176, 177].

A leading theory of IVIg's mechanism of action for immune-mediated diseases is that a minor fraction of IgGs in IVIg are sialylated, which may be responsible for its efficacy. In IVIg, α2,6 sialic acid linked Fc regions bind to DC-SIGN receptors on myeloid cells, which induces IL-33 production[178]. Myeloid IL-33 production causes IL-4 production by basophils that up-regulates the inhibitory FcγRIIB, which suppresses auto-antibody-mediated macrophage inflammatory responses in mice[178]. This theory has been demonstrated in a murine serum-induced model of RA, but other studies using the same murine model of RA have not supported this theory[178, 179]. The study that contradicted this theory showed that sialylation or basophils are not required for the effectiveness of IVIg, and instead demonstrated the necessity of the Fc portion of IgGs in IVIg[178, 179]. Studies using passive antibody transfer models of ITP in mice have both supported the requirement of Fc sialylation and the specific ICAM3 grabbing nonintegrin-related 1 SIGN-R1 (the murine orthologue of DC-SIGN) and refuted the requirement of Fc sialylation, for the suppression of autoantibody responses[180-182]. This theory has not been observed in people receiving IVIg, as no increase in FcγRIIB expression has been seen in monocytes after IVIg infusion, and sialylation levels of IgGs did not affect the response to IVIg in KD[183-185].

1.5 Inflammatory bowel disease (IBD)

IBD is a chronic, incurable, relapsing, or remitting disease, characterized by inflammation of the intestine[186]. IBD encompasses Crohn's disease (CD) and UC. CD affects any part of the intestinal tract and the inflammation is usually discontinuous and is transmural[187]. UC affects only the rectum and colon, and the inflammation is continuous[187]. In UC the inflammation is within the mucosa, rather than transmural, as in CD[187]. Diagnosis of IBD is usually between the ages of 15 and 30 years old[188]. Symptoms of IBD include fever, abdominal pain, weight loss, and diarrhea that can contain blood or mucus[189, 190]. People with CD can develop complications such as fistulas, fibrosis, stenosis, abscesses, as well as extra intestinal manifestations, such as uveitis[170]. People with UC have a higher likelihood of developing colon cancer[189, 191]. People with either CD or UC are at a higher risk of developing other immunological disorders, such as arthritis, psoriasis, or asthma[192].

1.5.1 Incidence and disease burden

The incidence of IBD is high in developed nations. In 2012, IBD affects approximately 233,000 Canadians; 129, 000 have CD and 104,000 have UC; with an overall incidence of 0.67% of the population[193]. In 2015, over 1 million people in the United States and over 2.5 million people in Europe were diagnosed with IBD [194]. The incidence in North America and Europe is stable, but since 1990, the incidence in developing countries, such as Taiwan and Brazil, has increased dramatically[195].

IBD incidence differs between sexes and races. In North America and Europe, males and females are equally as likely to have IBD but in Asian countries, IBD is more prevalent in males[196]. Males and females differ in prevalence of IBD related complications; males have a higher prevalence of primary sclerosing cholangitis and ankylosing spondylitis, whereas females have a higher prevalence of extra-intestinal manifestations of the eye and skin[196]. In the United States, the incidence of IBD is higher in people, who identify as white, whereas the incidence is lower in people, who identify as black or Hispanic, and the incidence is increasing in other minorities[197, 198].

IBD is a costly disease, both to society and to individuals. In 2012, IBD was estimated to cost $2.8 billion in Canada annually[193]. Direct medical costs, such as the cost of medications, hospitalizations, and doctor visits accounted for $1.2 billion, whereas $1.6 billion was attributed to indirect costs, such as short term disability costs from absence at work[193]. Additional important factors not included in cost estimates of disease burden, including impaired quality of life, social stigma, and negative effects on career aspirations[194].

1.5.2 Pathogenesis of inflammatory bowel disease

Evidence suggests that environment, genetics, the gut microbiome, and the immune response all contribute to the pathogenesis of IBD (Figure 1.6). The gastrointestinal tract is a barrier to the outside world, which promotes tolerance to commensal bacteria but also promotes appropriate inflammatory responses to pathogenic organisms[199]. The cause of disease in IBD is not fully known, but it is thought to occur due to an overactive immune response to commensal microorganisms in the gut in genetically susceptible individuals[199].

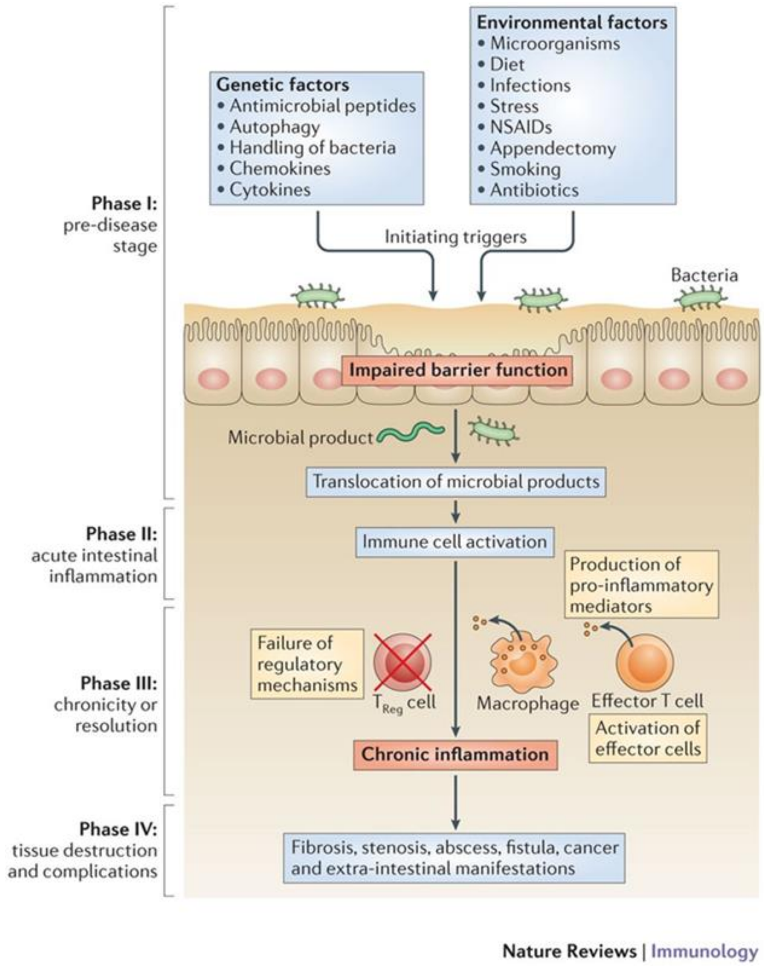

Figure 1.6 Pathogenesis of inflammatory bowel disease. IBD is caused by a combination of genetic and environmental factors, which result in impaired barrier function. With impaired barrier function, bacteria translocate below the epithelial layer, and cause activation of innate immune cells, such as macrophages, and activation of effector T cells. The production of pro-inflammatory mediators, and failure of regulatory mechanisms, such as the activation of Tregs, results in chronic inflammation. In IBD, the inflammation can cause tissue destruction and complications, such as fibrosis or cancer. Reproduced with permission from Nature Publishing Group: Neurath M, Nature Reviews Immunology 2014[200].

1.5.3 The role of the environment in inflammatory bowel disease

Many environmental factors contribute to the pathogenesis of IBD, including stress, pollution, diet, and vitamin D levels. Stress is said to play a role in the development of inflammatory bowel disease. Both adverse life events and chronic stress contribute to the development of disease, with psychological stress influencing the development of CD and depressive symptoms playing a part in the development of UC[201, 202]. Stress may result in increased epithelial permeability, which exacerbates inflammation in murine models of IBD[202]. High levels of air pollutants correlate with higher risk of both CD and UC, and higher amounts of air pollution are associated with higher levels of pro-inflammatory cytokines in plasma of people with IBD[203, 204]. The incidence of IBD is increasing in developing nations in Asia and correlates with high amounts of air pollution[205]. Diet is also a factor in the development of IBD, which could also be increasing in incidence in developing nations, due to the "westernization" of diet, including eating less fruits and vegetables and more proteins, fat, and sugar[206, 207]. A high fat or high sugar diet is associated with higher intestinal permeability, that could lead to uncontrolled inflammation in the gut[206]. Vitamin D levels are low in people newly diagnosed with IBD in Canada, and low vitamin D levels could contribute to higher incidence of IBD in populations where there are lower levels of sun exposure[208, 209]. Smoking has been associated with the development of CD, but not UC[200]. The use of non-steroidal anti-inflammatory drugs (NSAIDs) use have also been linked to higher risk of developing IBD[200].

1.5.4 The role of genetics in inflammatory bowel disease

The earliest genetic studies noticed a higher risk of IBD in family members of people with IBD, with a complex, non-Mendelian model of inheritance[210]. Genome wide association studies (GWAS) have dramatically advanced our knowledge of the role of genetics in the pathogenesis of IBD. In 2012, 163 single nucleotide polymorphisms (SNPs) were identified that confer a higher susceptibility to IBD, with 30 being CD- and 23 UC- specific, and 110 associated with both diseases[211]. Currently, there are 241 SNPs identified for IBD susceptibility[212].

Knowledge of genetic contributions to disease has prompted new research into mechanism of disease, which have contributed to the development of novel therapeutics and subtyping of people with IBD for personalized medicine approaches to treatment.

Polymorphisms in genes associated with autophagy and cytokine signalling have been associated with a higher risk of IBD. *NOD2* was the first IBD susceptibility SNP identified for CD[213]. The NOD2 protein is a NLR, which binds muramyl dipeptide (MDP), a bacterial peptidoglycan, and induces autophagy, regulates antigen presentation, and regulates innate and adaptive immune responses[214]. Other autophagy-related SNPs have been identified, including *ATG16L1* and *IRGM*, indicating the important role autophagy has in the regulation of immune responses in the gut[214]. SNPs have been identified in cytokine receptor genes, such as *IL23R* or *IL12B*, which bind the pro-inflammatory cytokines or subunit IL-23 and IL-12/23p40, respectively[214]. This has led to the connection of this cytokine to the pathogenesis of IBD and the development of the drug ustekinumab, which binds to the IL-12/23 common subunit, IL-12/23p40[215].

1.5.5 The role of the microbiota in inflammatory bowel disease

Dysbiosis, which is a microbial imbalance typically accompanied by reduced microbial diversity, has been associated with IBD[216, 217]. In the intestine, there are more than 10^{14} microbes, which is about 3x the number of bacterial cells as in the human body and 30x the amount of genetic material compared to the human genome[218, 219]. Commensal bacteria in the gut digest foods and provide essential nutrients, train the immune system, modulate the epithelial barrier, and control neuronal motor functions[216, 220]. In UC, there is low diversity and instability of the microbiome[221]. There are also lower amounts of *Clostridium* spp. and higher amounts of

Escherichia coli (*E. coli*), compared to healthy controls[221]. In CD, there is an overabundance of enterobacteria and a lower amount of Firmicutes and Bacteroidetes phyla, compared to healthy controls[214, 222]. Adherent invasive *E. coli* (AIEC) that can invade epithelial cells and survive within macrophages, and mucosa-associated *E. coli* have been found in some people with CD[223, 224]. Although gut bacteria are altered in people with IBD, it is unclear whether in the inflammatory environment drives dysbiosis or bacterial dysbiosis triggers inflammation[225].

1.5.6 The role of the epithelial barrier in inflammatory bowel disease

Intestinal permeability is increased in people with IBD, which increases exposure of immune cells in the lamina propria to the microbiota, promoting inflammation[226]. Some studies indicate that increased gut permeability, which can be due to environmental factors or genetics, can be an initiating factor in the development of IBD[199]. The intestinal epithelial barrier is a physical barrier of cells lining the intestinal tract in mice and humans[199, 227]. In the intestine of people with IBD, defective expression of tight junction proteins, such as β-catenin and claudins, have been observed[228, 229]. There are associations between SNPs for epithelial junction proteins, such as E-cadherin, and a higher risk of developing UC[211, 230]. The intestinal epithelium provides an additional barrier of mucus and anti-microbial peptides (AMPs) that are secreted by Goblet and Paneth cells, respectively in mice and humans[199, 227]. There are associations between SNPs for mucus proteins, such as the mucin 19 (Muc19) protein, and a higher risk of developing UC[211, 230]. Defective expression of AMPs has been seen in the intestines of people with IBD[228, 229]. Epithelial cells in mice and humans also express TLRs that promote barrier function by inducing epithelial cell proliferation, inducing production of mucins, AMPs, and secretory IgA[213]. Dysfunctional TLR4 signalling can result in intestinal inflammation in mice, through increased barrier permeability[228]. A *TLR4* SNP is associated with a higher risk of IBD in humans, due to impaired barrier function[231, 232].

1.5.7 The role of the immune system in inflammatory bowel disease

In addition to epithelial barrier defects, defects in the innate and adaptive immune system are associated with the development of IBD (Figure 1.6). The role of IL-10, which regulates the innate and adaptive immune system in the intestine, is discussed in section 1.5.10.

1.5.8 The innate immune response in inflammatory bowel disease

Innate immune cells, such as neutrophils, innate lymphoid cells, DCs, and macrophages contribute to the pathogenesis of IBD. The innate immune cells are the first responders of the murine and human immune system and promote an inflammatory response to DAMPs and PAMPs, through PRRs expressed on their cell surfaces or intracellularly.

Neutrophils infiltrate into the intestinal mucosa and epithelial crypts early in IBD[232]. They impair barrier function, secrete pro-inflammatory mediators, and damage tissue through proteolytic activity and oxidative damage in mice and humans[233]. Neutrophils are present throughout disease in humans, and the amount of infiltration correlates with disease symptoms and tissue damage[233].

Innate lymphoid cells (ILCs) from the mucosa of people with IBD secrete higher amounts of IL-23 and IL-17 than ILCs from the mucosa of healthy people[232, 234]. Few studies exist linking ILCs to the pathogenesis of IBD in humans, but there are three distinct groups of ILCs identified in mice that promote inflammation, regulate microbial communities, and control tissue damage and repair[235].

DCs sample luminal contents in the intestine, and either promote tolerance or inflammation in mice and humans. During homeostasis DCs are hypo-responsive to bacterial antigens, a potential mechanism is through epithelial cell production of thymic stromal lymphopoietin (TSLP)[236]. In mice, epithelial TSLP and TLR agonists induce DC IL-10 production, without production of IL-12 or IL-6, and promote Th2 rather than Th1 responses[236]. In people with CD, low levels of TSLP are expressed in intestinal epithelial cells, and DCs produce more IL-12 and IL-6 than DCs from non-inflamed individuals[236, 237].

Inflammatory macrophages are implicated in the pathogenesis of IBD. Like DCs, macrophages can regulate innate and adaptive immune responses in the gut of mice and humans. Macrophages are also in an anergic state during homeostasis, to promote tolerance to commensal microbes[238, 239]. *In vitro*, human intestinal macrophages do not produce IL-10, IL-1, IL-6, IL-12, or TNF in response to bacterial ligands, but still have phagocytic and bactericidal activity[240]. Genomic analysis has revealed that the majority of IBD susceptibility loci contain monocyte/macrophage genes, involving responses to pathogens[241]. In people with CD, $CD14^+$ macrophages have high production of IL-23 and TNFα, which contributes to higher IFNγ production by lamina propria mononuclear cells[24]. Human $CD14^+$ lamina propria macrophages from people with CD, but not from healthy controls, induce Th17 cells in the presence of commensal bacterial antigens[242]. Macrophages from the mucosa of people with IBD produce TNFα, IL-1α, and IL-1β, but are still responsive to the suppressive effect of IL-10 or IL-4[243].

Macrophages are heterogeneous cells, including in the inflamed intestine, where they have a role in tissue restitution. Macrophages, which express CD206, a marker for M(IL-4), are higher in the inflamed mucosa of people with chronic UC compared to people with newly diagnosed UC[244]. In CD, fibrosis is a common complication that can occur, due to excessive wound healing. There are high numbers of macrophages present in fibrotic lesions of people with CD[230]. Expression of macrophage matrix metalloproteinase 2 (MMP2), which breaks down extracellular matrix, is higher in the mucosa of people with CD[245, 246].

1.5.9 The adaptive immune response in inflammatory bowel disease

The adaptive immune response, involving T cells and B cells, is slower to develop but is more specific and has longer-term memory than the innate immune response. The adaptive immune response may be important in the development of chronic intestinal inflammation[199].

Th1, Th2, Th17, and Th9 cells are involved in the pathogenesis of IBD[199]. APCs present antigens to naïve $CD4^+$ T cells, and along with cytokines and other stimuli, direct T cells to different fates in mice and humans[247]. Th1 cells in mice and humans are induced by IL-12 and IL-27, and secrete IFNγ, TNF, and IL-2[247]. Th2 cells are induced by IL-4, and secrete IL-4, IL-5, and IL-13 in mice and humans[247]. Although now controversial, a Th1 response has been found to be prominent in CD, whereas a Th2 response was found to be more prominent in UC, due to higher mucosal IFNγ and IL-12 in CD and higher IL-5 and IL-13 secretion by mucosal T cells in UC[248-250]. However, Th17 cell involvement in IBD has changed the paradigm. Th17 cells in mice and humans are induced by IL-6 and transforming growth factor β (TGFβ), and expansion of these cells is induced by IL-23[247, 251]. They can produce IL-17A and IL-21 in mice and humans [251]. IL-17A can induce recruitment of neutrophils and more Th17 cells, whereas IL-21 promotes secretion of IL-17A and IFNγ, which are higher in inflamed tissues from people with CD[199, 252, 253]. In both CD and UC, mucosal Th17 cells and serum IL-17A are high[199]. Inhibiting both IL-12 and IL-23 with the drug, ustekinumab, may prevent both Th1/Th17 skewing[199]. Additionally, people with IBD also have higher numbers of IL-9 producing Th9 cells and people with severe

UC have higher mucosal IL-9 levels than people with mild disease[254]. IL-9 acts as a growth factor for immune cells, and disrupts epithelial barrier function in mice[255].

Tregs have a protective role in IBD. Tregs are antigen specific suppressive $CD4^+$ T cells that express the transcription factor forkhead box protein 3 (Foxp3) and CD25 in mice and humans[256]. Tregs secrete IL-10 and TGFβ in mice and humans, which suppress DC and macrophage antigen presentation and their ability to activate T cells[199]. Spontaneous intestinal inflammation occurs when Tregs are absent in mice, which supports the essential role they have in maintaining intestinal homeostasis[257]. It is unknown whether Tregs are defective in people with IBD, as more Tregs are present in inflamed versus non-inflamed mucosa of people with IBD and mucosal Tregs have potent anti-inflammatory activity *in vitro*[258]. However, effector T cells may be resistant, or less responsive, to the effects of Tregs in people with IBD[259].

B cells are also implicated in the pathogenesis of IBD. There are hyper-activated B cells, which secrete high amounts of IL-8 and elevated levels of mucosal IgGs against bacterial antigens in people with IBD[260, 261]. IL-8 recruits neutrophils and IgGs opsonize bacteria, which promotes an inflammatory response[137, 262]. This supports the role of microbial antigens in the pathogenesis of IBD.

1.5.10 The role of IL-10 in inflammatory bowel disease

The importance of IL-10 in intestinal homeostasis was first demonstrated in 1993, when it was shown that IL-10 deficient mice develop severe spontaneous enterocolitis[263]. The inflammation is dependent on the microbiota, as germ free mice do not develop spontaneous colitis[264]. IL-10 effects on macrophages are important in intestinal homeostasis, as macrophage

IL-10 receptor deficient mice develop severe spontaneous colitis, which has been attributed to pro-inflammatory macrophage activation and antigen presentation to $CD4^+$ T cells[238, 265].

Deficiencies in IL-10 are associated with IBD in humans. Many years later, after the importance of IL-10 was realized in murine intestinal homeostasis, GWAS identified a SNP in the *IL10* gene promoter that was strongly linked to a higher incidence of UC[266]. A SNP in the *IL10* gene was also found that confers a higher risk of CD[211, 267]. Severe IBD develops early in life in people, who have mutations affecting either IL-10 or the IL-10 receptor, and hematopoietic stem cell transplantation induced remission in these people[268-270].

Serum IL-10 levels are different based on disease severity in IBD. Studies have shown that IL-10 is high in people with CD or UC during active disease, compared to healthy controls[271, 272]. In CD, higher serum IL-10 levels are associated with lower disease severity, compared to lower IL-10 levels, which are associated with more severe disease[273, 274]. IL-10 may be important to initiate remission in IBD. IL-10 is high in the serum of people with UC during the initial phase of remission, while C- reactive protein and IL-6 levels are high during acute inflammation and are reduced during remission[273]. There is little research showing intestinal mucosal IL-10 levels; however, in CD, low ileal IL-10 is associated with higher recurrence of disease after bowel resection[275].

The role for IL-10 in the pathogenesis of IBD prompted clinical trials using recombinant human IL-10 (rhIL-10) to treat CD. However, the results were disappointing as only modest improvements were seen in people[74, 276, 277]. A possible reason for the disappointing results can be a lack of participant selection based on disease severity and serum IL-10 levels because people with more severe disease and lower IL-10 levels would be predicted to benefit most[277]. A sub-optimal IL-10 delivery to the mucosa due to systemic administration could also be possible, as many non-immune cells, such as fibroblasts and epithelial cells, express IL-10 receptors[61]. Finally, the dose of IL-10 given in trials could also result in immune-stimulatory effects, as IL-10 can promote B cell and NK cell responses[61].

The pathogenesis of IBD is complex; environmental, microbial, and immune factors combine to cause IBD in a genetically susceptible host (Figure 1.6). Defects in epithelial barrier function can increase translocation of bacteria into the lamina propria and cause inflammatory responses from innate immune cells that promote tolerance under homeostatic conditions. Innate immune cells direct the subsequent adaptive immune response, which together, contribute to the chronicity of the inflammation. IL-10 plays an essential role in preventing intestinal inflammation. Understanding the pathogenesis of disease and genetic contributions has led to new, more effective treatments for IBD.

1.5.11 Therapeutic options for inflammatory bowel disease

The goals of treatment for IBD are to induce and maintain remission, to promote mucosal healing, and to improve the quality of life for people with IBD as there is no cure for this complex disease[278]. The treatment given is based on disease location, severity, age of person, efficacy of the drug, and side effects[279]. For mild to moderate IBD, aminosalicylates, such as 5-aminosalicylic acid (5-ASA) can be used, but when aminosalicylates fail, potent anti-inflammatory corticosteroids, such as prednisone, can be prescribed[280]. Corticosteroids have adverse effects if used long term, such as development of diabetes, infection, and bone disease, and do not work well for maintaining remission[281]. If a person fails aminosalicylates and is refractory to, or prefers not to depend on, corticosteroids; immunosuppressant drugs, such as azathioprine, can be prescribed[279]. Immunosuppressants also have serious side effects, such as a higher risk of infections and pancreatitis[279].

The recent advance of biologic antibody therapies has greatly improved long-term outcomes for people with IBD, with the reduction of relapse rates and induction of mucosal healing[282]. Anti-TNFα and other antibody drugs can be used for people with poor prognosis or when the person is refractory to other therapies[283]. These drugs can also be prescribed upon diagnosis of IBD, by some doctors[281]. There are currently two anti-TNFα drugs approved by the FDA for use in both CD and UC, infliximab and adalimumab[284]. Golimumab and certolizumab pegol, which are also anti-TNFα drugs, have been approved by the FDA for use in UC and CD, respectively[284]. There are two anti-integrin drugs, natalizumab and vedolizumab, approved by the FDA, which target the recruitment of immune cells into the intestine[284]. Natalizumab is approved for CD and vedolizumab is approved for use in CD and UC[284]. In addition, there are now infliximab and adalimumab biosimilars approved by the FDA for use in CD and UC[285]. The anti-IL-12/23p40 antibody, ustekinumab, has been recently approved for use in CD by the FDA[286].

Although they have revolutionized treatment for IBD, anti-TNFα drugs do not work for everyone. They are relatively safe but can have serious side effects, such as higher risk of infections like tuberculosis, and higher risk of certain cancers[287]. Up to 40% of people with IBD are, or will become, refractory to anti-TNFα therapy. Approximately half treatment refractory people have not developed anti-drug antibodies, and the reason for their loss of response is unknown[288]. Thus, new treatments are needed and are being developed. These include tofacitinib, a JAK1-JAK3 inhibitor, and fecal microbiota transplantation[186]. Despite advances in the treatment of IBD, surgery can be the only therapeutic option for some people. 25-35% of people with UC will eventually require surgery to control symptoms or complications[289]. It is estimated that 70-90% of people with CD undergo surgery related to their disease[289]. This further emphasizes the need to develop new treatments for IBD and the need to determine why some people are unresponsive to antibody-based therapies.

1.6 Thesis hypothesis and objectives

1.6.1 Summary of rationale

Macrophages are plastic cells, which lie on spectrum of activation states[1]. Macrophages have a role in promoting inflammation in response to infection or injury, but also have an equally important role in stopping inflammatory responses to prevent tissue damage, which can occur through IL-10 production. IL-10 has potent anti-inflammatory effects on both innate and adaptive immune responses, by inhibiting pro-inflammatory cytokine production and antigen presentation[61]. Macrophages activated with Ic and LPS, M(Ic + LPS) are the best characterized example of this anti-inflammatory activation state[27]. The defining characteristic of this activation state in mice is high IL-10 production and very low or no production of pro-inflammatory IL-12, in response to LPS, a normally inflammatory stimuli. Murine M(Ic + LPS) produce high amounts of IL-10, through Ic crosslinking of the FcγRI, which results in activation of the MAPKs, Erk1/2 and p38[27, 38].

IVIg is a drug made up of pooled polyclonal antibodies. It is used at high doses as an anti-inflammatory therapy for a wide variety of autoimmune and inflammatory diseases[290]. The mechanism(s) of action for IVIg is not well understood. The goal of Chapter 2 was to determine whether IVIg can skew murine macrophages to an anti-inflammatory, IL-10 producing activation state in mice *in vitro,* similar to M(Ic + LPS). I tested whether IVIg can induce IL-10 production in murine BMDMs, through Fcγ receptors and the activation of MAPKs. I also investigated whether high IL-10 production and low IL-12/23p40 production can be induced in murine peritoneal macrophages activated with IVIg + LPS *in vivo*.

IBD is a chronic, incurable disease characterized by inflammation along the gastrointestinal tract[279]. The development of monoclonal antibody drugs, which block TNFα, has revolutionized treatment of this disease. However, it is predicted that up to 40% of people are, or will become, unresponsive to these drugs[288]. Because of this, and side effects of anti-TNFα therapies, the goal of Chapter 3 was to determine whether IVIg can be used to limit intestinal inflammation, using the dextran sulfate sodium (DSS)-induced murine model of colitis. I also investigated whether IVIg can ameliorate symptoms of induced colitis, by inducing IL-10 production by macrophages.

Since there are differences in Fcγ receptors and monocytes/macrophages in mice and humans, in Chapter 4, I determined whether IVIg can induce an anti-inflammatory, IL-10 producing activation state in human monocytes *in vitro*. Through multiple biochemical techniques, I tested the role of Fcγ receptors and MAPKs in IVIg-induced IL-10 production. Humans have a gene variant in the FcγRIIA, which has a high affinity for IgG antibodies. This gene variant is associated with a higher risk of inflammatory diseases such as IBD and KD, as well as poor responses to antibody-based drugs[151, 158, 291, 292]. By genotyping people for this polymorphism, I also tested whether this FcγRIIA gene variant affects monocyte anti-inflammatory responses to IVIg, as this may explain the higher risk of developing disease and poor performance on antibody-based drugs in people harboring this gene variant.

1.6.2 Hypothesis and objectives

I hypothesize that IVIg can be used to treat intestinal inflammation like that which characterized IBD, by inducing an IL-10 producing, anti-inflammatory activation state in murine macrophages and human monocytes.

Aim 1 (Chapter 2): To determine whether IVIg can activate murine macrophages to produce IL-10 *in vitro* and *in vivo*, and whether the production of IL-10 *in vitro* is dependent on Fcγ receptors and MAPK activation.

Aim 2 (Chapter 3): To determine whether IVIg can reduce intestinal inflammation during DSS-induced colitis in mice by activating macrophages *in vivo* to produce IL-10.

Aim 3 (Chapter 4): To determine whether IVIg can induce an anti-inflammatory activation state in human monocytes *in vitro*, and whether a disease-associated FcγRIIA gene variant affects macrophage responses to IVIg.

Chapter 2: Intravenous immunoglobulin skews murine macrophages to an anti-inflammatory IL-10-producing activation state *in vitro* and *in vivo*

2.1 Introduction and rationale

A hallmark of macrophage biology is their "plasticity," that is, their ability to respond to cues in their local microenvironment to mount an appropriate response. As such, macrophages are a highly heterogeneous cell type[3]. Two well characterized murine and human macrophage activation states are M(IFNγ + LPS) and M(IL-4)[10]. M(IFNγ + LPS) have distinct pro-inflammatory functions that are critical in host defense against invading pathogens[10]. M(IL-4), have properties consistent with their role in wound healing and tissue restitution[12].

Intriguingly, macrophages, which can produce large amounts of the anti-inflammatory cytokine, IL-10, have been described in mice and humans. IL-10 is an important cytokine involved in the restoration of tissue homeostasis because it can stop intrinsic and extrinsic inflammatory signalling from innate and adaptive immune pathways[3, 61]. Anti-inflammatory, IL-10-producing macrophages require two external stimuli, one of them being pro-inflammatory[13]. The best characterized example of this activation state in mice and humans are M(Ic + LPS)[11, 293, 294]. They are distinct from murine M(IL-4) in that murine M(Ic + LPS) do not promote the production of extracellular matrix, and do not express M(IL-4) markers, such as ArgI or FIZZ1[49]. The best marker for these macrophages in mice is their ability to produce very high levels of IL-10 and very low, or no, pro-inflammatory IL-12[295].

Ic activate murine macrophages by binding to the high affinity, activating FcγRI[27]. This leads to activation of the MAPKs, Erk1/2 and p38, both of which are required for IL-10 production by M(Ic + LPS)[38]. Erk1/2 causes phosphorylation of ser10 on histone 3 in the *Il10* promoter opening it up for transcription, and p38 drives the transcription of *Il10*[38].

It is interesting to note that treatment of macrophages with antibodies has been reported to activate them to produce high amounts of IL-10, in response to what are normally considered pro-inflammatory stimuli. Human macrophages treated with anti-TNFα antibodies have been shown to produce high amounts of IL-10 in response to LPS and suppress T cell proliferation[35]. Serum, which contains high amounts of antibody, has also been used to activate macrophages with T cell suppressive capacity via induction of iNOS in murine macrophages and IDO expression in human macrophages[36, 296, 297].

The mechanism by which IVIg works to suppress autoimmune and inflammatory responses is not completely understood. IVIg can reduce autoantibody-mediated inflammation in mice because a minor fraction of sialylated Fc fragments within the pooled IgGs binds to DC-SIGN receptors on myeloid cells causing up-regulation of the inhibitory FcγRIIB, which then suppresses autoantibody-mediated inflammation[298,178]. It has also been suggested that IVIg may block activating FcγRs or saturate the FcRn, but evidence from some studies contradicts these theories[290,299].

Based on the high dose of IVIg antibodies required to treat autoimmune and inflammatory diseases, and examples of antibody activation of IL-10 producing macrophages, I asked whether IVIg may work, in part, by activating macrophages to produce high levels of IL-10 in response to an inflammatory stimulus, *in vitro* and *in vivo*. I also sought to determine whether Fcγ receptors and MAPK activation are required for IVIg-induced IL-10 production, as with M(Ic + LPS).

2.2 Materials and methods

Mice. Wild type C57BL/6 mice were used to prepare bone marrow-derived macrophages for the majority of experiments, except where indicated. Eight-week-old male and female mice were used. Wild type C57BL/6 mice were housed at the BC Children's Hospital Research Institute (Vancouver, Canada), a barrier facility that is both *Helicobacter*-free and specific pathogen free. Experiments were performed in accordance with Canadian Council on Animal Care guidelines with approval from institutional animal care committees (A13-0014 and A17-0076). *Fcgr1*$^{-/-}$ murine femura and tibiae were obtained from Dr. Sjef Verbeek at the Leiden University Medical Center, Leiden the Netherlands. Mice were maintained on a C57BL/6 background and wild type C57BL/6 mice were used to derive *Fcgr1*$^{+/+}$ macrophages for experimental controls. *Fcgr2b*$^{-/-}$ mice were on a B6129SF2/J background and *Fcgr3*$^{-/-}$ mice were on a C57BL/6J background. Femura and tibiae from each of these knockout mice and control strains were purchased from Jackson Laboratories (Bar Harbor, ME, USA). *Il10rb*$^{-/-}$ on a C57BL/6 background were provided by Dr. Megan Levings at the University of British Columbia (Vancouver, BC, Canada) and C57BL/6 mice were used to derive *Il10rb*$^{+/+}$ macrophages for experimental controls. Femura and tibiae from *Il10*$^{-/-}$ mice on a BALB/c background and BALB/c *Il10*$^{+/+}$ control mice, which were bred and maintained at the University of Alberta animal care facility, were provided by Dr. Karen Madsen and used for macrophage derivations.

Macrophage derivation. Bone marrow macrophages were derived from bone marrow aspirates of femura and tibiae from all mice. Following adherence depletion for 1 hour (hr) at 37°C, bone marrow aspirates were resuspended in Iscove's modified Dulbecco's medium (IMDM), 10% fetal bovine serum (FBS), and penicillin/streptomycin at a concentration of 0.5×10^6 cells/mL for 10 days in the presence of 5 ng/mL MCSF (StemCell Technologies, Vancouver, BC, Canada), with complete media changes at day 4 and 7. Adherent cells were removed after 10 days, by incubating with cell dissociation buffer (Invitrogen, Carlsbad, CA, USA).

Cell stimulations. Cells were plated at a density of 1.0×10^6 cells/mL (100 μl/well in 96-well plates) and stimulated with either 10 ng/mL of LPS (*E. coli* serotype 127:B8, Sigma Aldrich, St. Louis, MO, USA), 30 mg/mL of IVIg (or indicated concentration; Gamunex® Immune Globulin Intravenous 10% solution for infusion; Transfusion Medicine, BC Children's Hospital, Vancouver, BC, Canada), or both IVIg + LPS. Cells were incubated for 24 h. After incubation, cell supernatants were harvested and clarified by centrifugation for analysis. For inhibitor studies, inhibitors were added 1 h prior to stimulations, at final concentrations of: dimethyl sulphoxide (DMSO) (vehicle control; 0.1%), SB203580 (SB) (10 μM, Cell Signaling Technology, Danvers, MA, USA), BIRB-796 (BIR) (180 nM, Cayman Chemical, Ann Arbor, MI, USA), PD98059 (PD) (50 μM, Cell Signaling Technology), SCH772984 (SCH) (1 μM, MedChem Express, Princeton, NJ, USA), XMD8-92 (XMD) (5 μM, Axon Medchem, Groningen, the Netherlands), and BIX02189 (BIX) (20 μM, Axon Medchem). Recombinant murine IL-10 (rIL-10) was used at a final concentration of 5 ng/mL (Affymetrix eBioscience, San Diego, CA, USA).

Cytokine measurements. Cytokines were assayed by enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions. ELISA kits for murine IL-10, IL-12/23p40, IL-6, and TNF were obtained from BD Biosciences (Mississauga, ON, Canada).

SDS-PAGE and western blotting. Macrophages were stimulated for 0, 10, 40, 120; 0, 20, and 80 minutes (mins); or 0, 4, 8, and 24 h, as indicated. After stimulation, macrophages were placed on ice and rinsed twice with cold phosphate-buffered saline (PBS). Whole cell lysates were prepared for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) by lysing in 1×Laemmli's digestion mix, DNA was sheered using a 26-guage needle, and samples were boiled for 1 min. Cell lysates were separated on a 12% (Figure 2.4A) or 10% polyacrylamide gel (Figure 2.4E and 2.5), and western blotting was carried out, as described previously[300]. Antibodies used for western blot analyses were anti-pErk1/2 (Cell Signaling Technology), anti-pp38 (Cell Signaling Technology), anti-pErk5 (Cell Signaling Technology), and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Fitzgerald Industries International, Acton, MA, USA).

Gene expression analyses. RNA was prepared from murine cells using the QIAGEN RNeasy Plus Mini Kit with DNAse I digestion (Toronto, ON, Canada) and reverse transcribed using qScript cDNA SuperMix (Quanta BioSciences, Gaithersburg, MD, USA), according to manufacturers' instructions. Gene expression was measured by quantitative polymerase chain reaction (qPCR) using the Sso Advanced Universal SYBR Green Supermix (BioRad, Mississauga, ON, Canada). *Il10* and *Il12b* gene expression were normalized to gene expression for *Gapdh*. PrimePCR SYBR Green Assay primers were obtained from BioRad. The catalog number for primers is 100-25636 with the following unique identification numbers qMmuCID0015452 (*Il10*), qMmuCID0022424 (*Il12b*), and qMmuCED0027497 (*Gapdh*).

***In vivo* murine studies.** Wild type C57BL/6 mice, 8-10 weeks of age, were injected intraperitoneally with IVIg (2.5 g/kg) or an equal volume of PBS, as an injection control. Peritoneal macrophages were harvested 1 h post injection by flushing the peritoneal cavity 3× with 5 mL of PBS. Cells were resuspended in IMDM, 10% FBS, and penicillin/streptomycin. Macrophages were enriched by adherence to tissue culture plastic for 1 h, resuspended, and plated at density of 1.0×10^6 cells/mL (100 μl/well in 96-well plates)[301]. Cells were either unstimulated or stimulated with LPS (10 ng/mL) for 24 h and clarified cell supernatants were harvested for cytokine analyses.

Wild type C57BL/6 mice, 8-10 weeks of age, were also injected intraperitoneally with IVIg (2.5 g/kg) or an equal volume of PBS, as a control, together with LPS (0.2 μg/g body weight). Peritoneal macrophages were harvested 1 h post injection and peritoneal lavage fluid, conditioned medium from 1 h adherence, and 24 h culture supernatants were harvested and clarified supernatants were used for cytokine analyses.

Statistical Analyses. Unpaired Student's *t*-tests, one-way, and two-way analysis of variance (ANOVAs) with either Tukey's or Dunnett's corrections for multiple comparisons were applied as indicated. Analyses were performed using Graph Pad Prism Software version 6.03. Differences of $p < 0.05$ were considered significant.

2.3 Results

2.3.1 IVIg-treated macrophages produce high levels of IL-10 and low levels of IL-12/23p40, IL-6, and TNF in response to LPS

IVIg has been reported to reduce pro-inflammatory cytokine production by murine dendritic cells but the mechanisms for reduced pro-inflammatory cytokine production are not fully understood[302]. Antibody cross-linking has also been reported to activate macrophages, known as M(Ic + LPS), which produce high levels of anti-inflammatory IL-10 in response to the pro-inflammatory stimulus, LPS. Based on this, I asked whether IVIg-treated macrophages produce high levels of IL-10 in response to LPS. MCSF-derived bone marrow macrophages were stimulated with LPS, IVIg, or IVIg + LPS. LPS stimulated macrophages produced high levels of pro-inflammatory IL-12/23p40, whereas IVIg alone did not induce production of these cytokines (Figure 2.1A). IVIg + LPS suppressed IL-12/23p40 production completely at a dose of 30 mg/mL, and the effect was dose-dependent (Figure 2.1A). LPS treatment induced 1 ng/mL of IL-10, whereas IVIg treatment did not induce IL-10 production (Figure 2.1B). Intriguingly, concomitant treatment with IVIg + LPS induced a significant 3.5-fold increase in IL-10 production relative to treatment with LPS alone. The effect of IVIg co-treatment on IL-10 production was also dose-dependent (Figure 2.1B). IVIg + LPS treatment also decreased the production of pro-inflammatory cytokines, IL-6 and TNF, relative to treatment with LPS alone (Figure 2.1C).

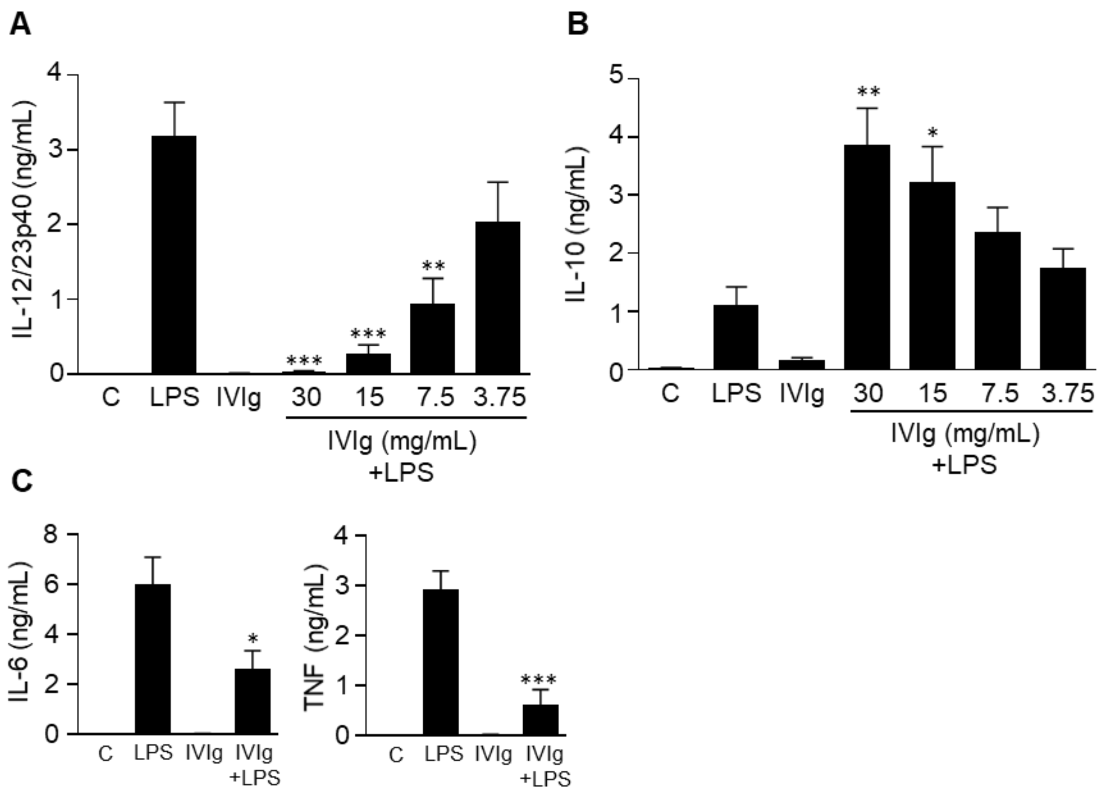

Figure 2.1 Macrophages co-stimulated with IVIg + LPS produce high levels of IL-10 and low levels of pro-inflammatory cytokines. MCSF-derived bone marrow macrophages were unstimulated (control (C)) or stimulated with LPS (10 ng/mL), IVIg (30 mg/mL), or co-stimulated with IVIg (at the dose indicated) and LPS (10 ng/mL) for 24 h. Clarified cell supernatants were assayed for (A) IL-12/23p40, (B) IL-10, and (C) IL-6 and TNF. Data are means ± SD of $n = 3$; macrophages were derived from 1 mouse for each of 3 independent experiments, and ELISAs were performed in duplicate. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.0001$ comparing (IVIg + LPS)-treated macrophages with macrophages treated with LPS alone. Statistical analyses were performed using a one-way ANOVA with Tukey's post-test for multiple comparisons.

2.3.2 Concomitant treatment with IVIg and LPS is not required for M(IVIg + LPS) to produce high levels of IL-10 and low levels of IL-12/23p40

Induction of IL-10 producing M(Ic + LPS) required concomitant treatment with Ic and LPS. Thus, next I asked if IVIg's impact on LPS-induced cytokine production required concomitant signals. BMDMs were pre-treated with IVIg (30 mg/mL) for 0, 0.5, 1, 2, 4, 8, or 24 h, and washed prior to stimulation with LPS. Macrophages were left unstimulated or were stimulated with LPS for an additional 24 h. IL-10 production was significantly higher with IVIg pre-treatment compared to stimulation with LPS alone at all time points (Figure 2.2A). Moreover, there was no change in the IVIg-dependent increase in LPS-induced IL-10 production over time (Figure 2.2A). IL-12/23p40 production in response to LPS was significantly lower at all pre-treatment time points (Figure 2.2B). However, the reduction of LPS-induced IL-12/23p40 production waned when IVIg treatment preceded LPS treatment by 24 h, compared to other time points. I then asked if IL-10 production can be induced and IL-12/23p40 production reduced by IVIg treatment when macrophages were stimulated with LPS prior to IVIg treatment. BMDMs were stimulated with LPS (10 ng/mL) for 0, 0.5, 1, 2, 4, 8, or 24 h. LPS was not removed from cultures and macrophages were left untreated or were treated with IVIg (30 mg/mL) for an additional 24 h. IL-10 production was significantly higher and IL-12/23p40 production was reduced when IVIg treatment was provided within 1 h of LPS stimulation (Figures 2.2C and D).

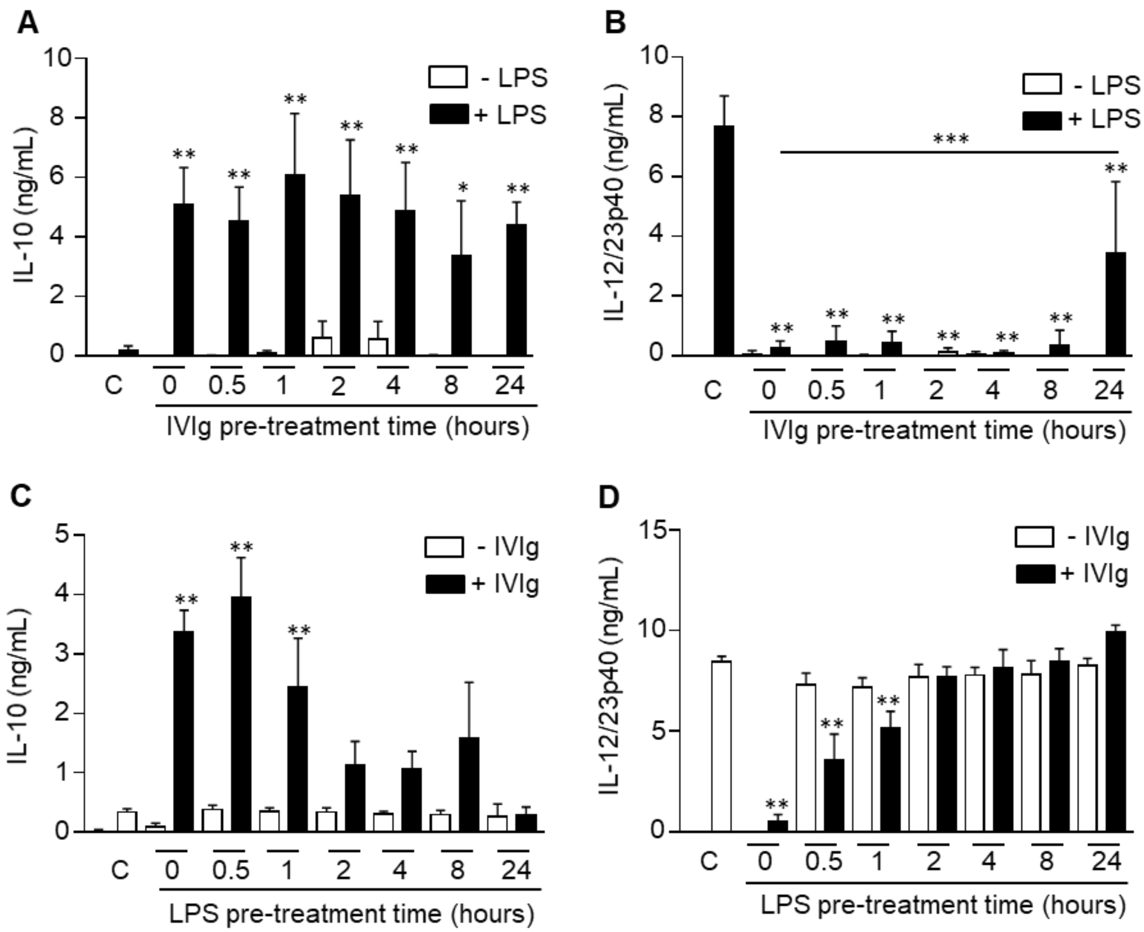

Figure 2.2 IVIg does not need to be provided at the same time as LPS to induce IL-10 or repress IL-12/23p40 production. MCSF-derived bone marrow macrophages were treated with IVIg (30 mg/mL) for 0, 0.5, 1, 2, 4, 8, or 24 h; washed 3 times with complete medium after the time indicated and then unstimulated or stimulated with LPS (10 ng/mL) for 24 h. Clarified cell supernatants were assayed for (A) IL-10 or (B) IL-12/23p40 by ELISA. MCSF-derived bone marrow macrophages were treated with LPS for 0, 0.5, 1, 2, 4, 8, or 24 h, and were left untreated or treated with IVIg (30 mg/mL) for 24 h. Clarified cell supernatants were assayed for (C) IL-10 or (D) IL-12/23p40 by ELISA. Data are means ± SD for $n = 3$; macrophages were derived from 1 mouse for each of 3 independent experiments, and ELISAs were performed in duplicate. *p < 0.001 and **p < 0.0001 comparing macrophages treated with IVIg + LPS to macrophages treated with LPS alone, and ***p < 0.001 comparing macrophages treated with IVIg + LPS to those treated with IVIg + LPS for t = 0 min. Statistical analyses were performed using a one-way ANOVA with Tukey's post-test for multiple comparisons.

2.3.3 The FcγRI, FcγIIB, or FcγIII alone, are not sufficient for IVIg-induced IL-10 production or reduced IL-12/23p40 production in response to LPS

The anti-inflammatory activity of IVIg has been attributed to sialylated IgGs within IVIg binding to the DC-SIGN receptors and up-regulating the FcγRIIB that binds autoimmune antibodies and inhibits immune responses, whereas the induction of IL-10 by M(Ic + LPS) is reported to act via the FcγRI[27, 303]. Thus, I next asked which FcγR(s) was(were) involved in increased IL-10 production and reduced IL-12/23p40 production by IVIg + LPS. We compared LPS and IVIg + LPS responses in macrophages deficient in the FcγRI, FcγRIIB, FcγRIII, or the FcR γ chain used by the FcγRI, FcγRIII, and FcγRIV; and their wild type counterparts. Wild type *Fcgr1*$^{+/+}$ and deficient *Fcgr1*$^{-/-}$ macrophages produced similar levels of IL-10 when stimulated with IVIg + LPS compared to LPS alone (Figure 2.3A, left). IL-12/23p40 production in response to LPS was lower in *Fcgr1*$^{-/-}$ macrophages compared to their wild type counterparts but IL-12/23p40 production was ablated in both genotypes upon treatment with IVIg + LPS (Fig 2.3A, right). *Fcgr2b*$^{-/-}$ macrophages produced more IL-10 in response to LPS and IVIg + LPS than their wild type counterparts and correspondingly, *Fcgr2b*$^{-/-}$ macrophages produced dramatically less IL-12/23p40 in response to LPS (Figure 2.3B). Consequently, the fold-induction of IL-10 in response to IVIg + LPS versus LPS alone was compromised in *Fcgr2b*$^{-/-}$ macrophages (Figure 2.3B, left), but IVIg + LPS treatment effectively ablated IL-12/23p40 production by both genotypes (Figure 2.3B, right). *Fcgr3*$^{+/+}$ and *Fcgr3*$^{-/-}$ macrophages produced similar levels IL-10 in response to LPS, which was induced in response to IVIg + LPS in both genotypes, though modestly higher in *Fcgr3*$^{-/-}$ macrophages (Figure 2.3C, left). *Fcgr3*$^{+/+}$ and *Fcgr3*$^{-/-}$ macrophages produced similar levels IL-12/23p40 in response to LPS and it was reduced in (IVIg + LPS)-treated macrophages (Fig 2.3C, right). *Fcer1g*$^{+/+}$ and *Fcer1g*$^{-/-}$ macrophages produced similar levels of IL-10 in response to LPS, which was reduced in response to IVIg + LPS in both genotypes (Figure 2.3D, left). IVIg + LPS was modestly less effective at reducing IL-12/23p40 production in *Fcer1g*$^{-/-}$ macrophages compared to their wild type controls (Figure 2.3D, right).

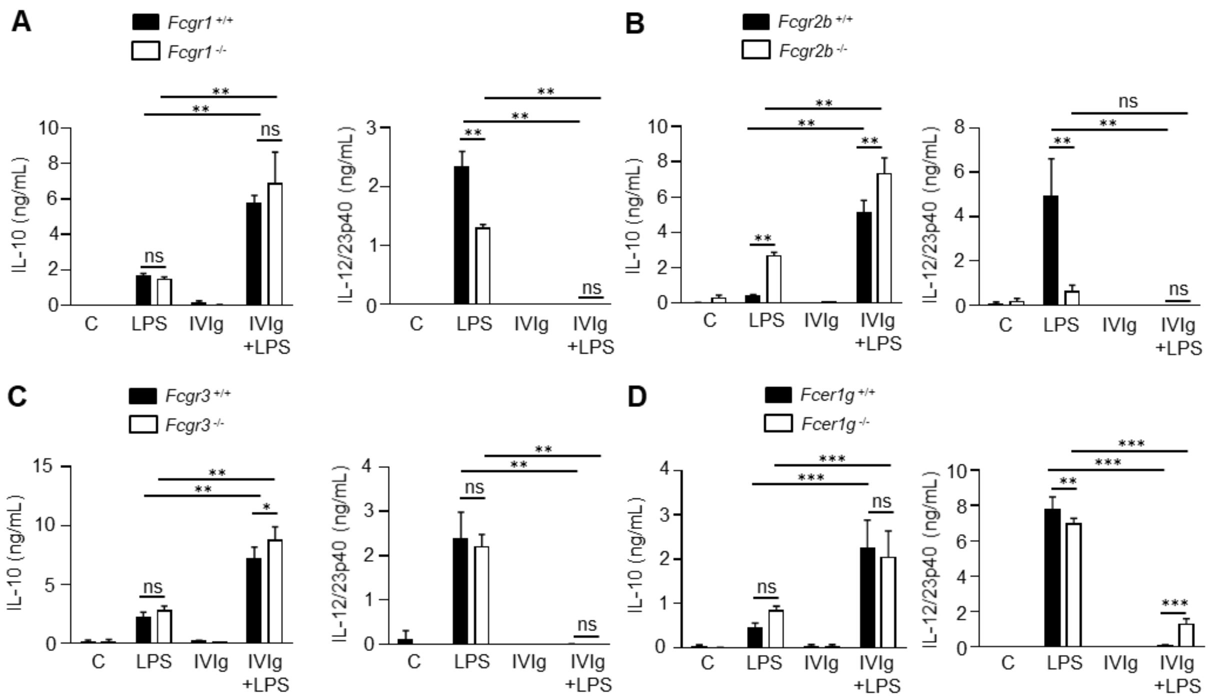

Figure 2.3 The FcγRI, FcγIIB, or FcγIII alone is not sufficient for IVIg-induced IL-10 production or reduced IL-12/23p40 production in response to LPS. MCSF-derived bone marrow macrophages were prepared from mice deficient in FcγR subunits and their wild type counterparts. Macrophages were unstimulated or stimulated with LPS (10 ng/ml), IVIg (30 mg/ml), or IVIg + LPS for 24 h, and clarified cell supernatants were assayed for IL-10 and IL-12/23p40 by ELISA. (A) *Fcgr1*$^{+/+}$ and *Fcgr1*$^{-/-}$ macrophages, (B) *Fcgr2b*$^{+/+}$ and *Fcgr2b*$^{-/-}$ macrophages, (C) *Fcgr3*$^{+/+}$ and *Fcgr3*$^{-/-}$ macrophages, and (D) *Fcer1g*$^{+/+}$ and *Fcer1g*$^{-/-}$ macrophages. Data are means ± SD for $n = 3$; macrophages were derived from 1 pair of mice for each of 3 independent experiments, and ELISAs were performed in duplicate. *p < 0.01, **p < 0.001, ***p < 0.0001, and ns = not significantly different for comparisons, as indicated. Statistical analyses were performed using a two-way ANOVA with Tukey's post-test for multiple comparisons.

2.3.4 MAPK signalling is required for IVIg-induced IL-10 production in response to LPS

I next determined whether IL-10 production by (IVIg + LPS)-stimulated macrophages required MAPK signalling, as has been reported for M(Ic + LPS)[38]. Macrophages were unstimulated or stimulated with LPS, IVIg, or IVIg + LPS for 0, 10, 40, and 120 min. Whole cell lysates were separated by SDS-PAGE, western blotted, and probed for pErk1/2, pp38, and GAPDH, as a loading control (Figure 2.4A). IVIg alone and (IVIg + LPS)-stimulated macrophages had earlier and prolonged phosphorylation of Erk1/2 compared to LPS-stimulated macrophages, which was evident by 10 min and maintained through 120 min. LPS-stimulated macrophages had strong p38 phosphorylation, which peaked at 40 min, whereas (IVIg + LPS)-stimulated macrophages had earlier p38 phosphorylation, evident by 10 min and maintained through 120 min. IVIg alone induced only modest levels of p38 phosphorylation, whereas it induced Erk1/2 phosphorylation at levels similar to IVIg + LPS. The impact of MAPK signalling on IL-10 and IL-12/23p40 production in response to IVIg + LPS was assessed using inhibitors. SCH is a novel and specific Erk1/2 inhibitor and PD inhibits the activation of the Erk1/2 kinase, MEK1[304]. IL-10 production was lower in (IVIg + LPS)-stimulated macrophages, in the presence of PD compared to vehicle control (Figure 2.4B, left). PD did not block the IVIg-induced suppression of LPS-induced IL-12/23p40 production (Figure 2.4B, right). The specific Erk1/2 inhibitor, SCH, also reduced IL-10 production in response to IVIg + LPS but was less effective (Figure 2.4B, left). SCH did not block IL-12/23p40 production (Figure 2.4B, right). p38 inhibitors, SB (inhibits p38α and p38β) and BIR (inhibits p38α) also significantly reduced IL-10 production in response to IVIg + LPS compared to solvent control (DMSO) (Figure 2.4C, left). These inhibitors also did not block the suppression of IL-12/23p40 production with IVIg + LPS (Figure 2.4C, right). The MEK1 inhibitor, PD was more effective in my assay and it has been reported to inhibit Erk5 (also known as BMK1)[305].Thus, to investigate whether the impact of PD was due to off-target effects on Erk5, I used the Erk5 inhibitor, XMD, and the MEK5 (Erk5 kinase) inhibitor, BIX, in my assay. Both XMD and BIX significantly deceased IL-10 production in response to IVIg + LPS relative to the vehicle control, DMSO (Figure 2.4D, left). XMD and BIX did not block the (IVIg + LPS)-induced suppression of LPS-induced IL-12/23p40 production (Figure 2.4D, right). Finally, BMDMs were stimulated with either LPS or IVIg + LPS for 0, 20, and 80 min. Whole cell lysates were separated by SDS-PAGE, western blotted, and probed with pErk5, pErk1/2, pp38, and GAPDH, as a loading control (Figure 2.4E). As previously demonstrated, Erk1/2 phosphorylation was stronger at both 20 and 80 min, and p38 phosphorylation was stronger at 20 min in (IVIg + LPS)-stimulated macrophages compared to those stimulated with LPS. Consistent with its role in IVIg + LPS macrophage activation, Erk5 phosphorylation was stronger in (IVIg + LPS)-stimulated macrophages compared to LPS-stimulated macrophages at 80 min. To determine whether these signalling events were maintained for 24 h, like the induction of IL-10 by IVIg-pre-treated macrophages, BMDMs were stimulated with either LPS, IVIg, or IVIg + LPS for 0, 4, 8 and 24 h (Figure 2.5). Whole cell lysates were separated by SDS-PAGE, western blotted, and probed with pErk5, pErk1/2, pp38, and GAPDH, as a loading control. Erk5, Erk1/2, and p38 phosphorylation were stronger in (IVIg + LPS)-stimulated macrophages compared to LPS or IVIg stimulations alone at all time points and were still elevated above background levels at 24 h post treatment (Figure 2.5).

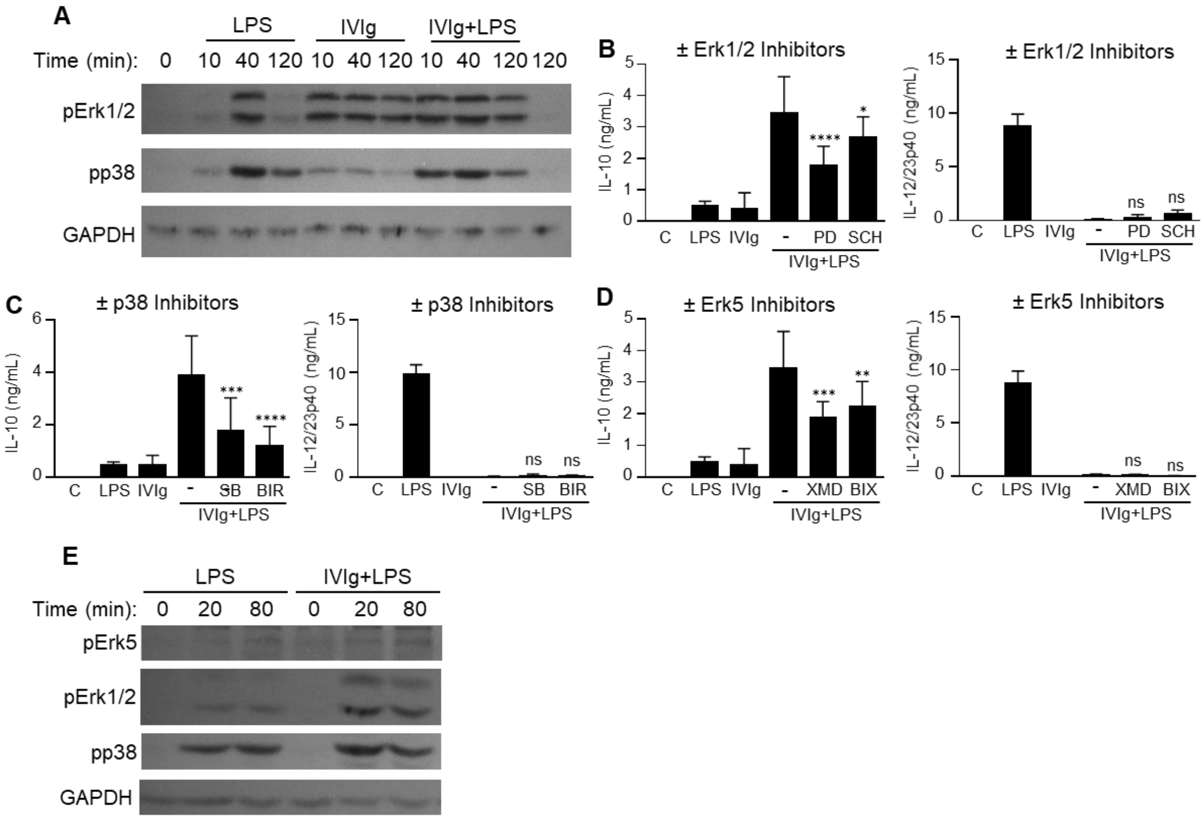

Figure 2.4 MAPKs are required for IVIg-induced IL-10 production in response to LPS. (A) MCSF bone marrow-derived macrophages were unstimulated or stimulated with LPS (10 ng/mL), IVIg (30 mg/mL), or IVIg + LPS for 0, 10, 40, or 120 min. Cell lysates (1.0×10^6 cells/time point) were prepared at the indicated times. Lysates were separated by SDS-PAGE and analyzed by western blotting using phospho-specific antibodies for Erk1/2, p38, and GAPDH as a loading control. Results shown are representative of $n = 3$ experiments; macrophages were derived from 1 mouse for each of 3 independent experiments. Macrophages were pre-treated for 1 h with an appropriate volume of DMSO, as a vehicle control, or (B) the Erk1/2 inhibitors, PD and SCH; (C) p38 inhibitors, SB and BIR; or (D) Erk5 inhibitors, XMD and BIX, and then were left unstimulated or stimulated with LPS (10 ng/ml), IVIg (30 mg/ml), or IVIg + LPS for 24 h. Clarified cell supernatants were analyzed for IL-10 and IL-12/23p40 by ELISA. (B-D) Values reported are means ± SD for $n = 4$ independent experiments, and ELISAs were performed in duplicate. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, and ns = not significantly different comparing macrophages treated with inhibitor and stimulated with IVIg + LPS to those treated with DMSO and stimulated with IVIg + LPS. Statistical analyses were performed using one-way ANOVA with Dunnett's post-test for multiple comparisons. (E) MCSF bone marrow-derived macrophages were unstimulated or stimulated with LPS or IVIg + LPS, westerns blots were prepared as in (A), and probed using phospho-specific antibodies for Erk5, Erk1/2, p38, and GAPDH, as a loading control. Results shown are representative of $n = 3$ independent experiments; macrophages were derived from 1 mouse for each of 3 independent experiments.

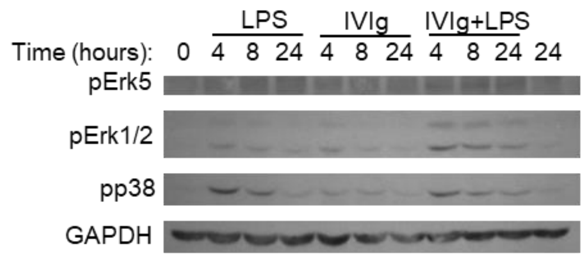

Figure 2.5 MAPK phosphorylation occurs after 4 hours of IVIg + LPS stimulation. MCSF-derived bone marrow macrophages were either unstimulated or stimulated with LPS (10 ng/mL), IVIg (30 mg/mL), or IVIg + LPS for 0, 4, 8, or 24 hours. Cell lysates (1.0×10^6 cells/time point) were prepared at the indicated times. Lysates were separated by SDS-PAGE and analyzed by western blotting using phospho-specific antibodies for Erk1/2, p38, and GAPDH, as a loading control. Results shown are representative of $n = 3$ experiments; macrophages were derived from 1 mouse for each of 3 independent experiments.

2.3.5 IL-10 is transcriptionally up-regulated rapidly in response to IVIg + LPS and in sufficient amount to reduce IL-12/23p40 transcription and production

To determine whether IL-10 produced in response to IVIg + LPS may contribute to reduced pro-inflammatory cytokine production, I examined the kinetics of IL-10 and IL-12/23p40 production and *Il10* and *Il12b* induction. Macrophages were stimulated with either LPS (Figure 2.6A, left) or IVIg + LPS (Figure 2.6A, right) for 0, 0.5, 1, 2, 4, 8, or 24 h. LPS-stimulated macrophages produced high amounts of IL-12/23p40 (closed squares) that reached a maximum of 7 ng/mL by 8 h, and low amounts of IL-10 that peaked at 4 h (open circles). In contrast, IVIg + LPS treatment caused a steep curve for IL-10 production that peaked at 8 h, and was 7-fold higher than that produced by LPS-treatment alone. Very low levels of IL-12/23p40 were produced relative to treatment with LPS alone. *Il10* and *Il12b* transcription showed similar kinetics. LPS stimulation caused induction of *Il12b* mRNA in macrophages (closed squares) and very little induction of *Il10* mRNA (open circles; Figure 2.6B, left). IVIg + LPS stimulation caused a dramatic spike in *Il10* mRNA levels (open circles) and very little induction of *Il12b* mRNA (closed squares; Figure 2.6B, right). To determine whether early and robust IL-10 production may contribute to reduced IL-12/23p40 production, I stimulated macrophages in the presence of recombinant murine IL-10 (rIL-10). Macrophages were either unstimulated or stimulated with LPS, in the presence or absence of rIL-10 (5 ng/mL). rIL-10 reduced IL-12/23p40 production in response to LPS (Figure 2.6B).

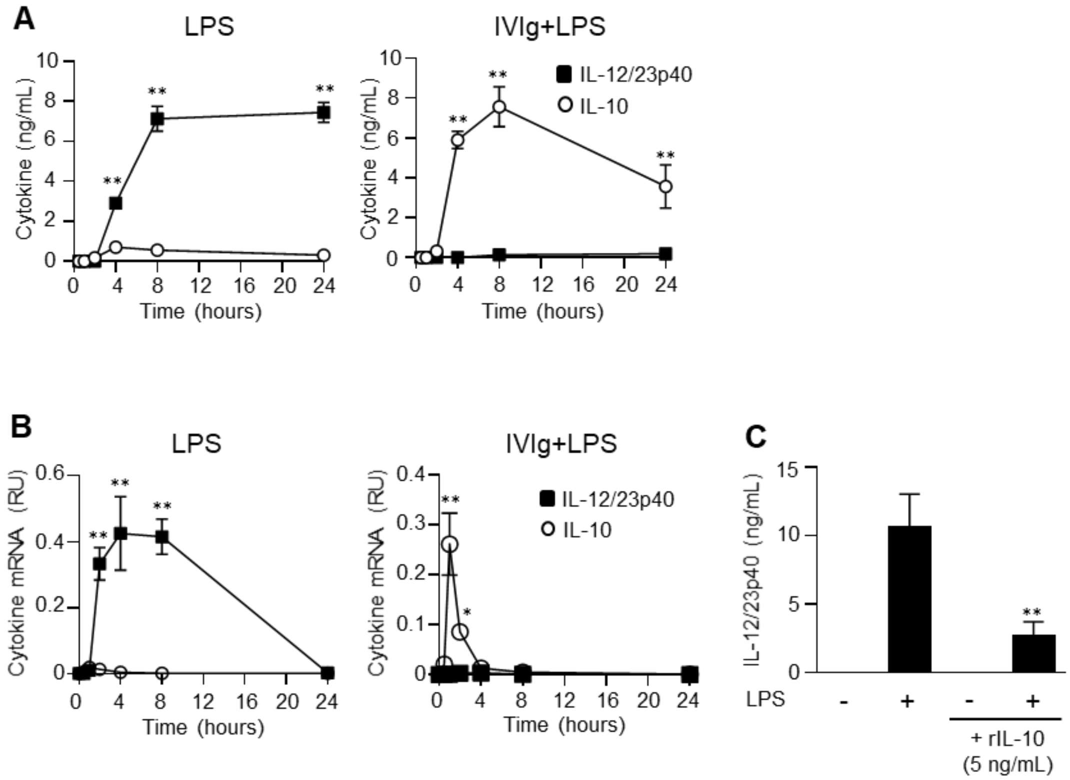

Figure 2.6 IL-10 and *Il10* are produced early in response to treatment with IVIg + LPS, and the amount of IL-10 produced is sufficient to inhibit IL-12/23p40 production in response to LPS stimulation. (A) MCSF-derived bone marrow macrophages were stimulated with LPS (10 ng/mL) or IVIg (30 mg/mL) + LPS (10 ng/mL) for 0, 0.5, 1, 2, 4, 8, or 24 h. Clarified cell supernatants were collected and assayed at each time point for IL-10 and IL-12/23p40 by ELISA. (B) MCSF-derived bone marrow macrophages were stimulated with LPS (10 ng/mL) or IVIg (30 mg/mL) + LPS (10 ng/mL) for 0, 0.5, 1, 2, 4, 8, or 24 h. Abundance of *Il10* or *Il12b* mRNA relative to *Gapdh* was analyzed by quantitative PCR. Data represent means ± SD for $n = 3$; macrophages were derived from 1 mouse for each of 3 independent experiments; ELISAs were assayed in duplicate (A), and quantitative PCR was assayed in triplicate (B). $^*p < 0.001$ and $^{**}p < 0.0001$ for treatment with IVIg + LPS compared with LPS alone. Statistical analyses were performed using a one-way ANOVA with Tukey's post-test for multiple comparisons. (C) Macrophages were unstimulated or stimulated with LPS (10 ng/mL), rIL-10 (5 ng/mL), or rIL-10 (5 ng/mL) + LPS (10 ng/mL) for 24 h. IL-12/23p40 production was assayed in clarified cell supernatants by ELISA. Data represent means ± SD for $n = 3$; macrophages were derived from 1 mouse for each of 3 independent experiments, and ELISAs were assayed in duplicate. $^{**}p < 0.0001$ for treatment compared with LPS stimulation. Statistical analysis was performed by use of one-way ANOVA.

2.3.6 IL-10 contributes to IVIg-induced suppression of pro-inflammatory cytokine production in response to LPS

To determine whether IL-10 contributes to reduced pro-inflammatory cytokine production in response to IVIg + LPS, I compared cytokine production in response to LPS, IVIg, or IVIg + LPS in wild type macrophages (*Il10rb*$^{+/+}$) and macrophages deficient in the IL-10Rβ chain (*Il10rb*$^{-/-}$), which is required for IL-10 receptor signalling. *Il10rb*$^{+/+}$ and *Il10rb*$^{-/-}$ BMDMs produced similar levels of IL-10 in response to IVIg + LPS (Figure 2.7A, top panel), and *Il10rb*$^{-/-}$ macrophages produced more IL-12/23p40 in response to LPS (Figure 2.7A, top panel). Importantly, in *Il10rb*$^{-/-}$ macrophages, deficient in IL-10 signalling, IVIg + LPS treatment was less effective at reducing IL-12/23p40 production compared to their wild type counterparts (Figure 2.7A, top panel). IL-6 production was higher in *Il10rb*$^{-/-}$ compared to *Il10rb*$^{+/+}$ for both LPS and (IVIg + LPS)-stimulated macrophages, however IL-6 production was not significantly reduced by IVIg treatment in these macrophages (Figure 2.7A, top panel). TNF production was comparable in *Il10rb*$^{+/+}$ and *Il10rb*$^{-/-}$ macrophages in response to LPS, and was dramatically reduced in wild type macrophages, but not *Il10rb*$^{-/-}$ macrophages (Figure 2.7A, top panel). To solidify a specific role for IL-10 in IVIg-induced suppression of LPS-induced pro-inflammatory cytokine production, I stimulated macrophages from *Il10*$^{+/+}$ and *Il10*$^{-/-}$ mice with LPS, IVIg, or IVIg + LPS. *Il10*$^{-/-}$ macrophages do not produce IL-10 (Figure 2.7B bottom panel), whereas *Il10*$^{+/+}$ macrophages produced high levels of IL-10 in response to IVIg + LPS compared to treatment with LPS alone. IL-12/23p40 production did not differ between *Il10*$^{+/+}$ and *Il10*$^{-/-}$ macrophages in response to LPS, but it was completely abrogated in *Il10*$^{+/+}$ macrophages and only partially reduced (35%) in *Il10*$^{-/-}$ macrophages (Figure 2.7B, bottom panel). Similarly, *Il10*$^{+/+}$ macrophages had reduced IL-6 production in response to IVIg + LPS compared to LPS, but *Il10*$^{-/-}$ macrophages did not (Fig 2.7B, bottom panel). *Il10*$^{-/-}$ macrophages produced more TNF in response to LPS than *Il10*$^{+/+}$ macrophages. TNF production was dramatically reduced by IVIg + LPS treatment relative to LPS treatment in *Il10*$^{+/+}$ macrophages, but IVIg-induced suppression of TNF production was severely compromised in *Il10*$^{-/-}$ macrophages (Figure 2.7B, bottom panel).

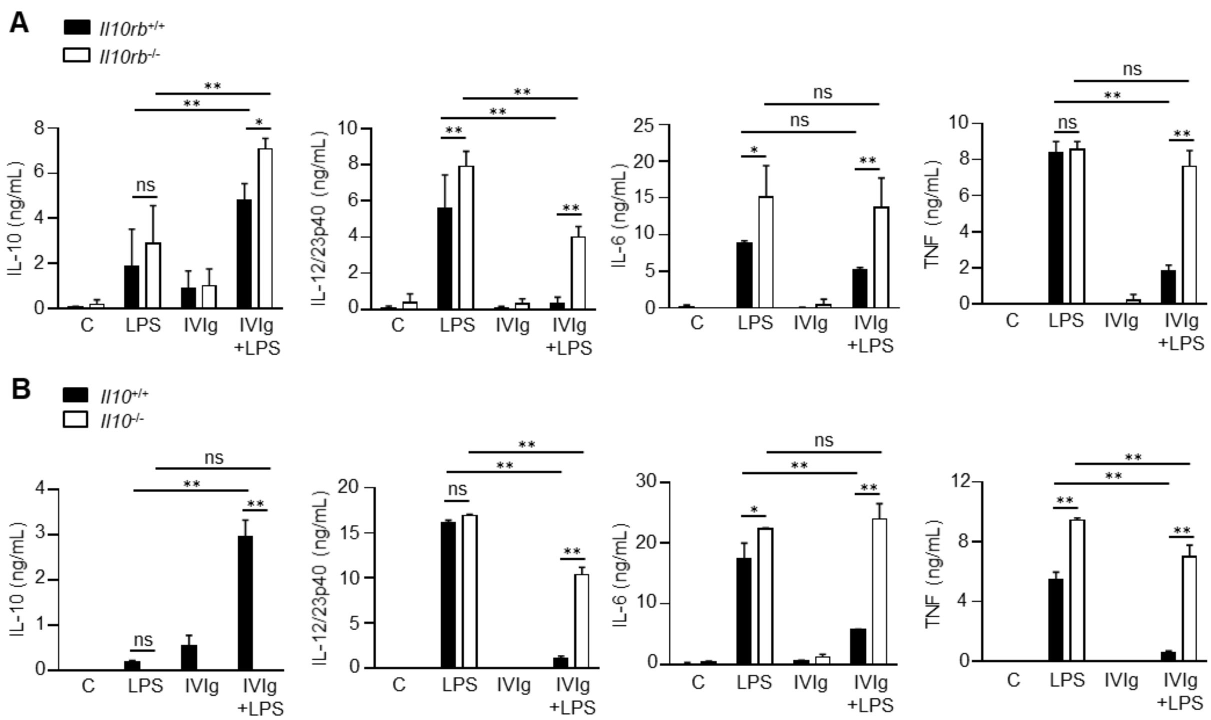

Figure 2.7 IL-10 produced in response to IVIg + LPS contributes to reduced pro-inflammatory cytokine production. MCSF-derived bone marrow macrophages from *Il10rb*$^{+/+}$ and *Il10rb*$^{-/-}$ mice (A) or *Il10*$^{+/+}$ and *Il10*$^{-/-}$ mice (B) were unstimulated or stimulated with LPS (10 ng/mL), IVIg (30 mg/mL), or IVIg + LPS for 24 h. Clarified cell supernatants were assayed for IL-10, IL-12/23p40, IL-6, or TNF by ELISA. Data are means ± SD for $n = 3$; macrophages were derived from 1 pair of mice for each of 3 independent experiments, and ELISAs were assayed in duplicate. *p < 0.01, **p < 0.001, and ns = not statistically significant for the comparisons indicated. Statistical analyses were performed by use of two-way ANOVA with Tukey's post-test for multiple comparisons.

2.3.7 IVIg skews macrophages to an anti-inflammatory IL-10-producing activation state *in vivo*

To determine whether IVIg could skew macrophages to an anti-inflammatory activation state *in vivo*, IVIg was given to mice intraperitoneally and its effect on peritoneal macrophages was assessed *ex vivo*. First, I compared peritoneal macrophage responses to LPS in mice that were either injected with IVIg or PBS. Peritoneal macrophages were harvested 1 h after injections. Peritoneal macrophages from IVIg-treated mice produced significantly more IL-10 than macrophages from mice treated with PBS, as an injection control (Figure 2.8A, left). Unstimulated peritoneal macrophages (no LPS) did not differ in the amount of IL-10 produced when treated *in vivo* with IVIg or PBS. Peritoneal macrophages from mice that received either IVIg or PBS did not produce detectable levels of IL-12/23p40 (Figure 2.8A, right).

In a second series of experiments, mice were injected intraperitoneally with IVIg + LPS or PBS + LPS and peritoneal macrophages and lavage fluid were harvested 1 h after injection. Peritoneal lavage fluid from (IVIg + LPS)-treated mice contained high levels of IL-10 compared with that harvested from (PBS + LPS)-treated mice (Figure 2.8B, left). IL-12/23p40 levels were not significantly lower in the lavage fluid of (IVIg + LPS)-treated mice compared to those treated with PBS + LPS (Figure 2.8B, right). Conditioned medium from peritoneal lavage cells cultured for 1 h to select macrophages by adherence was also assayed for IL-10 and IL-12/23p40. Conditioned medium from peritoneal cells harvested by lavage from mice treated with IVIg + LPS had higher levels of IL-10 compared with that from (PBS + LPS)-injected controls, with neither producing detectable levels of IL-12/23p40 (Figure 2.8C). Peritoneal macrophages isolated from (IVIg + LPS)-injected mice produced 5-fold more IL-10 and significantly lower

IL-12/23p40 after 24 h in culture, compared to macrophages that were isolated from mice injected with PBS + LPS (Figure 2.8D).

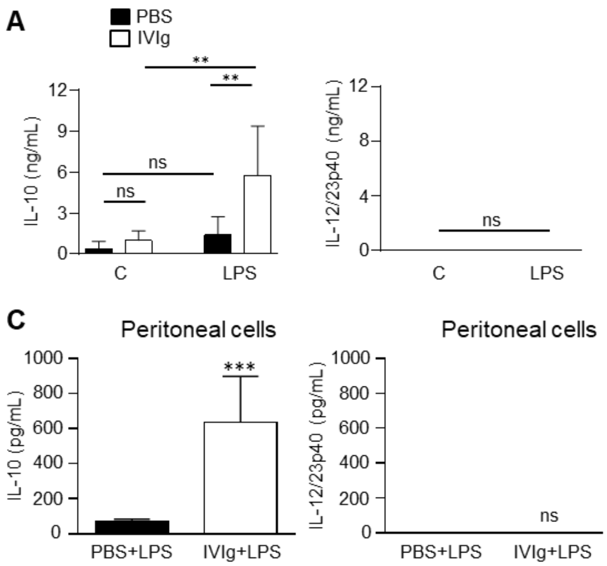

Figure 2.8 IVIg skews macrophages to an anti-inflammatory, IL-10-producing activation state *in vivo*. (A) Wild type C57BL/6 mice were given IVIg (2.5 g/kg) or an equal volume of sterile PBS intraperitoneally, and peritoneal macrophages were isolated after 1 h. Macrophages enriched by adherence to tissue-culture plastic were unstimulated or stimulated with LPS (10 ng/ml) for 24 h. Clarified cell supernatants were assayed for IL-10 and IL-12/23p40 by ELISA. (B–D) Wild type C57BL/6 mice were given IVIg (2.5 g/kg) + LPS (0.2 μg/g body weight) or an equal volume of PBS + LPS (0.2 mg/g body weight). Mice were euthanized for peritoneal lavage after 1 h and isolated cells were enriched for macrophages by adherence to tissue-culture plastic for 1 h. Clarified peritoneal lavage fluid (B), clarified conditioned medium from 1 h adherence step (C), and clarified conditioned medium from 24 h macrophage (Mϕ) cultures (D) were assayed for IL-10 and IL-12/23p40 by ELISA. (A) Data are means ± SD for $n = 5$ mice/group treated in 3 independent experiments with ELISAs assayed in duplicate. (B–D) Data are means ± SD for $n = 5$ mice/group treated in 3 independent experiments with ELISAs assayed in duplicate. (A) **p < 0.01, and ns = not significantly different for the comparisons indicated. (B–D) *p < 0.05, **p < 0.01, ***p < 0.001, and NS = not significantly different for mice injected with IVIg + LPS compared with mice injected with PBS + LPS. Statistical analyses were performed using a two-way ANOVA with Sidak's post-test for multiple comparisons for (A) and using a Student's *t* test for (B–D).

2.4 Discussion

Herein, I report a novel mechanism by which IVIg inhibits inflammation. IVIg activates macrophages to produce large amounts of IL-10 in response to a pro-inflammatory stimulus, LPS. The IL-10 produced then acts in an autocrine or paracrine fashion and contributes to reduced IL-12/23p40, IL-6, and TNF production by the macrophages. The two signals, IVIg and LPS, did not need to be given simultaneously. Increased IL-10 production required MAPKs; Erk1/2, p38, and Erk5, were all required for the induction of IL-10 downstream of IVIg. IL-10 production occurred rapidly in response to IVIg at a level that was sufficient to impact IL-12/23p40 production. Tissue resident peritoneal macrophages primed with IVIg *in vivo* produced high levels of IL-10 when stimulated with LPS *in vitro*. Moreover, mice given IVIg + LPS by intraperitoneal injection produced high levels of IL-10 and low levels of IL-12/23p40, which was evident in lavage fluid and cultured peritoneal macrophages, demonstrating IVIg's potent effect on macrophage activation *in vivo.*

Induction of IL-10 by murine macrophages treated with IVIg + LPS was dependent on the dose of IVIg used, with higher doses of IVIg leading to higher IL-10 production. This is consistent with a model wherein, if antibody produced by the acquired immune system is present in sufficient excess, it can feedback and inactivate the innate immune response. Similar to my data, for murine macrophages activated by Ic + LPS (M(Ic + LPS), i.e. sheep red bloods cells (SRBCs) coated with IgG, a density threshold of IgG on SRBCs was required to permit sufficient cross-linking of FcγRs for robust IL-10 production[39]. IVIg may act in a similar fashion and require FcγR cross-linking for its activity. Receptor cross-linking may occur because a portion of the IgGs within IVIg preparations, exist as dimers or multimers. Alternatively, receptor cross-linking may be enabled at high doses of IVIg by saturation of cell surface FcγRs, which is sufficient to induce cell signalling, as for IgE[306]. The molecular events that lead to IVIg-induced FcγR signalling remain to be determined, but in either case, my results do account for the high doses of IVIg (25-35 mg/mL) that is required to suppress inflammation in people with autoimmune or inflammatory diseases[307, 308].

M(IVIg + LPS) also produced less IL-12/23p40, as well as less TNF and IL-6. Reduction of pro-inflammatory cytokine production by murine M(IVIg + LPS) was profound and may be unique to IVIg treatment. Murine M(Ic + LPS) produce low levels of IL-12/23p40 but TNF and IL-6 production is not affected by Ic activation[295].

Another important distinction between murine M(IVIg + LPS) and M(Ic + LPS) is that IVIg and LPS did not need to be given simultaneously to activate macrophages to produce high IL-10 and low IL-12/23p40-levels. M(Ic + LPS) are somewhat unique among macrophage activation states in that Ic do not reprogram macrophages, which are then assessed by an inflammatory insult. Rather, M(Ic + LPS) activation is achieved by providing both Ic and LPS simultaneously[295]. To compare M(IVIg + LPS) to M(Ic + LPS), I provided IVIg to murine macrophages for various times up to 24 h prior to LPS stimulation. I found that IL-10 production in response to LPS, remained high even 24 h after IVIg treatment; reduced IL-12/23p40 was also evident, though the impact of IVIg waned at the 24 h time point. This is consistent with sustained elevation of phosphorylation of MAPKs observed 24 h after IVIg + LPS treatment. I also found that IVIg induced high IL-10 production and prevented IL-12/23p40 production if provided within 1 h of LPS stimulation. To my knowledge, this is the first demonstration of a sustained high IL-10, low/no IL-12/23p40 producing activation state in macrophages.

The effect of IVIg reducing pro-inflammatory cytokine production by murine macrophages has been attributed to signalling through the DC-SIGN receptor[178]. In addition, the

FcγRI has been implicated in IL-10 production by murine M(Ic + LPS) because IL-10 production was lost in macrophages deficient in the FcR γ chain (required for signalling though the FcγRI, FcγIII, and FcγIV), but not in the FcγRIIB or FcγIII deficient macrophages[27]. My study is the first direct test of the role of the FcγRI in enhanced IL-10 production by macrophages because FcγRI knockout mice were not available when M(Ic + LPS) were first described[27]. My data suggest that neither the FcγRI, FcγRIIB, nor FcγRIII are sufficient for IL-10 induction in response to IVIg + LPS or decreased IL-12/23p40 in response to IVIg + LPS. However, the induction of IL-10 in response to IVIg + LPS compared to IVIg was compromised in FcγRIIB$^{-/-}$ macrophages, because of higher IL-10 production in response to LPS alone. This correlated with reduced IL-12/23p40 production in response to LPS stimulation, which was further reduced and inversely correlated with higher absolute levels of IL-10 produced in response to IVIg + LPS treatment. My data demonstrating that FcR γ chain signalling was not required for IVIg induction of IL-10 further support a role for the FcγRIIB in this process. Alternatively, multiple FcγRs could be involved in IL-10 induction and IL-12/23p40 suppression in response to IVIg + LPS due to overlapping activity and/or there could compensatory effects of the Fcγ receptor deficiencies in the single gene knockout mice. For example, *Fcgr2b*$^{-/-}$ and *Fcgr3*$^{-/-}$ mice have higher amounts of FcγRIVs on their cell surface[105].

I found that MAPKs were required for IL-10 production by murine M(IVIg + LPS). Erk1/2 and p38 activation occur earlier, are stronger, and are prolonged in (IVIg + LPS)-activated macrophages compared to LPS-activated macrophages. FcγRI signalling in murine M(Ic + LPS) requires Erk1/2 activation, which leads to chromatin modifications opening up the IL-10 promoter, and p38 activation, which drives transcription of IL-10[38, 39]. My data supports the same model in that IVIg + LPS and IVIg are both strong activators of Erk1/2 (IVIg priming cells for IL-10 production) and IVIg + LPS and LPS are strong activators or p38 (permitting promoter-dependent transcription of IL-10). Indeed, more IL-10 is produced by LPS-stimulated murine macrophages than dendritic cells, due to stronger Erk1/2 activation[80]. Activation of MAPKs was still evident 24 h after IVIg + LPS stimulation, which has been reported to lead to chromatin modifications, and may account for the longevity of the effect of IVIg on macrophages [38]. Erk1/2 inhibitors, SCH and PD, and p38 inhibitors, SCH and PD, reduced IL-10 production by M(IVIg + LPS). PD reduced IL-10 more effectively than SCH, which could be attributed to its off target effects on Erk5[305]. The potent and selective Erk5 inhibitor, XMD, and BIX, a MEK5 inhibitor, also effectively decreased IL-10 by M(IVIg + LPS)[309]. In macrophages, Erk5 phosphorylation was also stronger and prolonged post IVIg + LPS stimulation, mirroring the activation patterns of the other MAPKs. This provides evidence that Erk5 can have an anti-inflammatory role in macrophages in addition to its inflammatory effects[310].

My results suggest that IL-10 production by M(IVIg + LPS) contributes to reduced pro-inflammatory cytokine production. IL-10 production by M(IVIg + LPS) occurs rapidly and at sufficient levels to reduce IL-12/23p40 production. IL-10-induced reduction of pro-inflammatory cytokine production in response to pro-inflammatory stimuli, including IL-6, TNFα, and IL-1β, has been reported previously[58]. Consistent with activation of MAPKs leading to increased transcription of *Il10*, *Il10* mRNA was rapidly up-regulated in (IVIg + LPS)-stimulated macrophages 1 h post stimulation. Reduced IL-12/23p40 production also correlated with reduced transcription of *Il12b*, which is induced only after *Il10* transcription and may be dampened by production of IL-10[38]. In addition, I have used two independent genetic models to demonstrate that IL-10 is required for reduced pro-inflammatory cytokine production by M(IVIg + LPS). Macrophages from mice deficient in the IL-10 receptor β subunit (*Il10rb*$^{-/-}$), which can not signal in response to IL-10, or deficient in IL-10 itself (*Il10*$^{-/-}$), produced more IL-12/23p40, IL-6, and TNF when stimulated with IVIg + LPS, compared to their wild type littermates. Interestingly, IL-10 production was also higher in *Il10rb*$^{-/-}$ versus *Il10rb*$^{+/+}$, which can be attributed to loss of the IL-10 feedback mechanism that IL-10 uses to regulate its own production[80]. It is interesting to note that loss of IL-10 or IL-10 signalling did not abrogate the effects of IVIg reducing macrophage pro-inflammatory cytokine production suggesting that reduced pro-inflammatory cytokine production by M(IVIg + LPS) also occurs by one or more IL-10-independent mechanisms. This may be due to a direct effect of IVIg on pro-inflammatory cytokine production reported to occur downstream of Fcγ receptors[130]. IL-10 is more effective at reducing IL-12/23p40 production than production of IL-6 or TNF. The potency of its efficacy may account for the failure to note significant differences for other pro-inflammatory cytokine production by murine and human M(Ic + LPS), which also produce IL-10. Conditioned medium from M(Ic + LPS) is able to reduce IL-12p70 production completely when added back to macrophages treated with IFNγ + LPS and anti-IL-10 blocking antibodies abrogate the effect the conditioned medium[27]. Alternatively, this may also indicate other soluble factors produced by murine M(Ic + LPS), and by murine M(IVIg + LPS), contribute to reduction of IL-12/23p40 production.

My data also demonstrate the potent ability of IVIg to skew macrophages to an anti-inflammatory, IL-10 producing activation state *in vivo*. IVIg-primed macrophages produced high amounts of IL-10 in response to LPS *ex vivo* compared to those from mice primed with PBS, as a control. This is consistent with the ability of cultured peritoneal macrophages activated with Ic, M(Ic), and stimulated with LPS to produce high levels of IL-10[301]. However, I found that these peritoneal macrophages did not produce IL-12/23p40. IVIg + LPS injection in mice caused higher production of IL-10 than PBS + LPS injection in peritoneal lavage fluid and in media conditioned by peritoneal cells and enriched macrophages. Peritoneal macrophages from (IVIg + LPS)-injected mice also produced significantly lower amounts of IL-12/23p40 in 24 h compared to those from (PBS + LPS)-injected mice. These data are consistent with my *in vitro* observations in that IL-10 was produced rapidly in response to IVIg + LPS and reduced subsequent IL-12/23p40 production. These data also demonstrate that IVIg can induce macrophage IL-10 production *in vivo* and dampen down macrophage inflammatory responses to LPS. This is consistent with a previous report, which demonstrated that IgG + LPS promoted higher IL-10 and lower IL-12/23p40 levels in plasma of recombination-activating gene 1 (RAG1) deficient mice (deficient in mature T and B cells) compared to mice treated with LPS alone[27]. Current thinking is that reduction of mortality rates in clinical studies when polyclonal IVIg is given therapeutically to treat sepsis is due to the presence of antibodies directed against bacterial or cytokine antigens; however, increased production of anti-inflammatory IL-10 and reduced pro-inflammatory cytokine production by macrophages could also contribute to reduced mortality in sepsis[311, 312].

Chapter 3: IVIg or IVIg-treated macrophages reduce DSS-induced colitis in mice

3.1 Introduction and rationale

Biologic therapies, in the form of monoclonal antibodies directed against TNFα, have revolutionized the treatment for IBD. Unfortunately, 10-20% of people with IBD are unresponsive to anti-TNFα therapies and up to 40% become refractory to treatment over time, approximately half of whom do not have anti-drug antibodies[288, 313]. Anti-TNFα drugs also have deleterious side effects, including higher risk of infections and malignancy[287]. Thus, new treatments need to be developed.

Macrophages have an important role in maintaining intestinal homeostasis, by sampling the luminal bacteria and promoting a tolerogenic, rather than inflammatory, response[314]. Macrophages have been implicated in the pathogenesis of IBD by promoting an inappropriate response to bacteria, causing immune cell recruitment, and promoting inflammatory cytokine and ROS production[288, 314, 315]. M(IFNγ + LPS) are a well-studied example of macrophages that have similar properties to inflammatory intestinal macrophages in IBD[10, 24]. M(IL-4), which have wound healing properties, are protective in murine models of intestinal inflammation[316, 317]. Anti-inflammatory macrophages, which produce high amounts of IL-10, can also limit intestinal inflammation in mice[318, 319]. The best characterized example of this activation state are murine macrophages treated with Ic and LPS (M(Ic + LPS)) *in vitro*, which are distinct from murine M(IL-4) as they do not express markers, including ArgI or FIZZ1, and they do not promote the production of extracellular matrix[11, 49].

IL-10 induces a tolerogenic effect on innate and adaptive immune cells, by preventing their production of pro-inflammatory mediators and by promoting the production of anti-inflammatory mediators[61]. Dysregulated expression of IL-10 can lead to the development of IBD[277, 320]. IL-10-inducing therapeutics may provide an effective treatment for IBD with fewer side effects.

IVIg's immunosuppressive mechanism(s) are not well understood[308]. As an anti-inflammatory, IVIg is given at very high doses (1-2 g/kg), and few proposed mechanisms of action can explain this requirement[298]. One proposed mechanism that may explain this requirement, is that a minor fraction of Fc regions on IgGs are sialylated[303]. However, some studies contradict this theory and demonstrate that sialylated Fc regions are not required to limit inflammation in animal models for ITP and RA[20]. Evidence also contradicts this mechanism of action in humans, as FcγRIIB expression is not higher after IVIg treatment in people with KD[167, 321].

I have found that murine BMDMs can be activated to produce high levels of IL-10 and low levels of IL-12/23p40, IL-6, and TNF *in vitro*, when treated concomitantly with IVIg and LPS[322]. Based on this, I asked whether IVIg treated macrophages or IVIg treatment can ameliorate DSS-induced intestinal inflammation in mice by inducing macrophage IL-10 production.

3.2 Materials and methods

Mice. Wild type C57BL/6 mice, wild type BALB/c mice, $\mathit{Il10rb}^{+/+}$ and $\mathit{Il10rb}^{-/-}$ mice on a C57BL/6 background, $\mathit{Il10}^{egfp}\mathit{Foxp3}^{mrfp}$ mice on a C57BL/6 background, and $\mathit{Il10}^{flox/flox}\mathit{LysMCre}^{+/-}$ and $\mathit{Il10}^{flox/flox}\mathit{LysMCre}^{-/-}$ mice on a C57BL/6 background were used for

DSS-induced colitis experiments. Eight to 12-week-old male and female mice were used, except for DSS experiments with BALB/c mice, where only male mice were used due to lower susceptibility to DSS in female mice. Mice were housed and bred at the BC Children's Hospital Research Institute (Vancouver, BC, Canada), a barrier facility that is *Helicobacter*-free and specific pathogen free. Experiments were performed in accordance with Canadian Council on Animal Care guidelines with approval from institutional animal care committees (A13-0014, A13-0054, A17-0061, and A17-0076).

Wild type mice on a C57BL/6 background (8-12-week-old) were used to prepare BMDMs for adoptive transfer experiments into wild type C57BL/6 mice. $Il10^{+/+}$ and $Il10^{-/-}$ mice on a BALB/c background (8-12-week-old) were used to prepare bone marrow-derived macrophages for adoptive transfer experiments into wild type BALB/c mice.
Femura and tibiae from $Il10^{+/+}$ mice and $Il10^{-/-}$ mice, which were bred and maintained at the University of Alberta animal care facility (Edmonton, AB, Canada), were provided by Dr. Karen Madsen.

$Il10rb^{-/-}$ and $Il10^{egfp}Foxp3^{mrfp}$ mice were provided by Dr. Megan Levings at the University of British Columbia (Vancouver, BC, Canada), and were bred and maintained at the BC Children's Hospital Research Institute (Vancouver, BC, Canada). C57BL/6 mice were used as $Il10rb^{+/+}$ experimental controls and were bred and maintained at the BC Children's Hospital Research Institute (Vancouver, BC, Canada). $Il10^{flox/flox}LysMCre^{+/+}$ mice were provided by Dr. Masako Murai at the La Jolla Institute for Allergy and Immunology (La Jolla, CA, USA), and were used with wild type C57BL/6 mice, which were bred and maintained at the BC Children's Hospital Research Institute (Vancouver, BC, Canada), to generate $Il10^{flox/flox}LysMCre^{+/-}$ and $Il10^{flox/flox}LysMCre^{-/-}$ mice. $Il10^{flox/flox}LysMCre^{+/-}$ and $Il10^{flox/flox}LysMCre^{-/-}$ mice were bred to generate further *Il10*$^{flox/flox}$*LysMCre*$^{+/-}$ experimental mice and *Il10*$^{flox/flox}$*LysMCre*$^{-/-}$ control mice for experiments.

Macrophage derivation and activation. For adoptive transfer experiments, macrophages were derived from bone marrow aspirates from the femura and tibiae from wild type C57BL/6 mice and *Il10*$^{+/+}$ or *Il10*$^{-/-}$ mice on a BALB/c background, as described in Chapter 2.2. Following adherence depletion, bone marrow aspirates were resuspended in IMDM 10% FBS, and penicillin/streptomycin at a concentration of 0.5×10^6 cells/ml for 10 days in the presence of 5 ng/ml MCSF (Stemcell Technologies, Vancouver, BC, Canada), with complete media changes at days 4 and 7. Macrophages were either left unstimulated or co-stimulated with 30 mg/mL IVIg (Gamunex Immune Globulin Intravenous 10% solution for infusion; Transfusion Medicine BC Children's Hospital, Vancouver, BC, Canada) and 10 ng/ml LPS (Escherichia coli serotype 127:B8; Sigma-Aldrich, St. Louis, MO, USA) for 24 h prior to adoptive transfer.

DSS-induced colitis. DSS (1.5%, 2.5%, or 5% as indicated; MW 36–50 000; MP Biomedicals, Solon, OH, USA) was dissolved in drinking water and given to mice *ad libitum*. DSS was provided for 6 or 7 days with daily monitoring of weight loss, rectal bleeding, and stool consistency. Scores of 0-4 for weight loss, rectal bleeding, and stool consistency were given for each mouse daily and combined for an individual Disease Activity Index score (DAIs). Scoring criteria, using a 12-point scale, is shown in Table 2.1. For rectal bleeding, non-visible blood was detected using hemoccult paper (Beckman Coulter, Mississauga, Canada). Once euthanized, colons were excised, and samples were taken for histological analysis and for colonic explant cultures, where indicated.

Table 2.1 Disease Activity Index scoring

Score	Weight loss	Rectal bleeding	Stool consistency
0	0%	None	Normal
1	1-3%	Blood detectable by hemoccult paper only	Soft stool
2	3-6%	Small amount of blood visible in stool	Very loose stool
3	6-9%	Large amount of blood in stool	Watery diarrhea (no pellets formed)
4	>9%	Extensive blood in stool and blood visible at the anus	No formed stool

Macrophage adoptive transfer. Adherent unstimulated or stimulated BMDMs were incubated with cell dissociation buffer (Thermo Fisher Scientific, Waltham, MA, USA) for 5 min at 37°C to remove from flasks. 1×10^6 cells, or PBS, as an injection control, were injected into the tail vein of mice on days 0 and 4 during DSS treatment.

IVIg treatment. 1 g/kg of body weight IVIg or an equivalent volume of PBS, as an injection control, was given to mice, where indicated, intraperitoneally on days 0, 2, 4, and day 6 of DSS treatment.

Histopathology analyses. Colons were fixed in PBS-buffered 10% formalin (Fisher Scientific, Ottawa, Canada). Colons were embedded in paraffin, cross-sectioned, and stained with hematoxylin and eosin (H&E) at the BC Children's Hospital Research Institute histology facility. Histological damage was scored by two individuals blinded to the experimental conditions using a 16-point scale as described in Table 2.2[316].

Table 2.2 Histological damage scoring

Damage Component	Score
Loss of architecture	0 = none
	1 = <25% loss
	2 = 25%-50% loss
	3 = 50%-75% loss
	4 = >75% loss
Immune cell infiltration	0 = none
	1 = occasional immune cell in lamina propria
	2 = increased immune cells in lamina propria
	3 = confluent immune cells in lamina propria and breaching mucosa
	4 = immune cell infiltration throughout the section
Goblet cell depletion	0 = none
	1 = <50% depletion
	2 = >50% depletion
Ulceration	0 = none
	1 = intermediate ulceration
	2 = substantial ulceration
Edema	0 = none
	1 = <50% of section
	2 = >50% of section
Muscle thickening	0 = none
	1 = intermediate thickening
	2 = substantial thickening

Colon explant cultures. Colons were excised from *Il10rb*$^{+/+}$ and *Il10rb*$^{-/-}$ mice on day 6 of DSS treatment, 1.5 h post PBS or IVIg injection. Sections (0.5 cm) were taken, weighed, and cultured in IMDM 10% FBS, and penicillin/streptomycin for 24 h. After incubation, supernatants were harvested and clarified by centrifugation for cytokine analysis by ELISA.

Histological analyses. Excised colons were formalin-fixed, paraffin embedded and sectioned at the BC Children's Hospital Research Institute histology facility. Tissues from *Il10*^{egfp}*Foxp3*^{mrfp} mice treated with either PBS or IVIg, and treated with DSS 7 days were deparaffinized, rehydrated, and stained with 4′,6-diamidino-2-phenylindole (DAPI) nuclear stain (Thermo Fisher Scientific, Waltham, MA, USA). Colonic sections were taken on day 6 from wild type C57BL/6 mice given DSS and treated with PBS or IVIg 1.5 h prior. Sections were probed for *Emr1* (F4/80) and *Il10* (IL-10) mRNA, using an RNAScope kit (Advanced Cell Diagnostics, Newark, CA, USA), according to manufacturer's instructions. All solutions were provided by the manufacturer. Colonic sections (5 μm) were baked for 1 h at 60°C, to fix the tissue onto slides. The sections were then deparaffinized and air dried. Peroxidase activity was blocked using 1.5% hydrogen peroxide for 10 min at room temperature and using a protease solution for 30 min at 40°C. RNA target retrieval was performed by incubating samples with a target retrieval solution, for 15 min at 100°C. Probes were hybridized to *Il10* and *Emr1* mRNA targets for 2 h at 40°C. The probe signals were amplified by sequential hybridization and amplification, using amplification solutions. Red and green/teal signals were detected by incubating with chromogenic solutions provided for 10 min at room temperature. The colonic sections were counterstained with 50% hematoxylin (Sigma-Aldrich), dried at 60°C for 15 min, and mounted with VectaMount (Vector laboratories, Burlingame, CA, USA). Images were acquired and analyzed using a Zeiss Axiovert 200 microscope, AxioCam HR camera, and AxioVision 4.0 software. DAPI/ green fluorescent protein $(GFP)^+$ and *Emr1*/*Il10*$^+$ cells were quantified by counting cells from six representative fields at 20× or 40× magnification from six tissue sections per colon separated by ≥ 50 μm. Counting was performed by two individuals blinded to experimental condition.

CD11b$^+$ intestinal mononuclear cell isolation. Colons were excised from wild type C57BL/6 mice given DSS and treated with PBS or IVIg on day 7. Intestinal mononuclear cells were isolated using collagenase digestion[323]. Colons were flushed with 4°C PBS 5% FBS to remove luminal contents. Flushed colons were cut longitudinally and into 0.5 cm segments, prior to vigorous rinsing with 4°C PBS 5% FBS for 2 × 10 min. The epithelial barrier was stripped by shaking colon segments for 1.5 h with prewarmed (37°C) PBS, 5% FBS, 2 mM ethylenediaminetetraacetic acid (EDTA), with solution changes every 30 min (Thermo Fisher Scientific, Waltham, MA, USA). EDTA was removed from the colons by washing with PBS, 5% FBS for 2 × 10min. Colon segments were minced into fine pieces and digested by incubating with 37°C Roswell Park Memorial Institute (RPMI) medium, 5% FBS, 1mg/mL type IV collagenase (Worthington Biochemical, Lakewood, NJ, USA) for 40 min at 250 rpm. The solution was strained and isolated cells in the supernatant were kept on ice. Undigested tissues were incubated with prewarmed (37°C) RPMI, 5% FBS, 1 mg/mL type IV collagenase for 30 min at 250 rpm and were strained and pooled with the first digest. Digested fractions were pooled from 2 mice per treatment, and CD11b$^+$ intestinal mononuclear cells were isolated using the EasySep murine CD11b positive selection kit, according to manufacturer's instructions (Stemcell Technologies, Vancouver, BC, Canada).

Cytokine and GFP analyses. Cytokines were assayed by ELISA, according to the manufacturer's instructions. ELISA kits for murine IL-10, IL-12/23p40, IL-β, and TNF were obtained from BD Biosciences (Mississauga, ON, Canada). GFP content was assayed by ELISA, according to manufacturer's instructions (Abcam, Cambridge, UK).

Statistical analyses. One- and two-way ANOVA with Tukey's or Sidak's corrections for multiple comparisons and two-tailed unpaired Student's *t*-tests were applied as indicated. Analyses were performed using GraphPad Prism software, version 6.03. Differences of $p < 0.05$ were considered significant.

3.3 Results

3.3.1 M(IVIg + LPS) reduce DSS-induced intestinal inflammation

We have previously reported that IVIg induces high IL-10 production and reduces pro-inflammatory cytokine production, in response to LPS, a normally inflammatory stimuli[322]. Herein, I asked whether adoptively transferred M(IVIg + LPS) could reduce intestinal inflammation, in mice during DSS-induced colitis. C57BL/6 mice were given 2.5% DSS in their drinking water for 7 days. Mice were given PBS or untreated macrophages (M(UnRx)), as controls, or macrophages treated with IVIg + LPS, (M(IVIg + LPS)), on days 0 and 4 during DSS treatment via tail vein injection. DAIs, which consist of scores for weight loss, rectal bleeding, and stool consistency, did not differ between mice treated with PBS or M(UnRx) (Figure 1A). Mice treated with M(IVIg + LPS) had significantly lower DAIs compared to both control mice treated with PBS or M(UnRx). Weight loss was not different between mice treated with PBS or M(UnRx); however, there was a slight reduction in weight loss in mice treated with

M(IVIg + LPS), that was statistically different from the M(UnRx) control mice only on day 7 ($p < 0.05$; Figure 3.1A). Rectal bleeding and stool consistency scores were not different between mice treated with PBS or M(UnRx), but were significantly lower in mice treated with M(IVIg + LPS), compared to both control groups (Figure 3.1A). At day 7, colons were excised, and sections were taken for histological analyses. Representative H&E-stained colon cross-sections from mice in each treatment group that were used for scoring histological damage are shown (Figure 3.1B). Histological damage scores, which include scores for tissue architecture, inflammatory infiltrates, muscle thickening, edema, and ulceration, were not different between mice treated with PBS or M(UnRx) (Figure 3.1B). Mice treated with M(IVIg + LPS) had significantly lower histological damage scores compared to both the PBS and M(UnRx) treated control mice (Figure 3.1B). Together, these results indicate that M(IVIg + LPS) reduce intestinal Together, these results indicate that M(IVIg + LPS) reduce intestinal inflammation in mice.

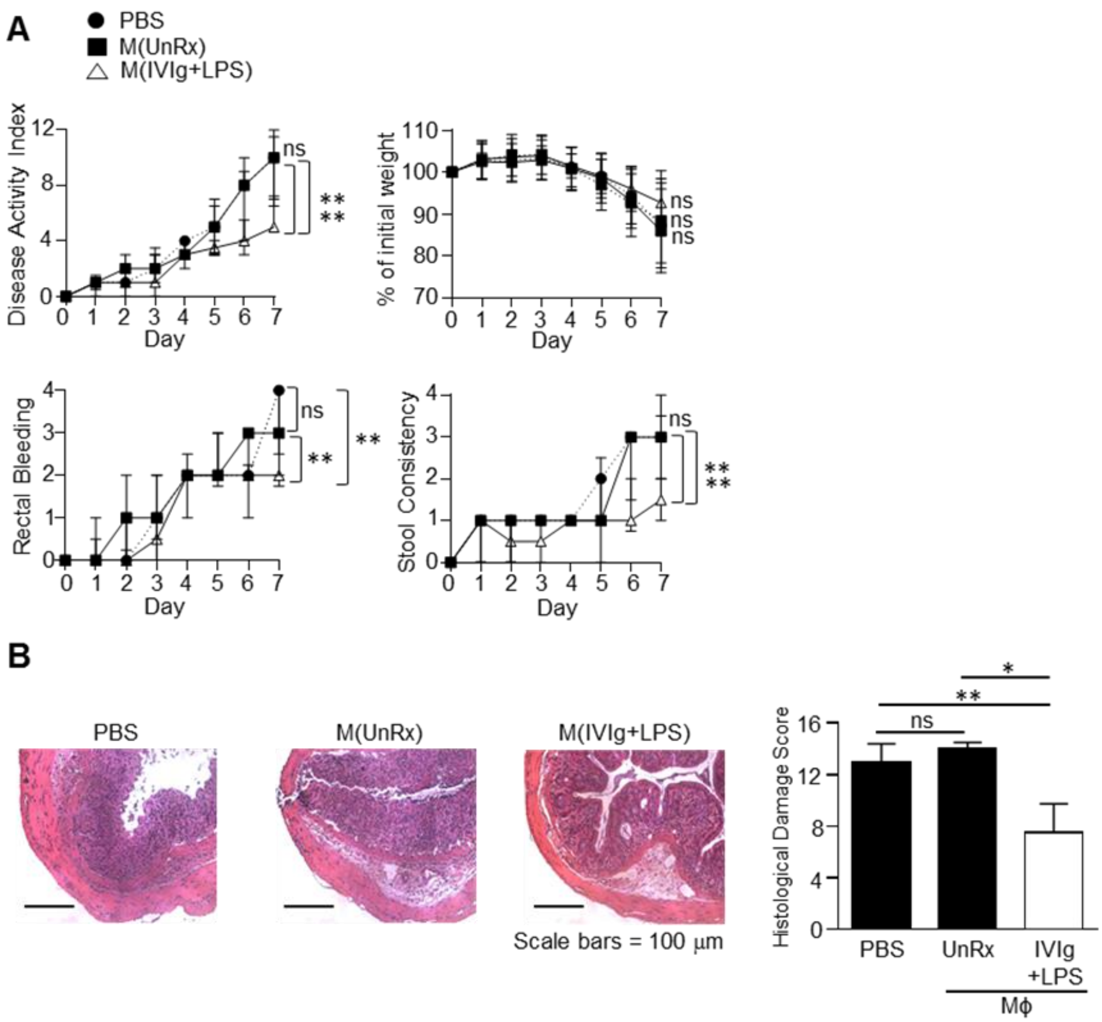

Figure 3.1 M(IVIg + LPS) reduce DSS-induced intestinal inflammation. C57BL/6 mice were given 2.5% DSS in their drinking water for 7 days. Mice were given PBS or untreated macrophages (M(UnRx)), as controls, or macrophages treated with IVIg + LPS (M(IVIg + LPS)) on days 0 and 4 during DSS treatment via tail vein injection. (A) DAIs, which consist of scores for weight loss, rectal bleeding, and stool consistency, were measured daily. DAI, rectal bleeding, and stool consistency are presented as median ± interquartile range, whereas weight is presented as mean ± SD. (B) Representative H&E-stained colons are shown and histological damage score was scored and is expressed as median ± interquartile range. Scale bars = 100 µm. Data from (A and B) are *n* = 11 or13 mice/group from 4 independent experiments. *p < 0.01, **p < 0.001, and ns = not statistically different for the comparisons indicated using a two-way ANOVA with Tukey's multiple comparisons test in (A), and a one-way ANOVA with Tukey's multiple comparisons test in (B).

3.3.2 Amelioration of DSS-induced colitis by M(IVIg + LPS) is IL-10-dependent

I next wanted to determine whether IL-10 contributes to M(IVIg + LPS)-induced protection during DSS-induced colitis. BALB/c mice were given 5% DSS in their drinking water for 7 days. Mice were also given PBS as an injection control, *Il10*$^{+/+}$ M(IVIg + LPS), or *Il10*$^{-/-}$ M(IVIg + LPS) on days 0 and 4 during DSS treatment via tail vein injection. *Il10*$^{+/+}$ M(IVIg + LPS) treated mice had significantly lower DAIs compared to the PBS treated mice, whereas *Il10*$^{-/-}$ M(IVIg + LPS) did not have improved DAIs compared to the PBS treated mice (Figure 3.2A). *Il10*$^{+/+}$ M(IVIg + LPS) treated mice had significantly lower scores for weight loss, rectal bleeding, and stool consistency compared to PBS treated mice, whereas *Il10*$^{-/-}$ M(IVIg + LPS) did not (Figure 3.2A). Representative H&E-stained cross-sections from mice in each treatment group are shown (Figure 3.2B). Mice treated with *Il10*$^{+/+}$ M(IVIg + LPS) had significantly lower histological damage scores compared to the PBS-treated mice, but reduced histological damage was not observed in mice treated with *Il10*$^{-/-}$ M(IVIg + LPS) (Figure 3.2B). Histological damage scores were not different between mice treated with *Il10*$^{-/-}$ M(IVIg + LPS) or PBS (Figure 3.2B). Together, these results indicate that IL-10 contributes to M(IVIg + LPS) amelioration of intestinal inflammation.

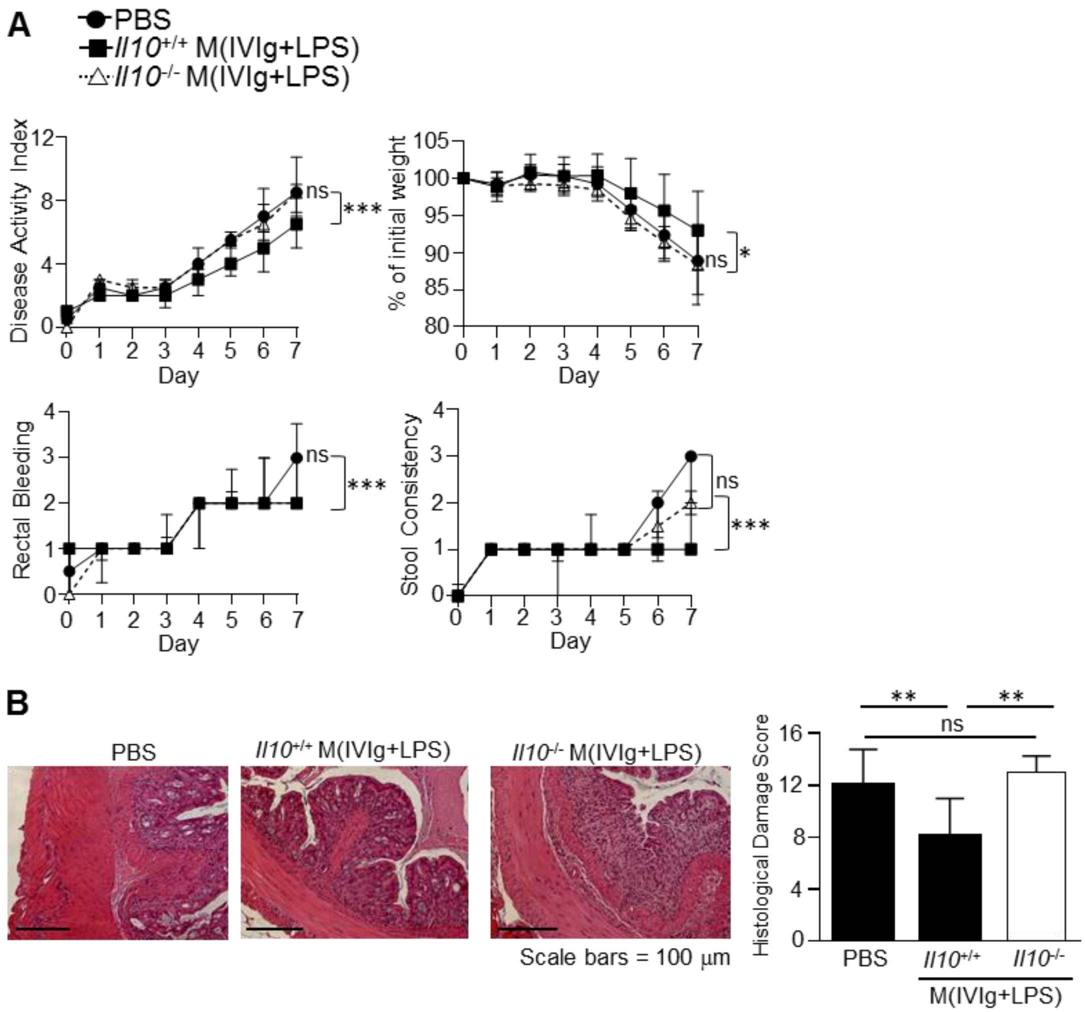

Figure 3.2 Amelioration of DSS-induced colitis by M(IVIg + LPS) is IL-10-dependent. BALB/c mice were given 5% DSS in their drinking water for 7 days. Mice were given PBS, *Il10*$^{+/+}$, or *Il10*$^{-/-}$ M(IVIg + LPS) on days 0 and 4 during DSS treatment via tail vein injection. (A) DAIs, including % of initial weight, rectal bleeding, and stool consistency, were measured daily. DAI, rectal bleeding, and stool consistency are presented as median ± interquartile range; weight is presented as mean ± SD. (B) Representative H&E-stained colons are shown and histological damage was scored and is expressed as median ± interquartile range. Scale bars = 100 µm. Data from (A and B) are *n* = 6-8 mice/group from 3 independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ns = not statistically different using a two-way ANOVA with Tukey's multiple comparisons test in (A) and a one-way ANOVA with Tukey's multiple comparisons test in (B).

3.3.3 IVIg treatment reduces inflammation during DSS-induced colitis

Since I have seen that M(IVIg + LPS) reduced inflammation during DSS-induced colitis, I asked whether IVIg treatment could also reduce intestinal inflammation. C57BL/6 mice were given 2.5% DSS in their drinking water for 7 days. Mice receiving DSS were injected intraperitoneally with PBS, as an injection control, or IVIg on days 0, 2, 4, and 6. Mice treated with IVIg had significantly lower DAIs compared to the PBS-treated control mice (Figure 3.3A). Each of weight loss, rectal bleeding, and stool consistency scores were lower in the IVIg-treated mice compared to control mice (Figure 3.3A). Representative H&E-stained colon cross-sections from mice in each treatment group that were used for scoring histological damage are shown (Figure 3.3B). Histological damage scores were also significantly lower in the IVIg-treated mice compared to the control mice (Figure 3.3B). Together, these results indicate that IVIg treatment reduces intestinal inflammation in mice.

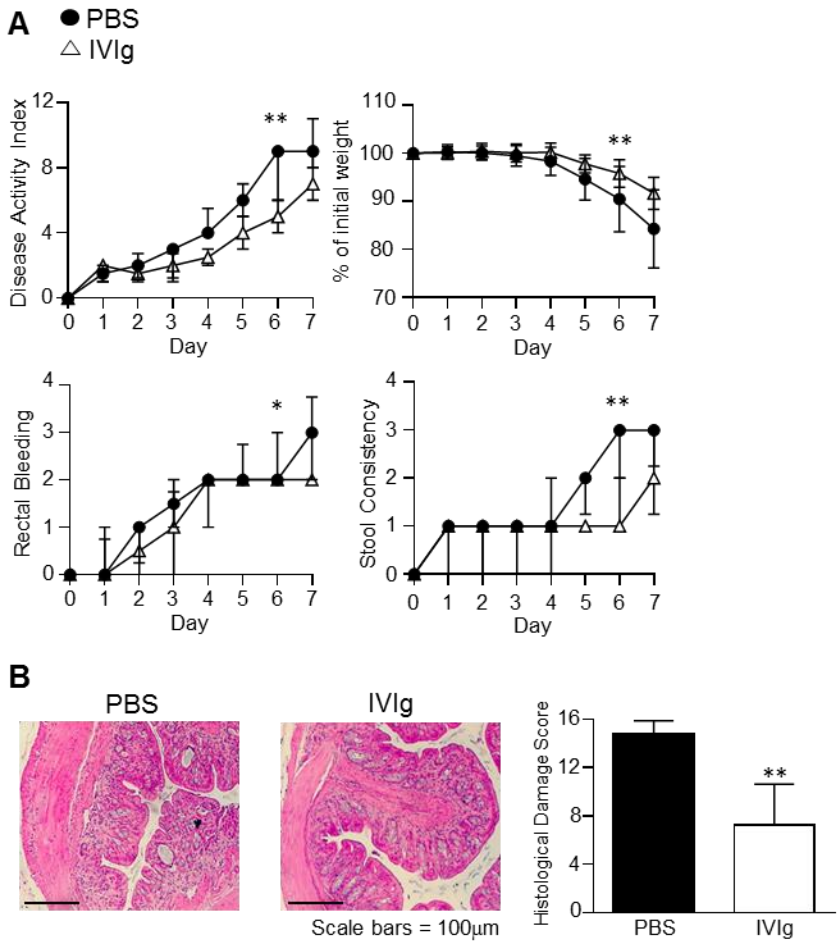

Figure 3.3 IVIg treatment reduces inflammation during DSS-induced colitis. C57BL/6 mice were given 2.5% DSS in their drinking water for 7 days. Mice were given PBS or IVIg (1 g/kg body weight) intraperitoneally on day 0, 2, 4, and 6 during DSS treatment. (A) DAIs, including weight loss, rectal bleeding, and stool consistency, were measured daily. DAI, rectal bleeding, and stool consistency are presented as median ± interquartile range, weight loss is represented as mean ± SD. (B) Representative H&E-stained colons are shown and histological damage was scored and is presented as median ± interquartile range. Scale bars = 100 µm. Data from (A and B) are $n = 12$ mice/group from 4 independent experiments. *$p < 0.01$, **$p < 0.001$, and ns = not statistically different for PBS-treated mice compared to IVIg-treated mice using a two-way ANOVA with Tukey's multiple comparisons test in (A) and a two-tailed Student's *t*-test in (B).

3.3.4 IVIg-mediated protection from DSS-induced colitis is IL-10-dependent

Since amelioration of DSS-induced colitis in mice treated with M(IVIg + LPS) was dependent on IL-10, I next wanted to determine whether protection by IVIg treatment was also dependent on IL-10. DSS concentration was titrated in the *Il10rb*$^{-/-}$ mice to achieve similar scores of clinical disease in the *Il10rb*$^{+/+}$ (wild type) and mice deficient for the IL-10 receptor β chain, *Il10rb*$^{-/-}$, on a C57BL/6 background. *Il10rb*$^{+/+}$ and *Il10rb*$^{-/-}$ mice were given 2.5% and 1.5% DSS, respectively, in their drinking water for 7 days. Mice receiving DSS were also injected with PBS, as an injection control, on days 0, 2, 4, and 6. *Il10rb*$^{-/-}$ mice were more sensitive to 2.5% DSS than *Il10rb*$^{+/+}$ mice; DAIs of PBS-injected *Il10rb*$^{-/-}$ mice were significantly higher than PBS-injected *Il10rb*$^{+/+}$ mice (Figure 3.4A). *Il10rb*$^{-/-}$ mice developed similar DAIs in response to 1.5% DSS as *Il10rb*$^{+/+}$ did in response to 2.5% DSS; DAIs of PBS-injected *Il10rb*$^{+/+}$ and *Il10rb*$^{-/-}$ did not differ significantly (Figure 3.4B).

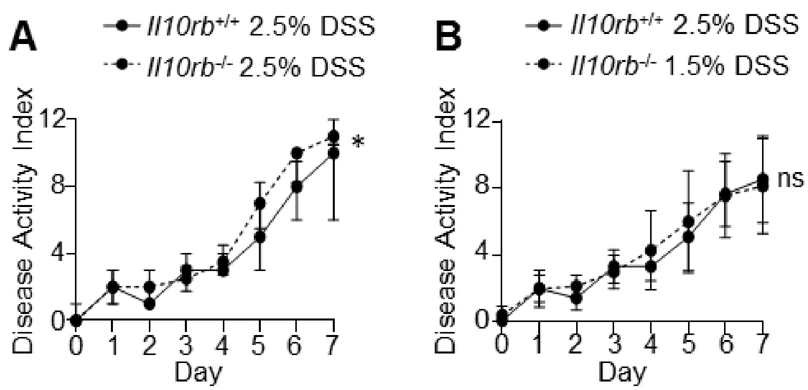

Figure 3.4 IL-10 receptor β chain deficient mice are more sensitive to DSS. *Il10rb*$^{+/+}$ mice on a C57BL/6 background were given 2.5% DSS in their drinking water and *Il10rb*$^{-/-}$ mice on a C57BL/6 background were given 2.5% or 1.5% DSS in their drinking water for 7 days. Mice receiving DSS were also given PBS (injection control) intraperitoneally on day 0, 2, 4, and 6. (A and B) DAIs were measured daily from *Il10rb*$^{+/+}$ mice given 2.5% DSS and (A) *Il10rb*$^{-/-}$ mice given 2.5% DSS and (B) *Il10rb*$^{-/-}$ mice given 1.5% DSS. (A and B) DAIs and are presented as median ± interquartile range. Data from (A) are n = 6 or 9 mice/group and from (B) are n = 7 or 9 mice/group from 3 independent experiments. *p < 0.01 and ns = not statistically different for the comparisons indicated using a two-way ANOVA.

Il10rb$^{+/+}$ and *Il10rb*$^{-/-}$ mice were given 2.5% and 1.5% DSS, respectively, in their drinking water for 7 days. Mice receiving DSS were injected intraperitoneally with PBS, as an injection control, or IVIg on days 0, 2, 4, and 6. Untreated *Il10rb*$^{+/+}$ and *Il10rb*$^{-/-}$ mice were also monitored to ensure that there was no spontaneous development of disease in the knockout genotype during the course of the experiment. *Il10rb*$^{+/+}$ and *Il10rb*$^{-/-}$ mice that were not given DSS did not shown signs of spontaneous clinical disease (*Il10rb*$^{-/-}$ mice are shown in Figure 3.5A; data not shown for *Il10rb*$^{+/+}$ mice). IVIg-treated *Il10rb*$^{+/+}$ mice had significantly lower DAIs compared to their PBS-treated counterparts. In contrast, IVIg treatment did not improve DAIs for *Il10rb*$^{-/-}$ mice, which were comparable to PBS-treated *Il10rb*$^{-/-}$ mice. At day 7, colons were excised, and sections were taken for H&E staining and histological analyses. Representative H&E-stained colon cross-sections from mice in each treatment group are shown (Figure 3.5B). *Il10rb*$^{+/+}$ and *Il10rb*$^{-/-}$ mice that were not given DSS did not have signs of histological disease (Figure 3.5B). Histological damage scores were significantly lower in IVIg-treated *Il10rb*$^{+/+}$ mice compared to the PBS-treated *Il10rb*$^{+/+}$ mice (Figure 3.5B). In contrast, IVIg-treated *Il10rb*$^{-/-}$ mice did not have improved histological damage scores compared to the PBS-treated *Il10rb*$^{-/-}$ mice. Together, these results indicate that IVIg-mediated reduction of intestinal inflammation is dependent on signalling through the IL-10 receptor β chain.

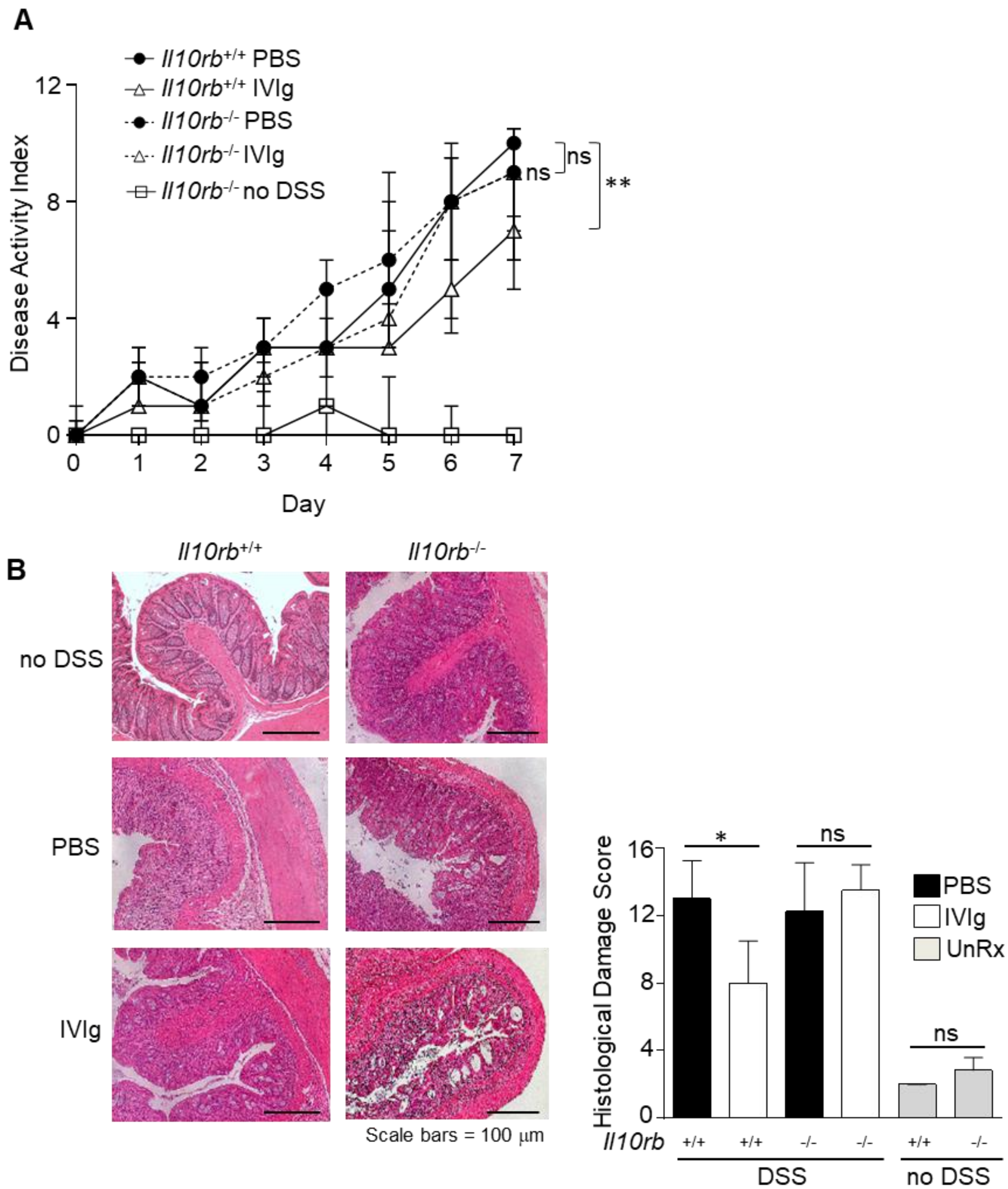

Figure 3.5 Amelioration of DSS-induced colitis by IVIg is dependent on the IL-10 receptor β chain. *Il10rb*$^{+/+}$ and *Il10rb*$^{-/-}$ mice on a C57BL/6 background were given 2.5% and 1.5% DSS, respectively, in their drinking water for 7 days. *Il10rb*$^{+/+}$ and *Il10rb*$^{-/-}$ mice were also given no DSS or treatment, as a control. Mice receiving DSS were given PBS (injection control) or IVIg (1 g/kg body weight) intraperitoneally on days 0, 2, 4, and 6. (A) DAIs were measured daily and are presented as median ± interquartile range. (B) Representative H&E-stained colon cross-sections are shown, and histological damage was scored and is expressed as median ± interquartile range. Scale bars = 100 μm. Data from (A) are *n* = 7-9 mice/group and from (B) are *n* = 5-9 mice/group from 3 independent experiments. *p < 0.05, **p < 0.01, and ns = not statistically different for the comparisons indicated using a two-way ANOVA with Tukey's multiple comparisons test in (A) and a one-way ANOVA with Sidak's multiple comparisons test in (B).

3.3.5 Colonic explants from mice treated with IVIg produce more IL-10 and less IL-12/23p40 and IL-1β, which is dependent on IL-10 receptor signalling

Since I showed that IVIg-mediated amelioration of DSS-induced colitis is IL-10-dependent, I next asked whether IL-10 production was higher and pro-inflammatory cytokine production was lower in IVIg-treated murine colons, and whether changes were dependent on IL-10 receptor β chain signalling. *Il10rb*$^{+/+}$ and *Il10rb*$^{-/-}$ C57BL/6 mice were given 2.5% and 1.5% DSS in their drinking water for 6 days. Mice given DSS were injected intraperitoneally with PBS or IVIg on days 0, 2, 4, and 6 and sacrificed on day 6, 1.5 h post injection. Colon sections were excised and cultured for 24 h. IL-10 production was 3-fold higher in IVIg-treated wild type mice, compared to PBS-treated control mice (Figure 3.6A). Pro-inflammatory cytokine production was reduced by IVIg treatment in the *Il10rb*$^{+/+}$ mice (Figure 3.6B). IL-12/23p40 and IL-1β production were significantly lower in IVIg-treated mice, whereas TNF was not statistically different (27% lower; $p = 0.126$; Figure 3.6B). In *Il10rb*$^{-/-}$ mice, IL-10 production was 2-fold higher in IVIg-treated mice compared to PBS-treated mice, although not statistically different ($p = 0.269$; Figure 3.6C). In contrast to *Il10rb*$^{+/+}$ mice, production of the pro-inflammatory cytokines IL-12/23p40, IL-1β, and TNF were not lower in *Il10rb*$^{-/-}$ mice treated with IVIg compared to PBS (Figure 3.6D). Taken together, these results indicate that IL-10 production is higher and pro-inflammatory cytokine production is lower in mice treated with IVIg during DSS-colitis, and that IL-10 signalling contributes to reduced pro-inflammatory cytokine production.

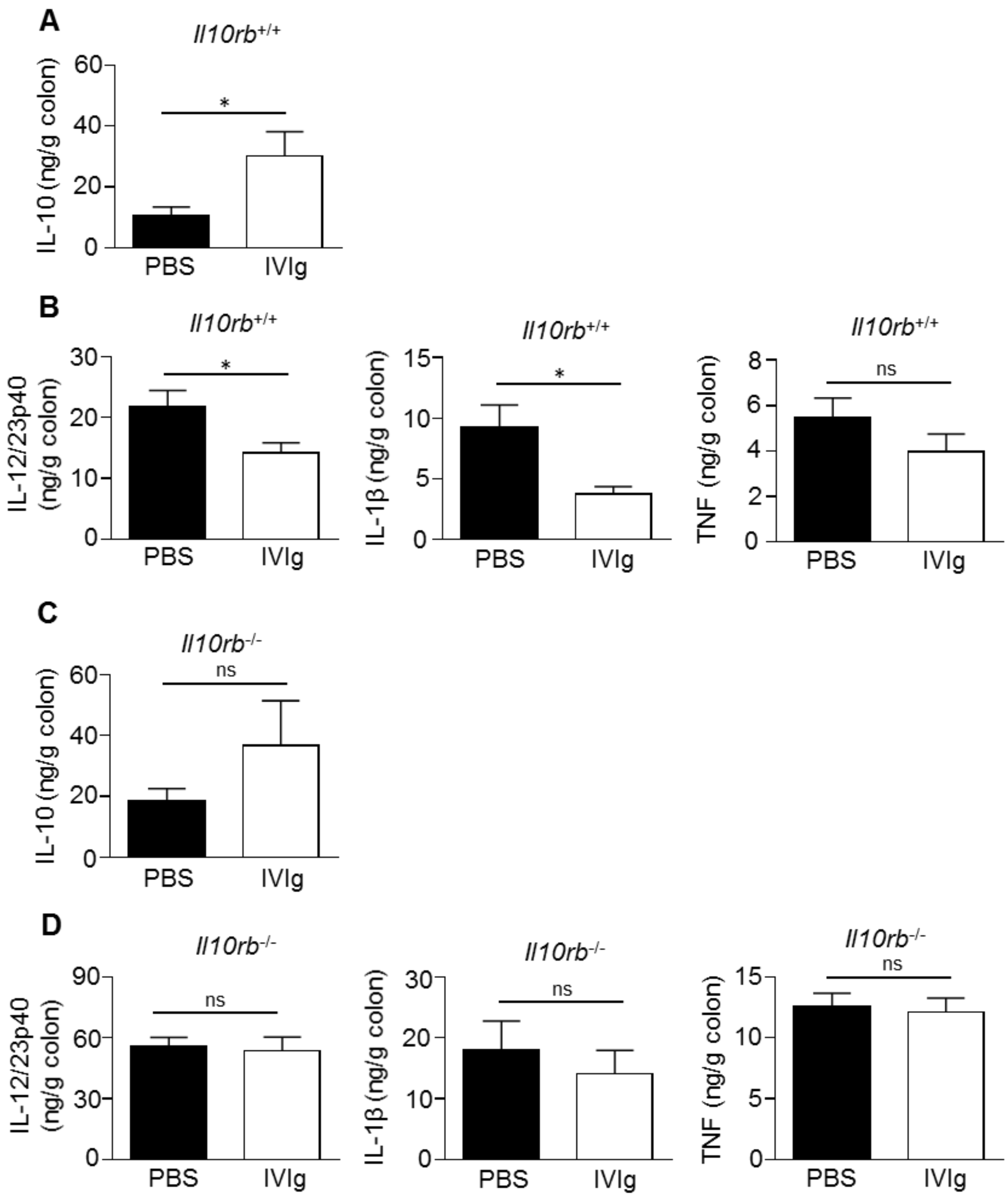

Figure 3.6 Colon explants from mice treated with IVIg during DSS-induced colitis have higher IL-10 production and reduced IL-12/23p40 and IL-1β production, which is dependent on IL-10 receptor β chain signalling. *Il10rb*$^{+/+}$ and *Il10rb*$^{-/-}$ C57BL/6 mice were given 2.5% and 1.5% DSS respectively in their drinking water for 6 days. Mice given DSS were treated with PBS or IVIg (1 g/kg body weight) intraperitoneally on day 0, 2, 4, and 6 and euthanized on day 6, 1.5 h post injection. Colon sections were excised and cultured for 24 h. ELISAs were performed to detect IL-10 (A and C), IL-12/23p40, IL-1β, and TNF (B and D). Data are expressed as mean ± SEM from n = 16 mice/*Il10rb*$^{+/+}$ group (A and B) and n = 10 mice/*Il10rb*$^{-/-}$ group (C and D) from 5 independent experiments. *p < 0.05 and ns = not statistically different for the comparisons indicated using a two-tailed Student's *t*-test with Welch's correction.

3.3.6 Macrophages are the source of IL-10 in IVIg-treated murine colons during DSS-induced colitis

Since we have published that *ex vivo* isolated peritoneal macrophages from mice treated with IVIg produce high amounts of IL-10, I asked whether macrophages were the source of IL-10 in IVIg-treated mice during DSS-induced colitis[322]. *Il10*$^{\text{egfp}}$*Foxp3*$^{\text{mrfp}}$ C57BL/6 mice were given 2.5% DSS in their drinking water for 7 days. Mice were given PBS or IVIg intraperitoneally on days 0, 2, 4, and 6 during DSS treatment. IVIg-treated mice had 2.3-times more GFP^{+} cells per field compared to PBS-treated mice, which indicates that IVIg treatment promotes higher numbers of IL-10 producing cells within the colon (Figure 3.7A). Next, C57BL/6 mice were given 2.5% DSS in their drinking water for 6 days. Mice were injected intraperitoneally with PBS or IVIg on days 0, 2, 4, and 6 during DSS treatment and sacrificed 1.5 h post injection on day 6. Colons were excised, and sections were taken for histological analyses. Colonic sections were probed for *Emr1* mRNA (encoding F4/80; red) and *Il10* mRNA (teal/green) using RNAScope. Representative probed colonic sections are shown (Figure 3.7B). The number of *Emr1*$^{+}$*Il10*$^{+}$ cells was 1.5-fold higher in IVIg-treated mice, compared to the PBS-treated control mice. The majority of *Il10* transcripts, 85%, were found within *Emr1*$^{+}$ cells in IVIg-treated mice. The amount of *Il10* staining was also higher per *Emr1*$^{+}$ cell in the IVIg-treated mice compare to the PBS control mice, which indicates a higher number of *Il10* transcripts present in the F4/80-expressing cells. This suggests that IVIg-treated mice have higher numbers of IL-10 producing macrophages than control mice, and that these macrophages produce more IL-10 than the macrophages within control mice. Finally, *Il10*$^{\text{egfp}}$ C57BL/6 mice were given 2.5% DSS in their drinking water for 7 days. Mice were injected intraperitoneally with PBS or IVIg on days 0, 2, 4, and 6 during treatment. GFP content was significantly higher in colonic CD11b$^+$ cells that were isolated from IVIg-treated *Il10*^{egfp} mice, (52% higher; Figure 3.7C) indicating that IL-10 production by macrophages is higher. Taken together, these results demonstrate that colonic macrophages are the source of IL-10 in IVIg-treated mice during DSS-induced colitis.

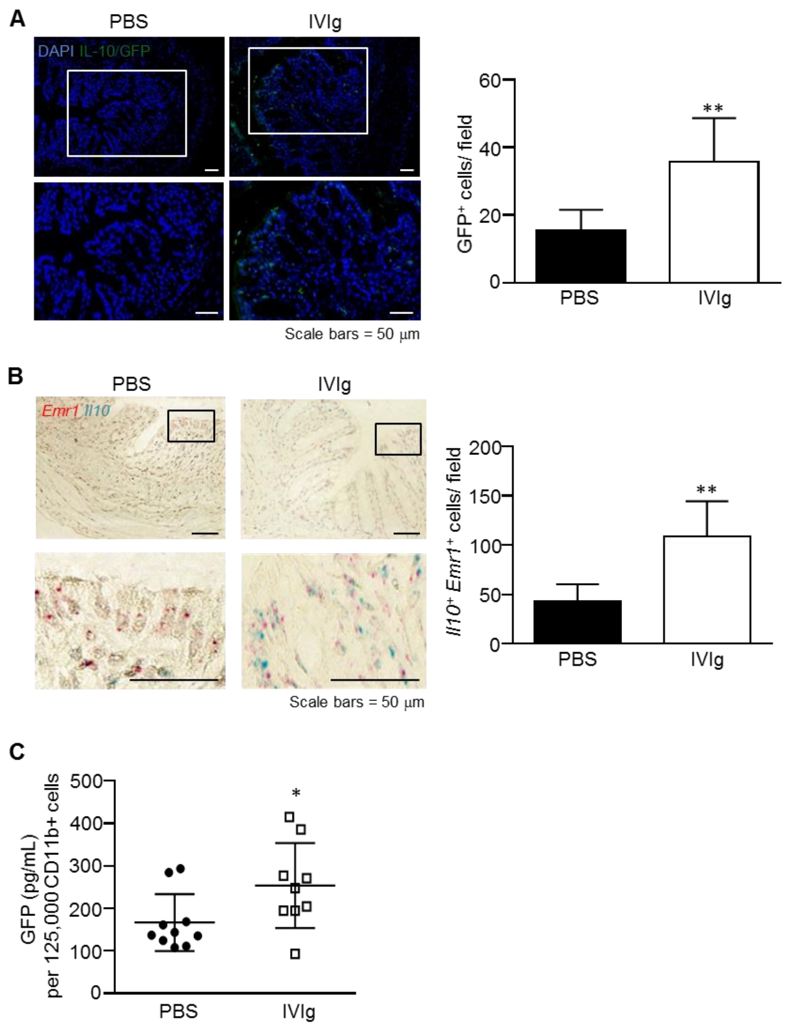

Figure 3.7 Macrophages are the source of IL-10 in IVIg-treated murine colons. *Il10*^{egfp}*Foxp3*^{mrfp} C57BL/6 mice were given 2.5% DSS in their drinking water for 7 days. Mice were given PBS or IVIg (1 g/kg body weight) intraperitoneally on day 0, 2, 4, and 6 during DSS treatment. (A) Colon sections were stained for DAPI (blue) and DAPI/GFP$^+$ cells were quantified from 3 independent experiments (n = 6-7 mice/group) at 20× magnification in six fields from six colonic sections/mouse, separated by ≥ 50 μM. Scale bars = 50 μm. C57BL/6 mice were given 2.5% DSS in their drinking water for 6 days. Mice were given PBS or IVIg (1g/kg body weight) intraperitoneally on day 0, 2, 4, and 6 during treatment and euthanized on day 6, 1.5 h post injection. (B) Colonic sections were probed for *Emr1* (F4/80) and *Il10* (IL-10) mRNA in red and teal/green respectively, using RNAScope. *Emr1*$^+$*Il10*$^+$ cells were quantified from 3 independent experiments, n = 5-6 mice/group at 40× magnification from six fields from six colonic sections/mouse, separated by ≥ 50 μM. (A and B) Scale bars = 50 μm. *Il10*^{egfp}*Foxp3*^{mrfp} C57BL/6 mice were given 2.5% DSS in their drinking water for 7 days. Mice were given PBS or IVIg (1 g/kg body weight) intraperitoneally on day 0, 2, 4, and 6 during DSS treatment. (C) Colonic CD11b$^+$ cells were isolated and GFP content was quantified by ELISA from 4 independent experiments, n = 9-10 mice/group. *$p < 0.05$ and **$p < 0.01$ using a two-tailed Student's t-test to compare PBS with IVIg treatment.

3.3.7 Amelioration of DSS-induced colitis by IVIg is dependent on macrophage IL-10 production

To determine whether IVIg-mediated attenuation of DSS-induced colitis was dependent on macrophage IL-10 production, I performed experiments in mice deficient in IL-10 expression specifically in the myeloid compartment. *LysMCre*$^{+/-}$*Il10*$^{flox/flox}$ and *LysMCre* $^{-/-}$*Il10*$^{flox/flox}$ (wild type control mice) on a C57BL/6 background were given 2.5% DSS in their drinking water for 7 days. Untreated *LysMCre*$^{+/-}$*Il10*$^{flox/flox}$ and *LysMCre* $^{-/-}$*Il10*$^{flox/flox}$ mice were also monitored during the course of the experiment to ensure that myeloid-specific IL-10 deficient mice did not develop spontaneous disease. Mice receiving DSS were injected intraperitoneally with PBS, as an injection control, or IVIg on days 0, 2, 4, and 6. *LysMCre*$^{+/-}$*Il10*$^{flox/flox}$ and *Il10*$^{flox/flox}$*LysMCre*$^{-/-}$ mice that were not given DSS did not develop spontaneous intestinal inflammation (*Il10*$^{flox/flox}$*LysMCre*$^{+/-}$ mice are shown in Figure 3.8A; data not shown for *Il10*$^{flox/flox}$*LysMCre*$^{-/-}$ mice). *Il10*$^{flox/flox}$*LysMCre*$^{+/-}$ and *Il10*$^{flox/flox}$*LysMCre* $^{-/-}$ mice were similarly sensitive to DSS; DAIs of PBS-injected mice did not differ significantly (Figure 3.8A). *Il10*$^{flox/flox}$*LysMCre* $^{-/-}$ (wild type) mice treated with IVIg had significantly lower DAIs compared to the *Il10*$^{flox/flox}$*LysMCre* $^{-/-}$ PBS-treated mice (Figure 3.8A). In contrast, IVIg treatment did not improve DAIs in *Il10*$^{flox/flox}$*LysMCre*$^{+/-}$ mice compared to PBS treatment (Figure 3.8A). At day 7, colons were excised, and sections were stained with H&E for histological analyses. Representative H&E-stained colonic cross-sections from mice in each treatment group are shown (Figure 3.8B). *Il10*$^{flox/flox}$*LysMCre*$^{-/-}$ and *Il10*$^{flox/flox}$*LysMCre*$^{+/-}$ mice that were not given DSS did not have signs of histological disease. Histological damage scores were significantly lower in IVIg-treated *Il10*$^{flox/flox}$*LysMCre* $^{-/-}$ mice compared to PBS-treated mice (Figure 3.8B). *Il10*$^{flox/flox}$*LysMCre*$^{+/-}$ IVIg-treated mice, however, did not have improved histological damage scores compared to their

PBS-treated counterparts. These results demonstrate that myeloid cell-derived IL-10 production limits DSS-induced colitis in IVIg-treated mice.

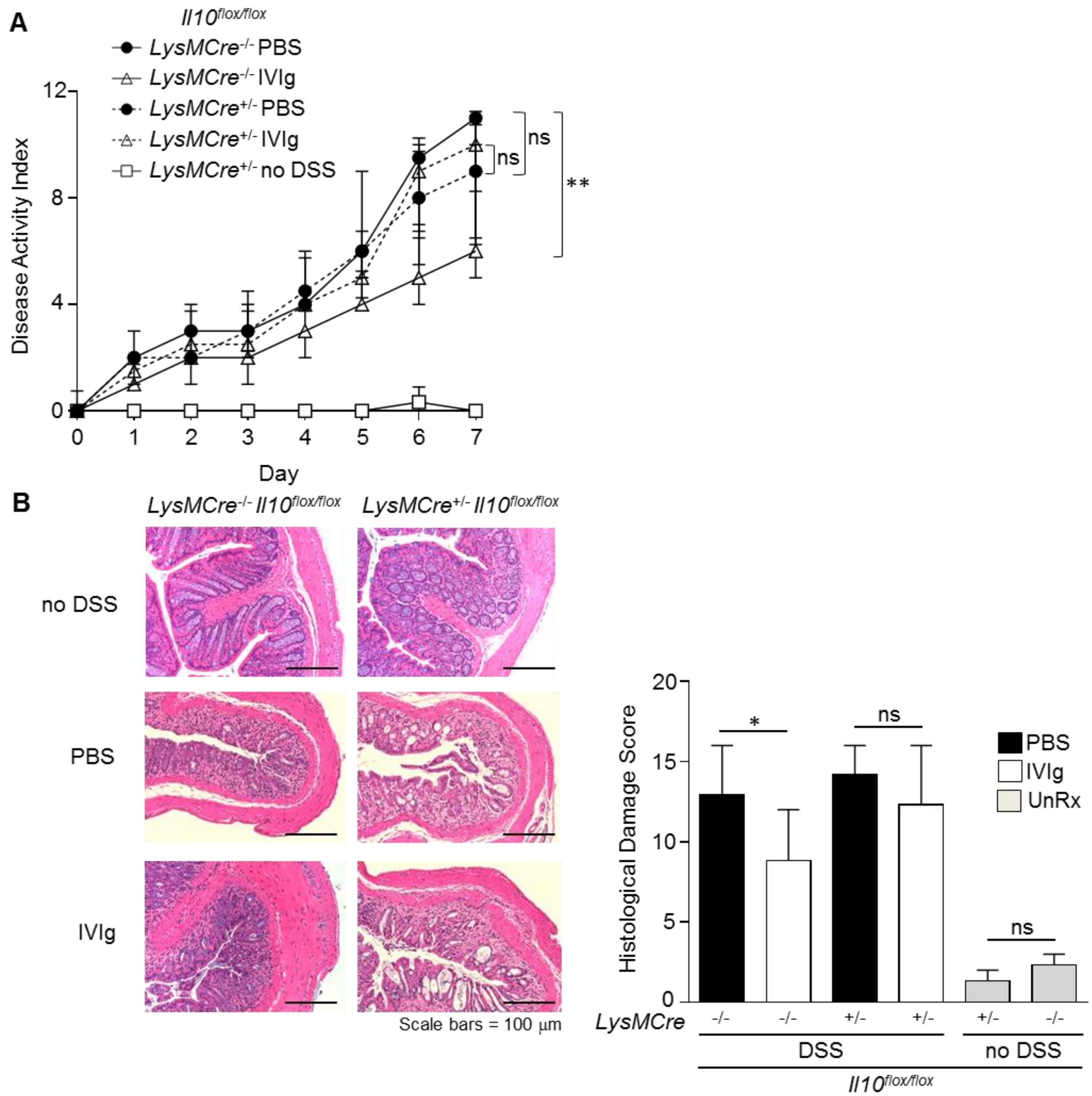

Figure 3.8 Amelioration of DSS-induced colitis by IVIg is dependent on myeloid-derived IL-10 production. *Il10*$^{flox/flox}$*LysMCre*$^{+/-}$ and *Il10*$^{flox/flox}$*LysMCre*$^{-/-}$ (wild type control) mice on a C57BL/6 background were given 2.5% DSS in their drinking water for 7 days. *Il10*$^{flox/flox}$*LysMCre*$^{+/-}$ and *Il10*$^{flox/flox}$*LysMCre*$^{-/-}$ mice were also given no DSS and were untreated (UnRx), as a control. Mice receiving DSS were given PBS (injection control) or IVIg (1 g/kg body weight) intraperitoneally on day 0, 2, 4, and 6. (A) DAIs were measured daily and are presented as median ± interquartile range. (B) Representative H&E-stained colons are shown and histological damage was scored and is expressed as median ± interquartile range. Scale bars = 100 µm. Data from (A) are *n* = 7-10 mice/group and from (B) are *n* = 7-10 mice/group from 3 independent experiments. *$p < 0.05$, **$p < 0.001$, and ns = not statistically different for the comparisons indicated using a two-way ANOVA with Tukey's multiple comparisons test in (A) and a one-way ANOVA with Sidak's multiple comparisons test in (B).

3.4 Discussion

I report a novel *in vivo* mechanism of IVIg-mediated immunosuppression. Adoptive transfer of M(IVIg + LPS) reduces intestinal inflammation during DSS-induced colitis and amelioration of inflammation is IL-10-dependent. IVIg treatment also reduces intestinal inflammation in DSS-treated mice, which correlates with reduced pro-inflammatory cytokine production by colon explants, and is dependent on IL-10 receptor β chain signalling. Moreover, I demonstrate that macrophages are the source of IL-10 production in IVIg-treated mice during DSS-induced colitis and that IVIg-mediated protection is reduced in mice with IL-10 deficiency in myeloid cells. Taken together, my data suggests that IVIg treatment can reduce intestinal inflammation by inducing macrophages to produce large amounts of anti-inflammatory IL-10.

IL-10 has tremendous potential for the treatment of IBD despite lack of success in early clinical trials[61, 277]. I have found that IVIg induces IL-10 production *in vivo*, which limits intestinal inflammation. I have also shown that IVIg reduces pro-inflammatory IL-12/23p40 and IL-1β production in colonic explant cultures *ex vivo*, through IL-10 signalling. IL-10 inhibits antigen presentation and the production of chemokines and inflammatory cytokines, such as IL-1β, IL-12, IL-23, and TNF[59, 61, 62]. IL-12 potently drives $CD4^+$ T cells into Th1 cells and induces IFNγ production, whereas IL-23 enhances the expansion of Th17 cells[14]. IL-1β drives accumulation of granulocytes and $CD4^+$ T cells and is a reliable marker of active inflammation[200, 324, 325]. IL-10 administered by gelatin microspheres reduces spontaneous colitis in IL-10 deficient mice and this correlates with reduced gene expression for *Il1B* and *Tnf* [326]. Similarly, in human models, IL-10 is constitutively produced by healthy human colonic explants, and depletion of IL-10 results in higher production of LPS-induced IFNγ, TNF, and IL-17, as well as increased epithelial damage and crypt loss[327]. In people with CD, IL-12, IL-23, and IL-1β are higher in the inflamed mucosa and serum compared to healthy controls[328, 329]. IL-10 may be important to reduce inflammatory mediators and initiate remission in people with IBD. Indeed, IL-10 is high in the serum of people with UC and CD during the initial phase of remission, whereas pro-inflammatory markers, C-reactive protein and IL-6, are high during acute inflammation and return to normal levels during remission[273]. Limiting the production of multiple pro-inflammatory cytokines, through IL-10 production, could provide a more efficacious treatment than targeting individual cytokines to limit inflammation in IBD.

My data suggests that macrophages are the source of protective IL-10 during IVIg treatment. Inducing IL-10 production by macrophages could be a useful strategy to treat IBD. Evidence from a variety of models of inflammation supports this theory. We have reported that (IVIg + LPS)-challenged peritoneal macrophages produce high levels of IL-10 and low levels of IL-12/23p40 *ex vivo*[322]. In an induced brain inflammation model in mice, microglial IL-10 production limits IL-12 and IL-1β production, via TLR4 and FcγRI activation *in vivo,* similar to murine M(Ic + LPS) and M(IVIg + LPS)[40]. In peripheral nerve injury in mice, macrophage IL-10 production potently stops inflammation, to allow the wound healing response to occur[330]. In an intestinal wounding model in mice, macrophage-derived IL-10 stops the inflammatory response, allowing for mucosal epithelial repair by WNT1 inducible signalling pathway-1 (WISP-1)[331]. Finally, macrophage IL-10 limits intestinal inflammation through the inhibition of IL-23 production in a *Citrobacter rodentium* infection model in mice[22]. Evidence in humans shows that inducing anti-inflammatory macrophages *in vivo* is beneficial for the treatment of IBD. Higher numbers of "regulatory macrophages", which produce high amounts of IL-10 in response to LPS *in vitro,* are found in the intestinal sections of people with IBD, who are infliximab responders, versus non-responders[35, 332]. Infliximab antibodies induce IL-10 production by human macrophages in an Fc-dependent manner *in vitro*, similar to IVIg[35]. Since IL-10 production by macrophages limits inflammation during disease, I suggest that IVIg-treated macrophage cell therapy or IVIg infusion could be effective treatments for IBD.

IVIg-treated macrophages could be a beneficial IL-10-based cellular therapeutic for IBD. Similar to what I have found with IVIg-treated macrophages in DSS-induced colitis, adoptive transfer of IVIg-treated CD11c^{+} DCs ameliorates ITP symptoms in mice and adoptively transferred M(Ic + LPS) alleviate the symptoms of endotoxin challenge and prevent death in mice, which is dependent on IL-10[302, 333]. Finally, IL-10 produced by adoptively transferred myeloid-derived suppressor cells attenuates inflammation in a collagen-induced arthritis model in mice by inducing Tregs and preventing T cell proliferation[334].

IVIg infusion could also be a beneficial therapeutic option for IBD. In mice, IVIg reduces symptoms of inflammation in models of RA, intracerebral hemorrhage, and ITP[129, 131, 178, 302, 335]. Although there are no studies using IVIg in intestinal inflammation models in mice, high doses of rat IgGs improve symptoms of DSS colitis in rats and this correlates with reduced immune cell recruitment[336]. There is also evidence in humans *in vitro* and *in vivo* that IVIg can induce IL-10 and reduce pro-inflammatory cytokine production. In human dendritic cells from healthy control participants, stimulation with IVIg and LPS causes higher IL-10 production and lower IL-12 production compared to LPS stimulation[337]. Importantly, *in vivo* IL-10 levels are higher in the serum and IL-10 production is higher by PBMCs from people with ITP after IVIg treatment compared to before treatment[338, 339]. IVIg treatment reduces serum TNF and IL-1β in people with GBS, although IL-10 levels were not measured[13]. In a retrospective chart review of people with medically refractory IBD, IVIg treatment improved clinical disease scores in people[340].

My study shows that IVIg ameliorates DSS-induced colitis symptoms through macrophage production of anti-inflammatory IL-10. This study provides a unifying mechanism explaining IVIg’s efficacy in a diverse set of diseases, as IL-10 has an important role in limiting innate and adaptive immune responses. Targeting macrophage activation with IVIg could be rapidly translated into therapy for IBD, as it is already licensed for use in people. Alternatively, cell therapy with IVIg-activated macrophages could provide a promising therapeutic strategy. Clinical trials should be performed to determine if IVIg is an effective treatment for IBD.

Chapter 4: IVIg induces IL-10 production by human monocytes, which is compromised by an FcγRIIA disease-associated gene variant

4.1 Introduction and rationale

Defects in macrophage function characterize many inflammatory diseases, which make macrophages key therapeutic targets[1]. Macrophages are well known for initiating inflammatory responses during infection or tissue injury, and directing the acquired immune response[1]. Macrophages also play an important role in suppressing the inflammatory response and resolving inflammation[3]. There are key differences between murine and human macrophages. For example, human M(IFNγ + LPS) and M(IL-4) do not produce NO or express Arg1, respectively, which are key features for these populations in murine macrophages[19]. Thus, it is critical that observations made in murine macrophages are confirmed in human monocytes/macrophages to translate murine work to human diseases.

A lesser studied but essential function of macrophages is to actively suppress inflammatory responses after insult or injury, and prior to the initiation of wound healing. Murine and human macrophages can adopt an anti-inflammatory activation state in which they secrete high amounts of IL-10, in response to stimuli that are normally pro-inflammatory[1]. The best characterized example of this activation state are murine M(Ic + LPS)[1, 333]. Human M(Ic + LPS) have been less thoroughly described; they have been shown to increase TLR-induced IL-10 production, but, unlike murine M(Ic + LPS), human M(Ic + LPS) do not decrease IL-12 production[29, 30].

The mechanism(s) of action of IVIg is not well understood[308]. A minor fraction of Fc portions of IgGs in IVIg are sialylated, which may be responsible for its efficacy, although evidence from human studies does not support this theory[167, 298].

The FcγRIIA has a relatively low affinity for IgG antibodies, and is found on the surface of myeloid cells and platelets[105]. A gene variant for FcγRIIA (rs1801274) exists, which can change the receptor from a relatively low affinity to high affinity for binding IgG antibodies. The low affinity gene variant for FcγRIIA-R131, or CC genotype, has an arginine at amino acid at position 131 that confers a low binding affinity for IgG1 and IgG3; whereas the FcγRIIA-H131, or TT genotype, has a histidine substituted at amino acid 131, which confers a higher binding affinity for IgG1 and IgG3, and confers binding affinity for IgG2 that is not present in the CC genotype[145-147, 341]. The disease-associated gene variant (TT) has been associated with a higher risk of inflammatory diseases in people, including UC and KD, compared to the non-risk gene variant (CC)[149-151]. It is also associated with a higher risk of failure to respond to therapy with the anti-TNFα antibody, infliximab, in people with RA[341]. Few *in vitro* mechanistic studies have been performed to explain the higher risk of disease and failure to respond to antibody-based therapies in people with the risk variant. PBMCs from people with the risk variant have higher IgG2-induced IL-1β production and neutrophils from people with the risk variant have higher levels of phagocytosis and degranulation in response to serum opsonized bacteria[159, 160].

I have found that murine bone marrow-derived and peritoneal macrophages can be activated to produce high levels of IL-10 and low levels of IL-12/23p40, similar to M(Ic + LPS), *in vitro* and *in vivo*, when co-treated with IVIg and LPS[322]. However, it is important to determine whether IVIg can induce a similar activation state in human monocytes or macrophages, since there are differences in Fcγ receptors and activations states between mice and humans. Based on this, I asked whether human monocytes have high IL-10 and low pro-inflammatory cytokine production when stimulated with IVIg + LPS, similar to murine macrophages. I also asked whether monocytes from people with the FcγRIIA risk variant have compromised ability to induce anti-inflammatory IL-10-producing monocytes in response to IVIg, which could explain the higher risk of inflammatory diseases and failure to respond to infliximab that is associated with the risk variant.

4.2 Materials and methods

Cell isolation and isolation of monocytes. PBMCs were isolated from healthy control blood by density gradient centrifugation, using Lymphoprep (StemCell Technologies, Vancouver, BC, Canada). Cells were washed and suspended in RPMI supplemented with 10% autologous serum and penicillin/streptomycin at a density of 2.0×10^6 cells /mL for 1.5 h. Non-adherent cells were washed away and adherent monocytes were re-plated at a density of 2.5×10^5 cells/mL for 24 h before use in assays.

Cell stimulations. Cells were plated at a density of 2.5×10^5 cells/mL (100 μL/well in a 96-well plates), and were left unstimulated or stimulated with 100 ng/mL LPS (*Escherichia coli* serotype 127:B8; Sigma-Aldrich, St. Louis, MO, USA), 5 mg/mL of IVIg (Gamunex Immune Globulin Intravenous 10% solution for infusion; Transfusion Medicine, BC Children's Hospital, Vancouver, BC, Canada), or both IVIg + LPS. After incubation, cell supernatants were harvested and clarified by centrifugation for analyses. For Fcγ receptor blocking experiments, antibodies were added 1 h prior to stimulations, at final concentrations of: IgG isotype control antibody (50 or 100 μg/mL; AB-108-C, R & D Biosystems, Minneapolis, MN, USA), FcγRI blocking antibody (100 μg/mL, AF 1257, R & D Biosystems), FcγRIIB/C blocking antibody (100 μg/mL, AF 1330, R & D Biosystems), and FcγRIII blocking antibody (50 μg/mL, AF 1597, R & D Biosystems). For IL-10 receptor blocking experiments, antibodies were added 1 h prior to stimulations, at final concentrations of 5 μg/mL for both the IgG isotype control antibody (clone RTK2758 BioLegend, San Diego, CA, USA) and IL-10 receptor blocking antibody (clone 3F9 BioLegend). For inhibitor studies, inhibitors were added 1 h prior to stimulations, at final concentrations of: DMSO (vehicle control; 0.1%), PD (50 μM, Cell Signaling Technology, Danvers, MA, USA), SCH (1 μM, MedChem Express, Princeton, NJ, USA), SB (10 μM, Cell Signaling Technology), or BIR (180 nM, Cayman Chemical, Ann Arbor, MI, USA),

Cytokine measurements. Cytokines were assayed by ELISA, according to the manufacturer's instructions. ELISA kits for human IL-10, IL-12/23p40, IL-6, and TNF were from BD Biosciences (Mississauga, ON, Canada).

SDS-PAGE and western blotting. Monocytes were stimulated for 0, 10, 40, or 120 min, as indicated. After stimulation, monocytes were placed on ice and rinsed twice with cold PBS. Whole cell lysates were prepared for SDS-PAGE by lysing in 1× Laemmli's digestion mix, DNA was sheered using a 26-guage needle, and samples were boiled for 1 min. Cell lysates were separated on a 10% polyacrylamide gel and western blotting was carried out, as described previously[300]. Antibodies used for western blot analyses for MAPK activation experiments were anti-pERK1/2 (Cell Signaling Technology, 9106), anti-pp38 (Cell Signaling Technology, 4631), and anti-GAPDH (Fitzgerald Industries International, 10R-G109a, Acton, MA, USA).

Antibodies used for western blot analyses for siRNA experiments were anti-FcγRI (Abcam, ab119843, Cambridge, UK), anti-FcγRIIB (Abcam, ab151497), anti-FcγRIII (Abcam, ab94773), anti-FcγRIIA (Abcam, ab167381), and anti-β-actin (Cell Signaling Technology, 4970). Densitometry was performed using ImageJ software (National Institute of Health, Bethesda, MA, USA).

Fcγ receptor siRNA. Monocytes were untreated (UnRx) for 48 h or pre-treated for 48 h with siRNAs using Lipofectamine RNAiMAX reagent (Thermo Fischer, Waltham, MA, USA) with 10 nM of a non-silencing small interfering RNA (siRNA) (ns; silencer select negative control siRNA #1,Thermo Fischer) or 2 different silencer select siRNAs (si1 or si2) to the FcγRI (s5069 and s5070, Thermo Fischer), FcγRIIA (s194408 and s223525, Thermo Fischer), FcγRIIB (s5073 and s5075, Thermo Fischer), or FcγRIIIA (s57398 and s223526, Thermo Fischer). Cells were harvested for western blot analyses or stimulated, and cell free supernatants were assayed for cytokines, as described above.

Genotyping the *FCGR2A* inflammatory disease susceptibility SNP, rs1801274. Blood samples were frozen at -20°C, and DNA was extracted using a commercially available kit, according to manufacturer's instructions (QIAGEN, Hilden, Germany). DNA was used to genotype the *FCGR2A* SNP, rs1801274, using a commercially available Taqman assay C_9077561_20; Thermo Fischer). SNPs were considered acceptable for analysis if they had call rates > 95% and frequencies did not deviate from Hardy-Weinberg equilibrium ($p < 0.05$). Analyses were performed on participants before stratification by genotype.

Statistical analyses. Parametric or non-parametric unpaired or paired two-tailed *t*-tests or repeated measures one-way ANOVAs with Dunn's multiple comparisons correction were used, where indicated. Graphpad prism software version 6.03 was used for analyses. Differences were considered significant at $p < 0.05$.

Human ethics approval. All experimental procedures were performed in accordance with ethical guidelines and approved by the University of British Columbia research ethics board (H13-03524 and H14-00622). All participants provided informed, written consent for blood collection for immune cell isolation and functional assays, DNA isolation, and genotyping.

4.3 Results

4.3.1 IVIg increases IL-10 production and reduces pro-inflammatory cytokine production by human monocytes stimulated with LPS

We have previously reported that IVIg-activated murine BMDMs produce high levels of the anti-inflammatory cytokine, IL-10, and low levels of pro-inflammatory cytokines in response to the inflammatory stimulus, LPS[322]. Herein, I asked whether IVIg-activated human monocytes also produce more IL-10 and less pro-inflammatory cytokines in response to LPS. Peripheral blood monocytes from healthy control participants were unstimulated (Control (C)) or stimulated with LPS, IVIg, or IVIg + LPS. Unstimulated or IVIg stimulated monocytes did not produce IL-10 (Figure 3.1A). LPS treatment induced modest amounts of IL-10 production, whereas IVIg + LPS treatment caused a 69% increase in IL-10 production (Figure 4.1A). Unstimulated or IVIg stimulated monocytes did not produce IL-12/23p40, IL-6, or TNF (Figure 4.1B). LPS induced high levels of IL-12/23p40 production, which was reduced by 98% when monocytes were co-stimulated with IVIg (Figure 4.1B). Similarly, IL-6 and TNF were produced by LPS-treated monocytes and their production was significantly reduced, 52% and 62% respectively, by concomitant treatment with IVIg (Figure 4.1B). These results show that IVIg activates human monocytes to produce IL-10 and reduce pro-inflammatory cytokine production in response to LPS.

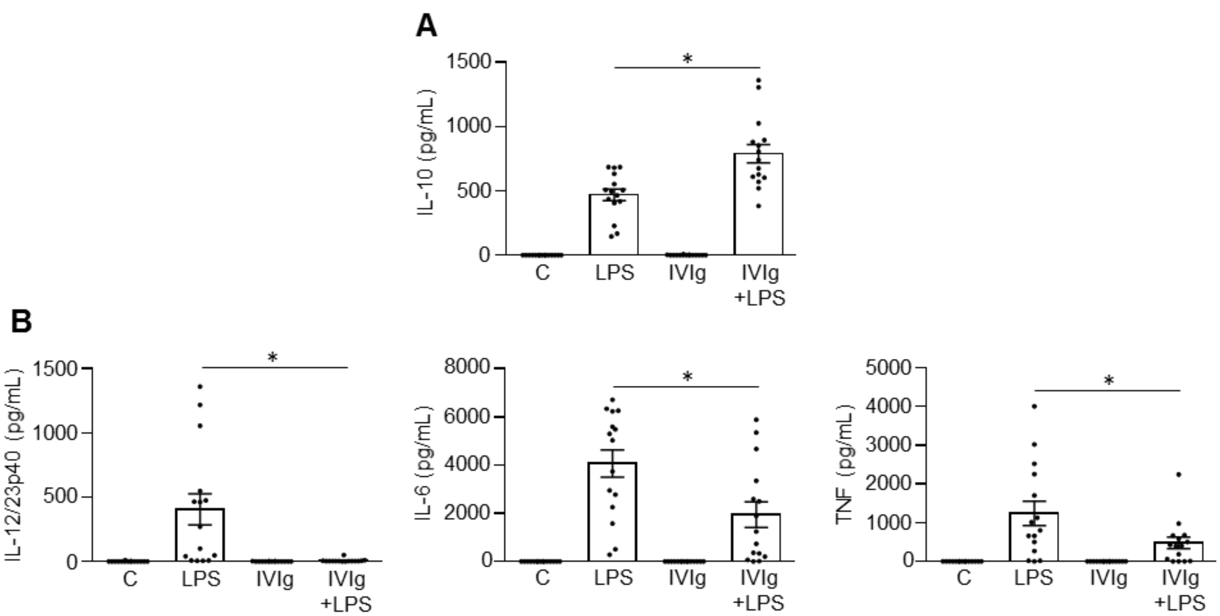

Figure 4.1 IVIg increases IL-10 production and reduces pro-inflammatory cytokine production in LPS-stimulated human monocytes. Monocytes from healthy control participants were unstimulated (Control (C)) or stimulated with LPS (100 ng/mL), IVIg (5 mg/mL), or both, for 24 h. Clarified cell supernatants were assayed for (A) IL-10, (B) IL-12/23p40, IL-6, or TNF by ELISA. Data are mean ± SEM from $n = 16$ participants performed as independent experiments, and assayed in duplicate. *$p < 0.001$ for cells treated with LPS compared to cells treated with IVIg + LPS. Statistical analyses were performed using a non-parametric paired *t*-test.

4.3.2 FcγRI and FcγRIIB are required for IVIg-induced IL-10 production in response to LPS

I next wanted to determine which Fcγ receptor(s) are required for IL-10 production by (IVIg + LPS)-activated monocytes. Monocytes were left untreated or pre-treated with blocking antibody to the FcγRI, FcγRIIB/C, or FcγRIII, or a common isotype control antibody (Figure 4.2A). Monocytes were then stimulated with LPS or IVIg + LPS. LPS-induced IL-10 production was not affected when either the FcγRI, FcγRIIB/C, or FcγRIII were blocked (Figure 4.2A, white bars). However, when stimulated with IVIg + LPS, blocking the FcγRI or FcγRIIB/C significantly decreased IL-10 production relative to the IgG control (82% of IgG control for FcγRI, 73% of IgG control for the FcγRIIB/C; Figure 4.2A). There was no change in (IVIg + LPS)-induced IL-10 production when the FcγRIII was blocked (Figure 4.2A).

To confirm these findings, I used siRNAs specific to the FcγRI, FcγRIIB, or FcγRIIIA, to determine the effect of receptor knockdown on (IVIg + LPS)-induced IL-10 production. Monocytes were untreated or pre-treated with a non-silencing siRNA control (ns) or two siRNAs (si1 and si2) to the FcγRI (Figure 4.2B), FcγRIIB (Figure 4.2C), or FcγRIIIA (Figure 4.2D). Cell lysates were prepared, separated by SDS-PAGE and analyzed by western blotting using antibodies for the FcγRI, FcγRIIB, or FcγRIIIA as well as β-actin, as a loading control. Monocytes were then left unstimulated or stimulated with LPS, IVIg, or IVIg + LPS, and IL-10 production was measured by ELISA. For each FcγR, the UnRx and ns siRNA-treated samples had similar levels of receptor expression. The siRNA 1 and 2 each knocked down the FcγRI by 40% compared to the ns siRNA control (Figure 4.2B, left). (IVIg + LPS)-induced IL-10 production was reduced significantly by siRNA1 and by siRNA2, compared to the ns siRNA control (31% siRNA1, 34% siRNA2; Figure 4.2B, right). The siRNA 1 and 2 knocked down the FcγRIIB receptor by 58% and 60%, respectively, compared to the ns siRNA control (Figure 4.2C, left). Compared to the ns siRNA control, (IVIg + LPS)-induced IL-10 production was reduced significantly by siRNA1 and by siRNA2 to the FcγRIIB (45% siRNA1 and siRNA2; Figure 4.2C, right). Compared to the ns siRNA control, the siRNA 1 and 2 reduced the FcγRIIIA receptor expression by 44% and 51%, respectively (Figure 4.2D, left). However, (IVIg + LPS)-induced IL-10 production was not reduced significantly during FcγRIIIA knockdown with either siRNA1 or siRNA2 ($p = 0.96$ and $p = 0.07$ Figure 4.2D, right). Taken together, these results suggest that the FcγRI and FcγRIIB are important for the induction of IL-10 in response to IVIg.

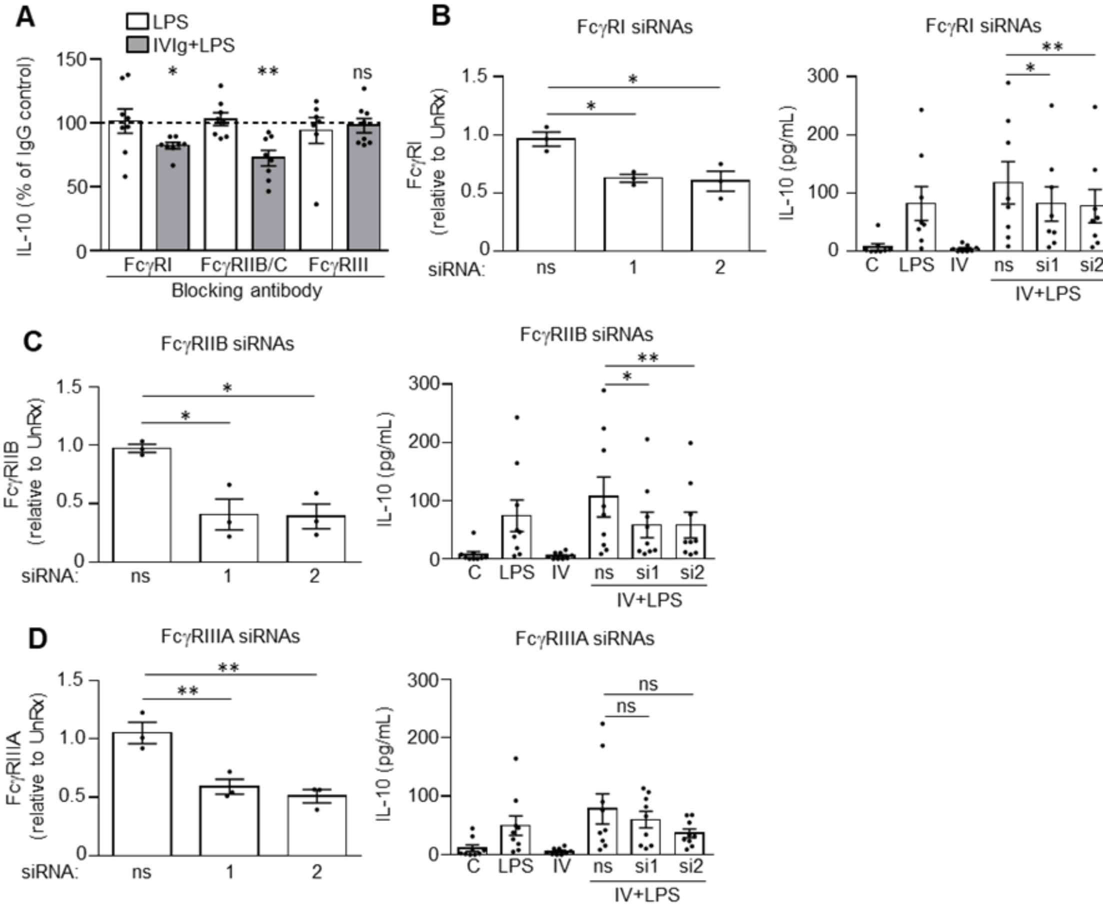

Figure 4.2 The FcγRI and FcγRIIB are required for IVIg-induced IL-10 production in response to LPS. (A) Monocytes were untreated or pre-treated for 1 h with an IgG isotype control (50 or 100 µg/mL) or a blocking antibody against the FcγRI (100 µg/mL), FcγRIIB/C (100 µg/mL), or FcγRIII (50 µg/mL). Cells were stimulated with LPS (100 ng/mL) or (IVIg (5 mg/mL) + LPS (100 ng/mL)) for 24 h. Clarified cell supernatants were assayed for IL-10. Data are mean ± SEM from n = 8 participants performed as independent experiments, and assayed in duplicate. (B-D left) Cells were untreated (UnRx) for 48 h or pre-treated for 48 h with a non-silencing siRNA (ns) or 2 different siRNAs (si1 or si2) to the FcγRI (B), FcγRIIB (C), or FcγRIIIA (D). Cell lysates were prepared, separated by SDS-PAGE and analyzed by western blotting. Densitometry for the FcγRI (B), FcγRIIB (C), or FcγRIIIA (D) are normalized to β-actin and relative to untreated control (UnRx). Densitometry data are mean ± SEM, representative of n = 3 independent experiments. Cells pre-treated with the ns siRNA control were unstimulated (control (C)) or stimulated with LPS (100 ng/mL), IVIg (5 mg/mL), or both, for 24 h, whereas the cells pre-treated with si1 or si2 were stimulated with IVIg (5 mg/mL) + LPS (100 ng/mL). Clarified cell supernatants were assayed for IL-10 (B-D, right). Data are mean ± SEM from n = 9 experiments. (A-D) Monocytes were isolated from 1 participant for each of 3, 8, or 9 independent experiments. (A) Statistical comparisons are for the IgG control to specific FcγR blocking antibody (raw data). *p < 0.05, **p < 0.01 and ns = not statistically different. Statistical analyses were performed using a repeated measures one-way ANOVA with Dunn's multiple comparisons test.

4.3.3 MAPK signalling is required for IVIg-induced IL-10 production in response to LPS in human monocytes

Since murine M(IVIg + LPS) and M(Ic + LPS) require MAPK signalling for IVIg- or Ic-induced IL-10 production in response to LPS, I next asked whether human M(IVIg) required MAPKs for IL-10 production in response to LPS[38, 322]. Monocytes were unstimulated or stimulated with LPS (100 ng/mL), IVIg (5 mg/mL), or both for 0, 10, 40, or 120 min. Cell lysates were prepared at the indicated times, separated by SDS-PAGE, and analyzed by western blotting using phosphospecific antibodies for ERK1/2, p38, and GAPDH, as a loading control (Figure 4.3A). LPS stimulation increased ERK1/2 activation compared to unstimulated cells. pERK1/2 was increased modestly with IVIg alone compared to unstimulated cells. (IVIg + LPS)-activated monocytes had significantly higher and prolonged ERK1/2 activation compared to LPS, with 1.7, 1.6, and 1.8-fold increases at 10, 40, and 120 min, respectively. LPS stimulation increased p38 activation compared to unstimulated cells. IVIg alone had little impact on pp38 levels compared to 0 and 120 min unstimulated controls. (IVIg + LPS induced significantly higher and prolonged p38 activation compared to LPS. (IVIg + LPS)-induced pp38 was increased 1.5, 1.7, and 1.8-fold at 10, 40, and 120 min, respectively, compared to LPS-induced pp38 at each time point.

To investigate the requirement for MAPK signalling on (IVIg + LPS)-induced IL-10 production, I used inhibitors to the MAPKs ERK1/2 and p38. PD inhibits the activation of MEK1, the ERK1/2 kinase and SCH is a novel ERK1/2 inhibitor. PD significantly reduced IL-10 production in response to IVIg + LPS compared to the DMSO (vehicle) control (51%; Figure 4.3B). SCH, which is a more specific ERK1/2 inhibitor, also reduced IL-10 significantly, although to a lesser extent (30%; Figure 4.3B). SB and BIR are potent and selective p38 inhibitors, SB inhibits p38α and p38β, whereas BIR inhibits p38α. Compared to the solvent control (DMSO), both SB and BIR significantly reduced (IVIg + LPS)-induced IL-10 production (38% SB, 35% BIR; Figure 4.3C). These results demonstrate that the MAPKs, p38 and ERK1/2, are required for (IVIg + LPS)-induced IL-10 production.

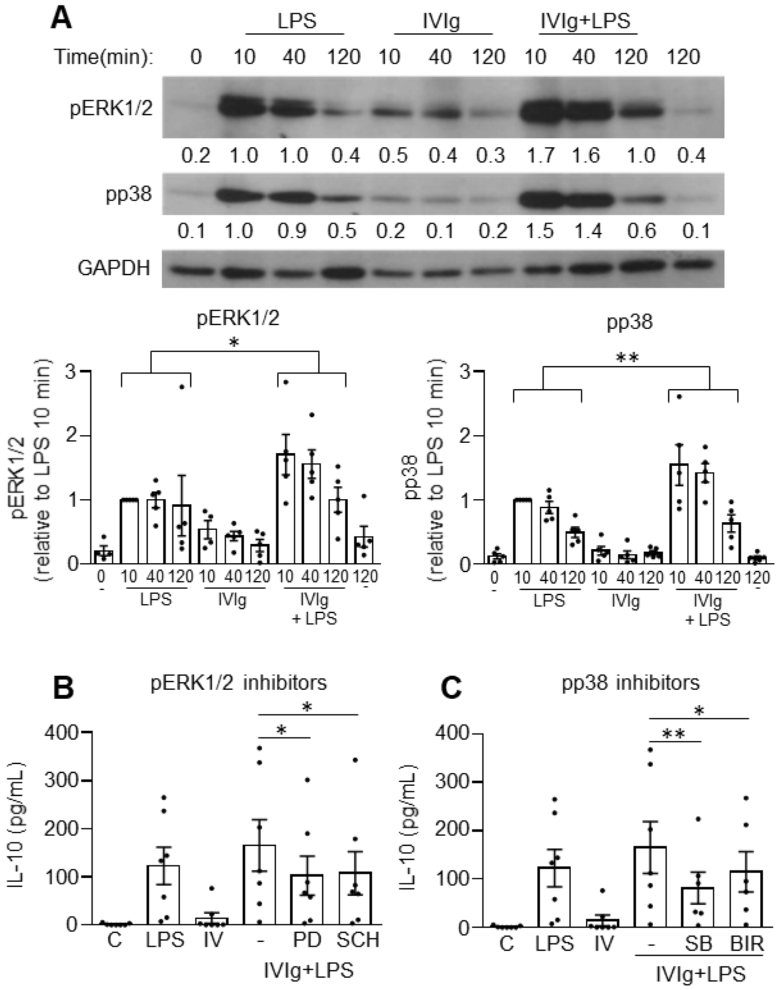

Figure 4.3 MAPKs are required for IVIg-induced IL-10 production in response to LPS. (A) Monocytes from healthy control participants were unstimulated or stimulated with LPS (100 ng/mL), IVIg (5 mg/mL), or both, for 0, 10, 40, or 120 min. Cell lysates (2.5×10^5 cells / time point) were prepared at the indicated times. Lysates were separated by SDS-PAGE and analyzed by western blotting using phosphospecific antibodies for ERK1/2, p38, and GAPDH, as a loading control. Results are representative of *n* = 5 experiments; Cells were isolated from 1 participant for each of 5 independent experiments. Densitometry for pERK1/2 and pp38; normalized to GAPDH and relative to LPS at 10 min; are averaged and shown below each band and values are graphed and reported as mean ± SEM. In (B) and (C), Cells were pre-treated for 1 h with an appropriate volume of DMSO, as a vehicle control, or (B) the ERK1/2 inhibitors, PD and SCH, or (C) the p38 inhibitors, SB and BIR, and then left unstimulated (C) or stimulated with LPS (100 ng/mL), IVIg (5 mg/mL), or IVIg + LPS for 24 h. Clarified cell supernatants were assayed for IL-10 by ELISA. (B and C) Values are reported as mean ± SEM for n = 7 participants performed as independent experiments, assayed in duplicate. *$p < 0.05$, **$p < 0.01$, and ns = not statistically different for the comparisons indicated. Statistical analyses were performed using a two-way ANOVA in (A) and repeated measures one-way ANOVA in (B) and (C) with Dunn's multiple comparisons correction.

4.3.4 IL-10 signalling reduces pro-inflammatory cytokine production by (IVIg + LPS)-activated monocytes

To determine whether IL-10 signalling contributes to lower pro-inflammatory cytokine production by (IVIg + LPS)-activated monocytes, I blocked IL-10 signalling with a blocking antibody against the IL-10 receptor during stimulation. Cells were either left untreated or pre-treated with an IgG isotype control antibody (IgG) or an anti-IL-10 receptor blocking antibody (IL-10R antibody), and stimulated with LPS or IVIg + LPS. IL-10 and pro-inflammatory cytokines produced were measured in cell supernatants by ELISA. For all treatments, the unstimulated control or IVIg alone did not cause cytokine production (data not shown). Monocytes pre-treated with the IL-10 receptor blocking antibody produced significantly more IL-10 when stimulated with LPS or IVIg + LPS compared to antibody control-treated monocytes (94% and 81%, respectively; Figure 4.4A). IL-10 receptor blockade increased pro-inflammatory cytokine production in LPS and (IVIg + LPS)-stimulated monocytes compared to the IgG control. In response to LPS or IVIg +LPS stimulation, respectively, IL-12/23p40 production increased 165% and 278% (Figure 4.4B), IL-6 production increased 62% and 123% (Figure 4.4C), and TNF production increased 80% and 144% (Figure 4.4D). These results suggest that IL-10 production contributes to the reduction of pro-inflammatory cytokines produced in response to IVIg + LPS.

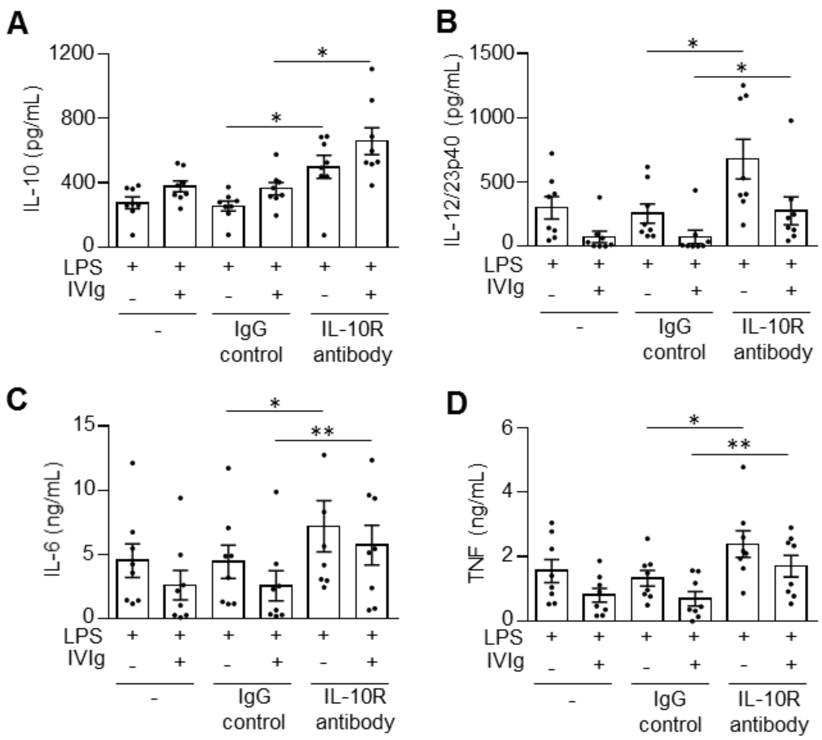

Figure 4.4 IL-10 signalling contributes to reduced LPS-induced pro-inflammatory cytokine production by IVIg-activated monocytes. Monocytes from healthy control participants were left untreated (-) or pre-treated for 1 h with an IgG isotype control (IgG) (5 µg/mL) or an IL-10 receptor (IL-10R) blocking antibody (5 µg/mL). Cells were then stimulated with LPS (100 ng/mL) or (IVIg (5 mg/mL) + LPS (100 ng/mL)) for 24 h. Clarified cell supernatants were assayed for (A) IL-10, (B) IL-12/23p40, (C) IL-6, and (D) TNF. Data are mean ± SEM from *n* = 8 participants performed as independent experiments, assayed in duplicate. *p < 0.05 and **p < 0.01 for the comparisons indicated. Statistical analyses were performed using a repeated measures one-way ANOVA with Dunn's multiple comparisons correction.

4.3.5 IVIg-induced anti-inflammatory macrophage activation is lower in monocytes from people with the high affinity FcγRIIA risk variant

People with the FcγRIIA high affinity gene variant have higher susceptibility to inflammatory diseases, such as ulcerative colitis[149, 150]. People with the risk variant have also been found to perform poorly on the antibody-based drug infliximab, which may work, in part, by activating macrophages to produce IL-10[35, 341]. Based on this, I wanted to investigate whether the FcγRIIA risk variant impacts monocytes anti-inflammatory responses to IVIg + LPS.

Monocytes from healthy control participants were unstimulated or stimulated with LPS, IVIg, or IVIg + LPS. Participants were genotyped for the FcγRIIA H131R polymorphism (rs1801274) and cytokine production was stratified to genotype. People with the CC genotype do not have the disease-associated gene variant, CT genotype are heterozygous for the risk variant, and the TT genotype are homozygous for the high affinity, disease-associated gene variant. Unstimulated monocytes or monocytes stimulated with IVIg alone did not produce IL-10, IL-12/23p40, IL-6, or TNF (data not shown). IVIg co-treatment significantly increased LPS-induced IL-10 production by 46% in monocytes from people with the non-risk variant (CC), but did not significantly increase IL-10 production from people with the risk variant genotype (TT) (Figure 4.5A). This suggests that the risk variant compromises (IVIg + LPS)-induced IL-10 production. When stimulated with LPS, monocytes from people with the risk variant genotype (TT) had modestly, but not significantly, lower IL-10 production compared to people with the non-risk variant genotype (CC) (30% less; $p = 0.13$; Figure 4.5A). Moreover, monocytes from people with the risk variant genotype (TT) produced significantly lower IL-10 in response to IVIg + LPS compared to those, who did not harbor the risk variant (42% less; Figure 4.5A). Monocytes heterozygous for the risk gene variant had a significantly higher IVIg-induced IL-10 in response to LPS, produced an intermediate amount of IL-10 when stimulated with LPS, and produced a similar amount of IL-10 as monocytes with the non-risk variant when stimulated with IVIg + LPS. These results suggest that monocytes from people with the TT risk variant genotype are less able to induce IL-10 production in response to IVIg.

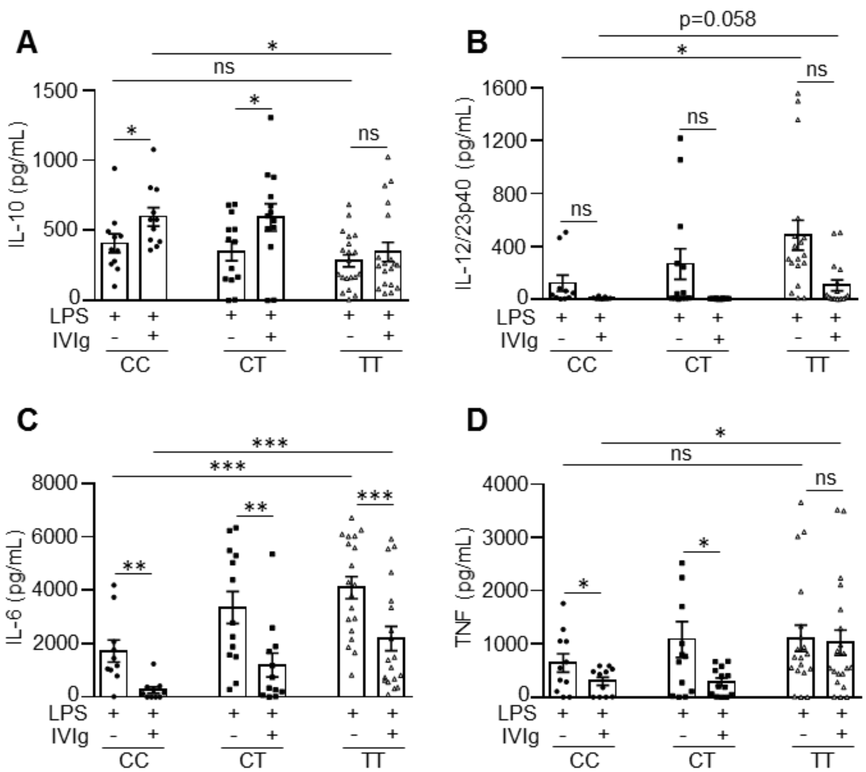

Figure 4.5 Monocytes from people with the FcγRIIA disease-associated gene variant have lower IVIg-mediated anti-inflammatory responses to LPS. Monocytes from healthy control participants were stimulated with LPS (100 ng/mL) or (IVIg (5 mg/mL) + LPS (100ng/mL)) for 24 h. Participants were genotyped for the FcγRIIA H131R polymorphism (rs1801274); CC = does not have the disease-associated gene variant (low affinity), CT = heterozygous for the disease- associated gene variant, and TT = homozygous for the disease-associated gene variant (high affinity), and responses were stratified based on genotype. Clarified cell supernatants were assayed for (A) IL-10, (B) IL-12/23p40, (C) IL-6, and (D) TNF. Data are mean ± SEM from n = 11 CC participants, n = 13 CT participants, and n = 20 TT participants performed as independent experiments, assayed in duplicate. *p < 0.05, **p < 0.01, ***p < 0.001, and ns = not statistically significant. Statistical analyses were performed using a repeated measures one-way ANOVA with Dunn's multiple comparisons correction.

Monocytes from people with the risk variant genotype (TT) had a reduced ability to limit pro-inflammatory cytokine production with IVIg and produced higher amounts of pro-inflammatory cytokines in response to LPS or IVIg + LPS, compared to monocytes with the non-risk variant (Figure 4.5B-D). Monocytes from people with the risk variant genotype (TT) produced significantly higher amounts of IL-12/23p40 when stimulated with LPS and monocytes from some individuals with the risk variant maintained the ability to produce IL-12/23p40 when stimulated with IVIg + LPS, which was ablated in monocytes from people with the non-risk variant (Figure 4.5B). Monocytes from participants with each of the gene variants were able to significantly reduce LPS-induced IL-6 production with IVIg co-treatment (Figure 4.5C). However, IL-6 production was significantly higher in both LPS and (IVIg + LPS)-stimulated monocytes from participants with the risk variant genotype (TT) compared to monocytes from people with the non-risk variant (Figure 4.5C). Monocytes heterozygous for the risk variant produced an intermediate amount of IL-6 when stimulated with LPS or IVIg + LPS. Monocytes from people with the non-risk variant significantly decreased LPS-induced TNF production with IVIg co-treatment (53%), whereas monocytes from people with the risk variant did not (Figure 4.5D). Monocytes from participants with the risk variant genotype (TT) did not have significantly higher TNF production in response to LPS ($p = 0.27$), but produced significantly more TNF in response to IVIg + LPS (Figure 4.5D). Monocytes heterozygous for the risk variant produced an intermediate amount of TNF when stimulated with LPS, had lower TNF production when stimulated with IVIg + LPS, and had significantly lower LPS-induced TNF production when co-treated with IVIg. These results suggest that monocytes from people with the TT risk variant genotype are less able to limit pro-inflammatory cytokine production in response to IVIg.

4.3.6 The high affinity FcγRIIA (TT) prevents (IVIg + LPS)-induced IL-10 production

Since I observed that monocytes from people with the disease-associated gene variant have reduced anti-inflammatory responses to IVIg, I wanted to determine whether the FcγRIIA plays a direct role in reducing IL-10 production. Monocytes were genotyped and UnRx or pre-treated with a ns siRNA control or two siRNAs to the FcγRIIA (si1 and si2). Cell lysates were prepared, separated by SDS-PAGE, and analyzed by western blotting by probing with antibodies for the FcγRIIA and β-actin, as a loading control. The untreated and ns siRNA treated cells had similar levels of FcγRIIA in both genotypes (Figure 4.6A). The siRNA1 and 2 knocked down the FcγRIIA protein to a similar extent in monocytes harboring the non-risk and risk variants, 56% and 59% for siRNA1 and 46% and 56% for siRNA2 (Figure 4.6A). To determine the effect of reduced FcγRIIA expression on anti-inflammatory responses, siRNA pre-treated monocytes were then unstimulated or stimulated with LPS, IVIg, or both. (IVIg + LPS)-stimulated monocytes from people with the non-risk variant genotype (CC) did not have increased IL-10 production when FcγRIIA was knocked down (Figure 4.6B, left). In contrast, (IVIg + LPS)-stimulated monocytes from people with the risk variant genotype (TT) had significantly increased IL-10 production with siRNA1 or with siRNA2 knockdown, compared to the ns siRNA control (39% increase si1, 68% increase si2; Figure 4.6B, right). This suggests that the high affinity FcγRIIA risk variant (TT) blocks IVIg-induced IL-10 production in monocytes.

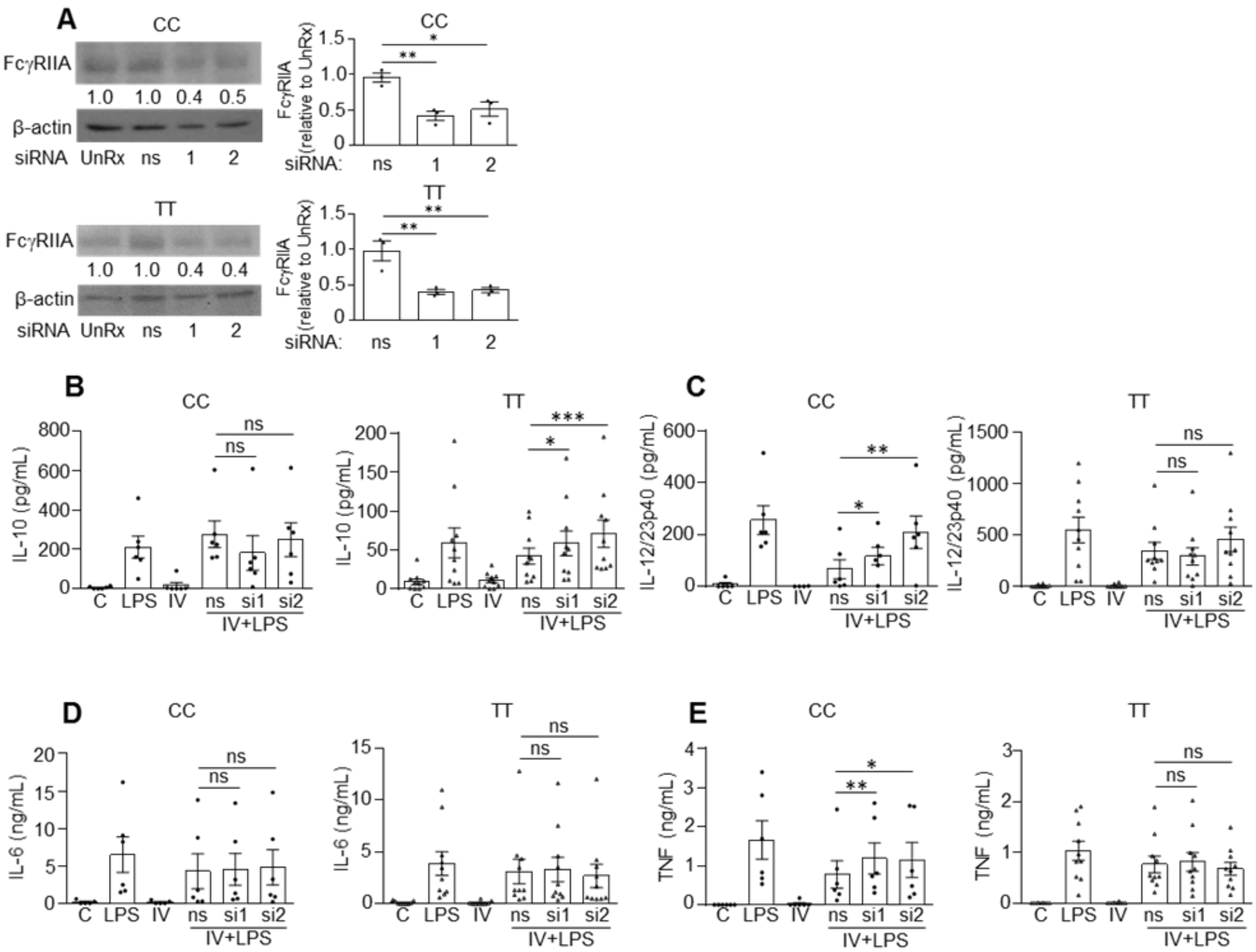

Figure 4.6 The FcγRIIA prevents IVIg-induced IL-10 production in monocytes from people with the disease-associated gene variant. Monocytes from healthy control participants with the non-risk genotype (CC) and risk genotype (TT) were untreated (UnRx) or pre-treated for 48 h with a non-silencing siRNA (ns) or 2 different siRNAs to the FcγRIIA (si1 or si2). (A) Cell lysates (2.5×10^5 cells/treatment) were prepared, separated by SDS-PAGE, and analyzed by western blotting with antibodies for the FcγRIIA and β-actin, as a loading control. Results are representative of *n* = 6 experiments for the non-risk genotype (CC) and *n* = 10 experiments for the risk genotype (TT); monocytes were derived from 1 participant for each independent experiment. Densitometry for the FcγRIIA; normalized to β-actin and relative to the control (UnRx); are averaged and shown below each band. (B-E) Monocytes pre-treated with the ns siRNA control were unstimulated (control (C)) or stimulated with LPS (100 ng/mL), IVIg (5 mg/mL), or both, for 24 h, and monocytes pre-treated with FcγRIIA si1 and si2 were stimulated with IVIg (5 mg/mL) + LPS (100 ng/mL). Clarified cell supernatants were assayed for (B) IL-10, (C) IL-12/23p40, (D) IL-6, and (E) TNF. Data are mean ± SEM and are representative of *n* = 6 experiments for the non-risk genotype (CC) and *n* = 10 experiments for the risk genotype (TT); monocytes were derived from 1 participant for each independent experiment, and assayed in duplicate. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ns = not statistically different for the comparisons indicated. Statistical analyses were performed using a repeated measures one-way ANOVA with Dunn's multiple comparisons correction.

FcγRIIA siRNAs increased production of pro-inflammatory cytokines by (IVIg + LPS)-stimulated monocytes from people with the non-risk variant but not by monocytes from people with the risk variant (Figure 4.6C-E). Specifically, IL-12/23p40 was increased significantly with siRNA1 and 2 in the non-risk variant monocytes (1.8-fold for siRNA1, 3.1-fold for siRNA2; Figure 4.6C, left). However, IL-12/23p40 production was not significantly increased by FcγRIIA knockdown in monocytes from people with the risk variant (Figure 4.6D, right). IL-6 production was not increased by FcγRIIA knockdown in either genotype (Figure 4.6D). In monocytes from people with the non-risk variant, (IVIg + LPS)-stimulated TNF production increased significantly with siRNA1 and with siRNA2, compared to the ns siRNA (1.5-fold for si1 and si2; Figure 4.6E, left). In contrast, there was no increase in (IVIg + LPS)-induced TNF production in monocytes from people with the risk variant when FcγRIIA was knocked down (Figure 4.6E, bottom right). Taken together, these results suggest that the high affinity FcγRIIA risk variant (TT) blocks IVIg-induced IL-10 production, but it does not impact pro-inflammatory cytokine production directly. In contrast, the low affinity non-risk variant genotype (CC) limits (IVIg + LPS)-induced pro-inflammatory cytokine production.

4.3.7 Monocytes from people with the FcγRIIA risk variant have dysregulated IVIg-induced MAPK phosphorylation

I have shown that MAPK activation is required for (IVIg + LPS)-induced IL-10 production. Since IL-10 production is compromised in people with the high affinity disease-associated FcγRIIA gene variant, I next asked whether this was due to a failure to activate MAPKs. Monocytes with either the non-risk or risk variant were unstimulated (0 and 120 min) or stimulated with LPS (100 ng/mL), IVIg (5 mg/mL), or both for 10, 40, or 120 min. Cell lysates from either genotype were analyzed by western blot for pERK1/2, pp38, and GAPDH, as a loading control. A representative western blot for the non-risk variant is shown and described in Figure 4.3A, and for the risk variant is shown and described in Figure 4.7A. Non-risk and risk variant western blot quantifications for n = 3 participants per variant are graphed in Figure 4.7A. ERK1/2 and p38 phosphorylation were induced in response to stimulation with LPS in monocytes from people with the risk variant, but were not significantly increased by concomitant treatment with IVIg, as for people with the non-risk variant (Figure 4.7A). I found in Figure 3 that monocytes from people with the non-risk variant had increased (IVIg + LPS)-induced ERK1/2 and p38 activation. In monocytes from people with the risk variant, ERK1/2 activation did not significantly increase upon stimulation with (IVIg +LPS) compared to LPS at 10 or 40 min and was only modestly higher at 120 min (Figure 4.7A). Similarly, monocytes from people with the risk variant did not have increased and prolonged p38 activation upon stimulation with IVIg + LPS compared to stimulation with LPS alone (Figure 4.7A).

Because MAPK activation was not induced by IVIg + LPS relative to either IVIg or LPS alone in monocytes from people with the FcγRIIA risk variant, I asked whether IL-10 production was MAPK-dependent. Monocytes were genotyped and unstimulated or stimulated with LPS, IVIg, or IVIg + LPS in the absence or presence of inhibitors for the MAPKs, ERK1/2 or p38. For ERK1/2 inhibition; in monocytes from people with the non-risk variant, PD and SCH significantly reduced IL-10 production in response to IVIg + LPS compared to the DMSO control (48% and 31%, respectively; Figure 4.7B, left). In contrast, monocytes from people with the risk variant did not have a statistically significant decrease in IL-10 when ERK1/2 was inhibited. Compared to the solvent control (DMSO), both p38 inhibitors SB and BIR significantly reduced (IVIg + LPS)-induced IL-10 production in monocytes from people with the non-risk variant (37% SB, 41% BIR; Figure 4.7C, left), whereas modest reductions in monocytes from people with the risk variant did not reach significance (23% SB, $p = 0.07$; 55% BIR, $p = 0.07$; Figure 4.7C, right). These results suggest that monocytes from people with the high affinity FcγRIIA risk variant fail to induce robust MAPK activation upon stimulation with IVIg + LPS, which may underlie their defect in (IVIg + LPS)-induced IL-10 production.

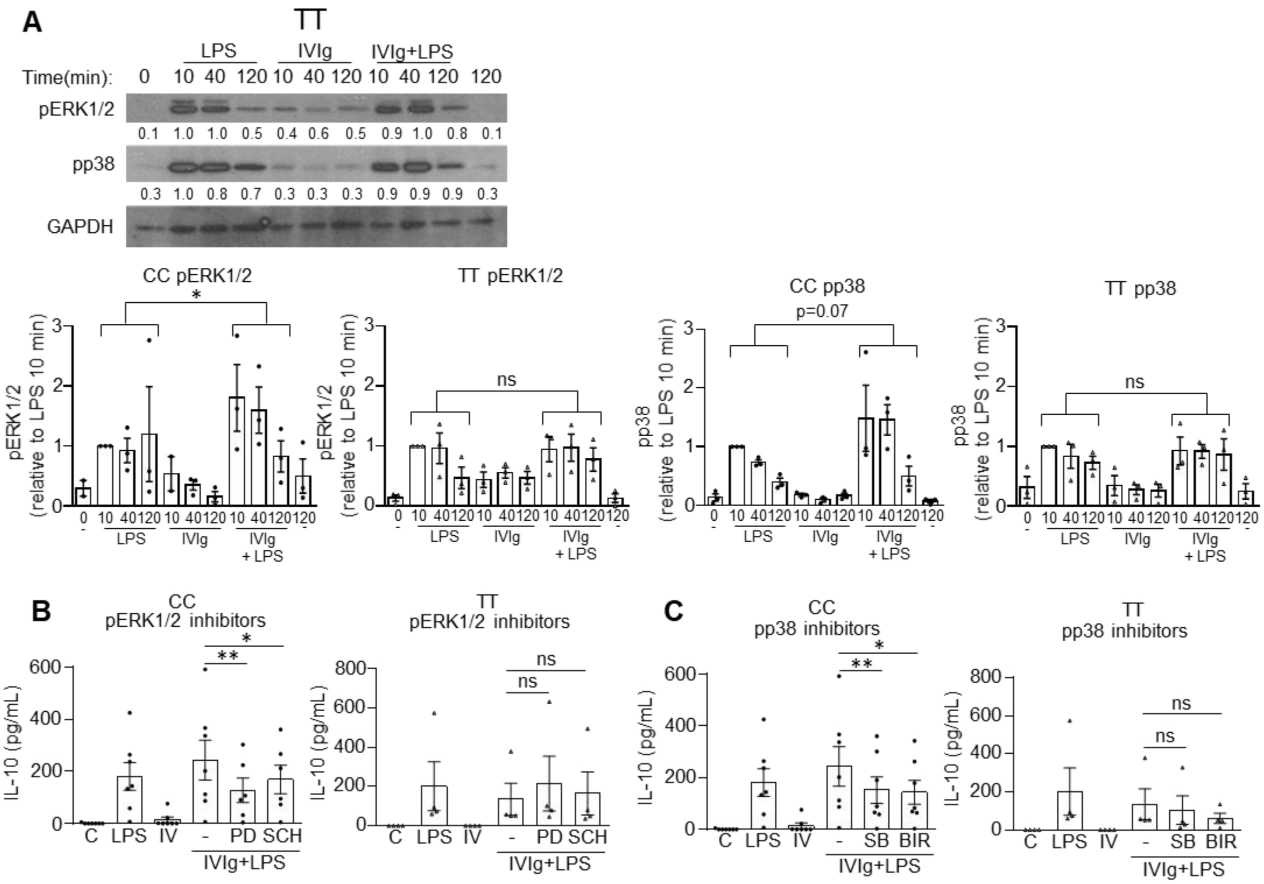

Figure 4.7 (IVIg + LPS)-induced MAPK phosphorylation is lower in monocytes from people with the FcγRIIA risk variant. (A) Monocytes from healthy participants with the risk genotype (TT) were unstimulated or stimulated with LPS (100 ng/mL), IVIg (5 mg/mL), or both, for 0, 10, 40, or 120 min. Cell lysates (2.5×10^5 cells / time point) were prepared at the indicated times. Lysates were separated by SDS-PAGE and analyzed by western blotting using phosphospecific antibodies for ERK1/2, p38, and using GAPDH, as a loading control. Representative western blot from participants with the TT genotype are shown in (A). Results are representative of $n = 3$ experiments per genotype; monocytes were derived from 1 participant for each of 3 independent experiments. Densitometry for pERK1/2 and pp38; normalized to GAPDH and relative to LPS 10 min; are averaged from $n = 3$ independent experiments and shown below each band and values are graphed as mean ± SEM for each genotype. Monocytes were pre-treated for 1 h with an appropriate volume of DMSO, as a vehicle control, or (B) the ERK1/2 inhibitors, PD and SCH; or (C) the p38 inhibitors, SB and BIR and then left unstimulated (C) or stimulated with LPS (100 ng/mL), IVIg (5 mg/mL), or both for 24 h. Clarified cell supernatants were analyzed for IL-10 by ELISA. (B and C) Values are reported as mean ± SEM for $n = 7$ participants for the CC genotype and $n = 4$ participants for the TT genotype performed as independent experiments, and assayed in duplicate. $^*p < 0.05$, $^{**}p < 0.01$, and ns = not statistically different for the comparisons indicated. Statistical analyses were performed using a two-way ANOVA in (A) and repeated measures one-way ANOVA in (B) and (C) with Dunn's multiple comparisons correction for (B-C).

4.4 Discussion

My data demonstrate that IVIg reduces monocyte-mediated inflammation by increasing production of anti-inflammatory IL-10 in response to LPS and reducing production of pro-inflammatory cytokines. Anti-inflammatory monocyte activation is mediated by the FcγRI and FcγRIIB and requires activation of the MAPKs, ERK1/2 and p38. I also report that monocytes with the high affinity, disease-associated FcγRIIA gene variant have lower IL-10 production and higher pro-inflammatory cytokine production than monocytes from people, who do not have the risk variant, and respond poorly to IVIg-mediated anti-inflammatory monocyte activation. My data is consistent with a model in which the high affinity FcγRIIA risk variant prevents anti-inflammatory monocyte activation by IVIg, by sequestering antibodies from the FcγRI and FcγRIIB, and thus failing to induce MAPK activation required for anti-inflammatory IL-10 production (Figure 4.8).

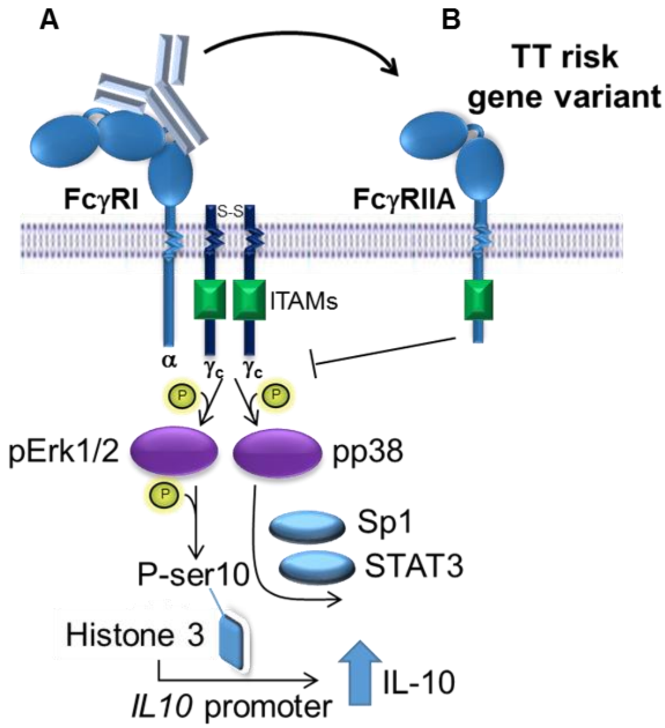

Figure 4.8 Proposed model for IVIg-induced IL-10 production in monocytes from people with the non-risk, low affinity FcγRIIA gene variant. (A) In monocytes from people with the low affinity, non-risk gene variant, the FcγRI induces increased IL-10 production in response to IVIg. ERK1/2 activation is increased, which primes cells for IL-10 production by phosphorylating ser10 on histone 3 opening up the *IL10* promoter and phosphorylating p38, which drives Sp1- and STAT3-mediated transcription of *IL10*. (B) The high affinity, risk variant FcγRIIA sequesters IVIg antibodies from the FcγRI in monocytes, which prevents activation of ERK1/2 and p38, and limits IL-10 production.

IVIg exerts its anti-inflammatory activity, at least in part, by production of IL-10, which is compromised in monocytes from people with the FcγRIIA risk variant. My study is the first to report that IVIg increases IL-10 production by human monocytes. *In vitro*, IVIg treatment has similarly been reported to increase IL-10 production by human dendritic cells in response to LPS, compared to LPS stimulation[337]. IVIg decreases IL-10 production by human MCSF-derived macrophages *in vitro*, although there are key differences in this study, including the type of serum used to culture cells and amount of IVIg used[342]. *In vivo*, IL-10 levels are higher in the serum and IL-10 production is higher by PBMCs from people with ITP post IVIg treatment[338, 339]. Other antibodies can also induce IL-10 production in human monocytes. Human M(Ic) and M(infliximab) increase IL-10 production in response to LPS *in vitro*[29, 30, 35]. Moreover, it has been suggested that infliximab activates intestinal macrophages to an anti-inflammatory or 'regulatory' phenotype *in vivo* specifically in treatment-responsive people with IBD, but not in non-responders[332]. In RA, people with the FcγRIIA risk variant are less responsive to the anti-TNFα drugs, infliximab and adalimumab, compared to people with the non-risk variant[292, 341].9-23% of people with KD are not responsive to IVIg therapy and up to 40% of people with IBD are, or will become, refractory to anti-TNFα therapy, approximately half of whom have not developed anti-drug antibodies[288, 343]. IVIg and anti-TNFα therapy non-responders may have the FcγRIIA risk variant, which suggests that FcγRIIA genotype may be useful in predicting responses to therapy.

I have found that IVIg reduces human monocyte production of LPS-induced pro-inflammatory cytokines IL-12/23p40, IL-6, and TNF, and reduction of these cytokines is impaired in monocytes from people with the FcγRIIA risk variant. LPS-induced IL-12 production is similarly decreased in human dendritic cells and macrophages treated with IVIg[337, 342]. Reduced IL-6 production by human M(IVIg + LPS) is similar to that reported for human M(Ic + LPS), however, human M(Ic + LPS) do not reduce IL-12/23p40 and TNF production[29]. My data is also consistent with *in vivo* observations wherein IVIg treatment reduces serum IL-6 levels and IL-6 production by LPS-stimulated whole blood in children with KD, and reduces serum TNF and IL-1β levels in people with GBS[344, 345]. Few studies exist linking the FcγRIIA gene variant to antibody-mediated immune responses, but previous findings are consistent with my data. Mononuclear cells with the risk variant have higher production of IL-1β when activated with IgG_2[112]. People with the risk variant receiving anti-D intravenous antibodies, which are antibodies to Rho(D) present on some RBCs, for ITP, have higher plasma levels of IL-6, TNFα, and monocyte chemoattractant protein-1 (MCP-1) post-infusion compared to people with the non-risk variant[339]. The reduction of potentially pathogenic pro-inflammatory cytokines, IL-12, IL-23, IL-6, and TNF, may be a unique characteristic of IVIg-induced immunosuppression, which is impaired in monocytes from people with the risk variant.

I have used two independent approaches, blocking antibodies and siRNA knockdown, to demonstrate that the FcγRI and FcγRIIB, but not FcγRIII, are involved in (IVIg + LPS)-induced IL-10 production. The FcγRI may activate MAPKs directly leading to IL-10 production, as described for murine M(Ic + LPS) and M(IVIg + LPS)[27, 322]. The FcγRIIB may contribute to this pathway and/or may act indirectly by inhibiting pro-inflammatory cytokine production thereby eliminating compensatory induction of IL-10 and/or pro-inflammatory cytokine-mediated negative regulation of IL-10 production[90, 134, 346]. In murine bone marrow-derived M(Ic + LPS), IL-10 production has been attributed to the FcγRI indirectly because IL-10 production is lost in macrophages deficient in the Fc receptor γ chain (required for signalling through the FcγRI, FcγRIII, and FcγRIV), but not in FcγRII or FcγRIII deficient macrophages[27]. *In vivo*, inhibitory signalling downstream of the FcγRIII (ITAMi signalling) has been implicated in the IVIg-dependent reduction of TNFα and MCP-1 in a murine unilateral uretral obstruction nephritis model; and the FcγRIIB has been implicated in IVIg's anti-inflammatory activity in a murine model of intracerebral hemorrhage, although IL-10 levels were not measured[130, 335]. Fcγ receptors, their binding affinities, and gene polymorphisms are different in mice and humans, so the involvement of specific Fcγ receptors in disease or treatment must be assessed in human cells. In whole blood from healthy humans, the FcγRI and FcγRIII are responsible for IVIg-induced IL-10 production in response to LPS, with larger reductions in IL-10 when both receptors are blocked[338]. The differences between this study and mine could be due to contributions or cross-talk from other cell populations present in whole blood. In human macrophages *in vitro,* infliximab can bind and act through the FcγRI, and can promote IL-10 production in response to LPS; and the FcγRI, FcγRIIA, FcγRIII, and FcγRIIB, are all reported to contribute to IL-10 production by human macrophages activated with Ic and Pam3CSK4, a bacterial lipoprotein[35, 347, 348]. Differences in FcγR requirement(s) reported may reflect differences in monocytes and macrophages, cell surface expression of FcγRs, as well as antibodies (isotypes or post-translational modifications).

I have shown that the FcγRIIA disease-associated gene variant limits both IL-10 production and reduction of pro-inflammatory cytokines by M(IVIg + LPS). This is the first study, to my knowledge, that directly shows the differential effects that this FcγRIIA genetic polymorphism has on macrophage IL-10 and pro-inflammatory cytokine production. The high affinity FcγRIIA risk variant could directly limit IVIg-induced IL-10 by sequestering IgG antibodies from FcγRI and FcγRIIB, which I have shown promote IVIg-induced IL-10 production. In addition, low level engagement of the non-risk FcγRIIA gene variant (low affinity) may drive ITAMi-mediated reduction of pro-inflammatory cytokine production, as is the case for the FcαRI and FcγRIII; whereas saturation of the high affinity FcγRIIA risk variant may promote ITAM activating signalling contributing to higher pro-inflammatory cytokine production[129, 349].

The activation of the MAPKs, ERK1/2 and p38, were required for (IVIg + LPS)-induced

IL-10 production in monocytes from people with the non-risk variant, as two distinct pharmacological inhibitors for each of ERK1/2 and p38, reduced IL-10 production. Murine M(Ic + LPS) require the FcγRI, which actives Erk1/2 and leads to phosphorylation of serine 10 on histone 3 opening up the *Il10* promoter, whereas p38 activation drives Sp1- and STAT3-mediated transcription of IL-10[38, 39]. My data supports a similar model for human M(IVIg + LPS), in which ERK1/2 is activated modestly in response to IVIg alone and robustly in response to IVIg + LPS, priming monocytes/macrophages for IL-10 production; whereas p38 is not activated by IVIg and robustly activated by co-stimulation with (IVIg + LPS) driving *IL10* transcription[38, 39]. Murine macrophages' ability to elicit stronger Erk1/2 activation than seen in dendritic cells, leads to their ability to produce higher amounts of IL-10[80]. Indeed, in murine M(Ic + LPS), the stronger the FcγRI signalling and Erk1/2 activation, the higher the IL-10 production[39, 80]. Human monocytes treated with LPS and ethanol have increased IL-10 production, which has been shown to be driven by increased activation of p38[350]. Interestingly, pharmacological inhibition of MAPKs did not block IL-10 production by monocytes from people with the risk variant and MAPK activation was not enhanced by IVIg + LPS compared to LPS alone in monocytes from people with the FcγRIIA risk variant. This data is consistent with my model in which the FcγRIIA risk variant sequesters antibodies from the FcγRI and perhaps FcγRIIB, preventing downstream activation of MAPKs required for IL-10 production (Figure 4.8).

Chapter 5: Conclusion and future directions

5.1 Conclusion

Macrophages can have potent anti-inflammatory activity, which limits inflammation and tissue damage. Despite being used to treat a variety of autoimmune and inflammatory diseases, the mechanism(s) of action of IVIg are not well understood. The induction of IL-10 producing, anti-inflammatory macrophages may be a valuable therapeutic strategy to treat inflammatory diseases, such as IBD.

In my thesis I addressed three questions: 1. Does IVIg induce IL-10 producing, anti-inflammatory murine macrophages *in vitro* and *in vivo*? 2. Can IVIg or IVIg-treated macrophages be used to treat intestinal inflammation in mice by inducing macrophage IL-10 production *in vivo*? 3. Does IVIg induce an anti-inflammatory activation state in human monocytes and is anti-inflammatory monocyte activation affected by an FcγRIIA gene variant?

Together, this thesis shows that IVIg activates IL-10 producing, anti-inflammatory murine macrophages and human monocytes, from people, who do not have the disease-associated FcγRIIA risk variant. It also shows that IVIg or IVIg-treated macrophages could be an effective therapy to treat IBD.

In Chapter 2, I showed that IVIg induces high IL-10 production and low pro-inflammatory cytokine production by murine macrophages, in response to LPS, and that IL-10 signalling limits the production of pro-inflammatory cytokines. Activation of Erk1/2 and p38 are required for murine IL-10 production, similar to M(Ic + LPS). Murine peritoneal macrophages are activated to a high IL-10 producing activation state *in vivo*, when mice are given IVIg + LPS by intraperitoneal injection. In Chapter 3, I demonstrated that IVIg treatment or adoptive transfer of M(IVIg + LPS) ameliorates symptoms of DSS-induced colitis in mice, which is dependent on macrophage-derived IL-10. Finally, in Chapter 4 I showed that IVIg-activated human monocytes, similar to murine BMDMs, produce higher levels of IL-10 and low levels of IL-12/23p40, IL-6, and TNF, in response to LPS. IVIg-induced IL-10 production in human monocytes requires FcγRI and FcγRIIB. IL-10 production in human monocytes requires the activation of MAPKs, ERK1/2 and p38, similar to in murine BMDMs. I found that IVIg-induced anti-inflammatory macrophage activation is impaired in monocytes from people with the FcγRIIA disease risk variant. Interestingly, the risk variant FcγRIIA prevents (IVIg + LPS)-induced IL-10 production and dysregulates MAPK activation.

This thesis shows that IVIg infusion may be an effective therapy for IBD. IVIg is already a safe, approved therapy for autoimmune and inflammatory diseases, and is used off-label for an even larger number of diseases[161]. Importantly, IVIg has been used safely for over 20 years to treat people with IBD, who were treatment refractory, had contraindication, such as respiratory fungal infections, or were receiving IVIg for other reasons[351, 352]. In a retrospective chart review of 24 people, who were difficult to treat, IVIg induced remission or clinical improvement in 79% of people with IBD and 62.5% had endoscopic improvement[351]. IVIg induces a very rapid and clinically significant remission in aminosalicylate and steroid resistant CD with maintenance of remission[353]. Unlike anti-TNFα therapies, IVIg is not associated with a higher risk of malignancies or infectious diseases[164, 287]. These studies suggest that large scale controlled clinical trials should be performed to determine whether IVIg can be used to treat IBD.

IVIg could provide an improved IL-10-based therapeutic for IBD. I have shown that IVIg treatment activates IL-10 producing, anti-inflammatory macrophages, which limit symptoms of DSS-induced colitis. This could occur by re-programming intestinal macrophages or infiltrating monocytes that are exposed to LPS in the permeable, inflamed intestine. This strategy could be more effective than systemic rhIL-10 administration, as IL-10 production would occur at the site of inflammation, where LPS is present[354]. IVIg treatment could be very effective for people with IBD, who have low IL-10 levels. In CD, a lower serum IL-10 level is associated with more severe disease, and in a trial of rhIL-10 for CD, people who had the highest disease severity responded best to the therapy[273, 274, 355]. IVIg therapy may not be beneficial to people with high serum IL-10, since high amounts of IL-10 can induce IFNγ[11, 59, 277].

IVIg could also be used in combination with anti-TNFα therapy. Infliximab binds to TNFα through its Fab regions, but also binds to Fcγ receptors, through its Fc region, which is one of its proposed mechanisms of action[347]. IVIg could be used to 'top up' antibody levels in people with IBD, who are non-responsive to therapy, and would be less expensive than using biologics[353]. In addition, people with the high affinity FcγRIIA gene variant (rs1801274) have a higher risk of failure to respond to therapy with the anti-TNFα antibody drugs, infliximab or adalimumab[144, 158]. IVIg could be used to block Fcγ receptors in people with the high affinity FcγRIIA gene variant before anti-TNFα antibody treatment, to make it more efficacious at blocking TNFα in these individuals[347].

Since IVIg is a limited resource, adoptive transfer of IVIg-treated, IL-10 producing macrophages could be used as a cellular therapy for IBD, reducing the amount of IVIg required for treatment. The use of autologous cell therapies in humans is increasing[356, 357]. Antigen specific Tregs, which produce IL-10, have been tested as a therapy for people with refractory CD with promising results[358]. Encouragingly, macrophage cell therapy has also been tested in human renal transplant recipients[297]. Immunosuppressive macrophages activated with serum, MCSF, and IFNγ (M(MCSF + IFNγ)) "regulatory macrophages" were found to be safe and well tolerated, with beneficial outcomes for people, though IL-10 production was not measured[297]. M(MCSF + IFNγ) are currently being tested in the ONE study, which is a multinational controlled clinical trial of cellular therapy for rental transplant[359].

A caveat for using *ex vivo*-activated macrophages as a therapy in humans is that macrophages are plastic cells[360]. Macrophages with anti-inflammatory properties could acquire signals in the inflamed intestine of people with IBD and convert to inflammatory macrophages[360]. However, epigenetic modifications to the *IL10* promoter could make IVIg-activated macrophages stable when used as a cell therapy in humans. Activation of Erk1/2 leads to transient epigenetic modifications to the *Il10* promoter in murine M(Ic + LPS), which makes the promoter more accessible to transcription factors and allows for high production of IL-10[27]. I have found that Erk1/2 is also activated by IVIg, in murine BMDMs and human monocytes (ERK1/2), which could cause the same epigenetic changes as in M(Ic + LPS), leading to a high IL-10 producing activation state. Alternatively, strategies using enzymes that epigenetically "fix" macrophages into a permanent high IL-10 producing activation state by promoting prolonged histone phosphorylation of the *IL10* promoter, could be developed for macrophage cell therapies in humans[360].

The activation of high IL-10 producing macrophages in the intestine could provide a more beneficial strategy than promoting wound healing macrophages[361]. Fibrosis is a common complication of CD that occurs due to excessive wound healing[245, 246, 314, 362]. Wound healing macrophages, although beneficial in models of intestinal inflammation, can promote fibrosis in some models of inflammation in mice[53, 54, 317]. There are high numbers of macrophages present in fibrotic lesions and high expression of MMP2, which breaks down extracellular matrix, in the mucosa of people with CD[245, 246]. I did not test directly whether M(IVIg + LPS) promote fibrosis *in vivo*. However, high IL-10 producing, anti-inflammatory macrophages do not promote wound healing directly, so are not predicted to promote excessive wound healing and fibrotic complications[49]. Also in support, IVIg treatment is not associated with the promotion of fibrosis in humans, and reduces gastrointestinal fibrosis in people with systemic sclerosis [164, 287, 363, 364]. IVIg infusion or IVI-treated macrophage cell therapy could provide an IBD therapy without the risk of fibrotic side effects.

IL-10 producing, anti-inflammatory macrophages have an important role in preventing the development of inflammatory diseases, which result from uncontrolled immune responses. My results suggest that people with the high affinity FcγRIIA may develop inflammatory diseases, such as UC and KD, due to impaired anti-inflammatory monocyte activation and higher inflammatory responses. Conversely, IL-10 producing monocytes/macrophages can also limit pathogen clearance, by the suppression of protective inflammatory Th1 cell, NK cell, and monocyte/macrophage responses[95]. Monocytes/macropahges in people with the high affinity FcγRIIA variant may be more effective at fighting infections, although they may promote the development of inflammatory diseases. Indeed, *H. influenzae* infections and meningitis are less severe in people with the high affinity gene variant than people with the low affinity gene variant[155-157]. With this knowledge, personalized treatments may be developed for infections and immune-mediated diseases in people with either of the FcγRIIA gene variants. Adjuvant therapies for infections that promote inflammatory monocyte/macrophage activation may provide better outcomes for people with the low affinity gene variant. Conversely, treatments for immune-mediated diseases that augment anti-inflammatory monocyte/macrophage activation may be useful for people with the high affinity gene variant.

Blocking or chemically inhibiting the FcγRIIA in people with the high affinity receptor, prior to the treatment with IVIg or anti-TNFα antibodies, could improve efficacy of these drugs for inflammatory diseases. My data are consistent with a model in which people with the high affinity FcγRIIA may respond poorly to antibody-based therapies, due to antibody sequestration from the FcγRI, which impairs ERK1/2 activation and limits IL-10 production by monocytes. FcγRIIA-specific blocking antibodies have been created, which ameliorate FcγRIIA-mediated thrombocytopenia in transgenic mice *in vivo*[365]. An FcγRIIA small molecule inhibitor has also been developed, which specifically binds to both variants of this receptor[366]. The inhibitor prevents Ic-induced TNFα production by a human macrophage cell line and ameliorates symptoms of collagen-induced arthritis in transgenic mice, which are mediated by the FcγRIIA[366]. Blocking or inhibiting the high affinity FcγRIIA in people, prior to the treatment with IVIg or anti-TNFα antibodies, could prevent this receptor from sequestering antibodies from the receptors that mediate IL-10 production by monocytes. This could cause higher IL-10 production by monocytes, which could result in improved therapeutic outcomes for people with the high affinity FcγRIIA gene variant. This research holds promise for people, who respond poorly to IVIg or anti-TNFα, and harbor the high affinity FcγRIIA gene variant.

Finally, this novel understanding of IVIg's mechanism of action may prompt the development of an alternative immunotherapy to replace IVIg, as it is a limited resource[367]. I have found that the FcγRI and FcγRIIB are required for IVIg-induced IL-10 production. This indicates that antibodies, which specifically activate the FcγRI and FcγRIIB, could be a beneficial replacement therapy, as they would be independent of the high affinity FcγRIIA gene variant genotype. FcγRI and FcγRIIB cross-linking may be a better therapeutic strategy than promoting MAPK activation, as ERK5, ERK1/2, and p38 activation are involved in cell proliferation, which can cooperate to promote tumor formation[305]. Although FcγRI agonist antibodies have not been developed yet, an FcγRIIB-specific agonist antibody exists, which triggers inhibitory signalling by human B cells *in vitro*[368, 369]. IgG Fc variants that have preferential and enhanced binding to both the FcγRI and FcγRIIB could also be created. For example, an engineered IgG1 Fc variant has been generated that has 200-fold higher binding affinity for the FcγRIIB, but does not bind to either of the FcγRIIA variants *in vitro*[370]. Data presented in this thesis could allow for the translation of this mechanistic understanding into the development of replacement therapies that will be effective for a larger proportion of the population.

Taken together, these studies suggest a unifying mechanism of action for IVIg. I have found that IVIg induces an anti-inflammatory IL-10 producing activation state in murine macrophages and human monocytes *in vitro*. The FcγRI and FcγRIIB and MAPK activation are required for IL-10 production by IVIg-activated human monocytes. IVIg also induces IL-10 production by macrophages *in vivo*, which ameliorate symptoms of DSS-induced colitis in mice. This suggests that IVIg could be an effective therapeutic strategy to treat people with IBD. Furthermore, IVIg-induced anti-inflammatory activation is impaired in monocytes from people with the disease-associated FcγRIIA gene variant, which could contribute to the development of inflammatory diseases and poor responses to antibody-based drugs. Adjuvant immunotherapies that block the high affinity FcγRIIA may make antibody therapies more effective in people with this gene variant. This knowledge of IVIg's mechanism could also be used to develop an IVIg substitute, which may be effective for people, independent of their FcγRIIA genotype.

5.2 Future directions

The long-term goal of this research is to promote the development of IVIg substitutes, which may be effective in treating people with IBD and other immune-mediated diseases, independent of the FcγRIIA. The critical next steps are to determine whether IVIg can reduce intestinal inflammation in humanized or transgenic mice and to determine whether IVIg induces IL-10 production by macrophages from people receiving this treatment. This research could also allow for a precision medicine approach to be used when treating people with IVIg or other antibody-based drug treatments.

I have demonstrated, using multiple techniques, that IVIg induces IL-10 production by macrophages, which limits intestinal inflammation during DSS-induced colitis. However, IVIg should be used in other models of intestinal inflammation, such as the T cell transfer model of colitis or *Citrobacter rodentium* infectious model of colitis, to determine with more certainty whether IVIg-induced IL-10 is an effective therapy for intestinal inflammation in mice.

In order for an IVIg replacement therapy to be developed, it must be determined which Fcγ receptor(s) are required for IVIg-induced IL-10 production by macrophages in a model of inflammation *in vivo*. Although I used Fcγ receptor deficient mice, blocking antibodies to receptors, and siRNA knockdown of receptor expression, different Fcγ receptors may be involved in a more complex *in vivo* environment. Future studies will address this limitation by showing whether IVIg can ameliorate inflammation in Fcγ receptor deficient mice.

An important step in determining whether IVIg could be used to reduce intestinal inflammation in people, is to determine whether IVIg can reduce intestinal inflammation in models using humanized or transgenic mice. Humanized mice, such as chimeric bone marrow liver thymic mice (BLT mice), have a humanized repertoire of Fcγ receptors and immune system, which will be useful as there are important differences in murine and human Fcγ receptors and macrophages. Although they have not been developed yet, transgenic mice, which have a full repertoire of human Fcγ receptors, would also be useful to test the effect of IVIg in models of intestinal inflammation.

I showed that the low affinity, activating FcγRIIA reduces pro-inflammatory cytokine production by (IVIg + LPS)-activated monocytes, whereas the high affinity FcγRIIA does not. Little is known about the differential effects that this receptor has on human immune responses. The role of ITAMi signalling in the IVIg-induced reduction of pro-inflammatory cytokines by monocytes from people with the low affinity gene variant should be investigated further, by immunoprecipitation for the FcγRIIA and western blotting for SHP-1 and SHIP. If IVIg induces ITAMi signalling in the low affinity FcγRIIA, targeting this receptor could also provide an effective anti-inflammatory treatment for people with this gene variant.

An advantage of my research is that it uses primary human cells rather than human cell lines to determine mechanistically how FcγRIIA gene variants affect immune responses. Primary human cells have different Fcγ receptor expression levels and post-translational modifications, which takes human variability into account. A disadvantage of these studies, however, is that they do not account for combinatorial effects with other Fcγ receptor gene variants, such as with FcγRIIIA gene variants. The FcγRIIIA has a gene variant, which also has a higher affinity for antibodies, and is associated with a higher risk of developing RA[153]. The combined effect of Fcγ receptor gene variants on anti-inflammatory macrophage activation with IVIg will be examined in the future.

It is important to validate my results using healthy control human monocytes with studies using monocytes derived from the peripheral blood of people receiving IVIg treatment. Future studies will be performed to determine whether IVIg infusion in people skews monocytes to an anti-inflammatory activation state, by the crosslinking of the FcγRI and FcγRIIB and activation of MAPKs. Monocytes will be isolated from blood taken before and after IVIg infusion in people, and LPS-induced cytokine production will be measured. Fcγ receptor blocking antibodies or siRNA, MAPK inhibitors, and western blotting will be used to validate this mechanism of action in monocytes from people, who have been treated with IVIg.

Finally, my study did not test the effect of FcγRIIA gene variants on the response to anti-TNFα therapies and the anti-$\alpha_4\beta_7$ integrin drug, vedolizumab, although what I demonstrated for IVIg may be relevant for these drugs as well. Infliximab, adalimumab, and vedolizumab are IgG1 antibodies, as are 60% of the antibodies in IVIg[164]. The influence of FcγRIIA genotype on macrophage anti-inflammatory activation with anti-TNFα and anti-$\alpha_4\beta_7$ integrin drugs, will be examined in future studies, to more accurately predict whether impaired monocyte/macrophage anti-inflammatory activation it is associated with poor response to these drugs. Further investigation is required to determine whether FcγRIIA (rs1801274) genotype can be used in a precision medicine approach to predict responsiveness to IVIg as well as other antibody-based biological therapies.

Taken together, the work in this thesis may allow for the translation of IVIg therapy to people, who are resistant to current treatments for inflammatory bowel disease. This research contributes to the understanding of the mechanism of action of IVIg, which will have an impact on the development of new immunotherapies that will be effective in people, independent of their FcγRIIA genotype.

References

1. Mosser, D. M.; Edwards, J. P., Exploring the full spectrum of macrophage activation. *Nat Rev Immunol* **2008,** *8* (12), 958-69.

2. Murray, P. J., Macrophage Polarization. *Annu Rev Physiol* **2017,** *79*, 541-566.

3. Murray, P. J.; Wynn, T. A., Protective and pathogenic functions of macrophage subsets. *Nat Rev Immunol* **2011,** *11* (11), 723-37.

4. Gordon, S.; Plüddemann, A.; Martinez Estrada, F., Macrophage heterogeneity in tissues: phenotypic diversity and functions. *Immunol Rev* **2014,** *262* (1), 36-55.

5. Gordon, S.; Plüddemann, A., Tissue macrophages: heterogeneity and functions. *BMC Biol* **2017,** *15* (1), 53.

6. Stremmel, C.; Schuchert, R.; Wagner, F.; Thaler, R.; Weinberger, T.; Pick, R.; Mass, E.; Ishikawa-Ankerhold, H. C.; Margraf, A.; Hutter, S.; Vagnozzi, R.; Klapproth, S.; Frampton, J.; Yona, S.; Scheiermann, C.; Molkentin, J. D.; Jeschke, U.; Moser, M.; Sperandio, M.; Massberg, S.; Geissmann, F.; Schulz, C., Yolk sac macrophage progenitors traffic to the embryo during defined stages of development. *Nat Commun* **2018,** *9* (1), 75.

7. Varol, C.; Mildner, A.; Jung, S., Macrophages: development and tissue specialization. *Annu Rev Immunol* **2015,** *33*, 643-75.

8. Chazaud, B., Macrophages: supportive cells for tissue repair and regeneration. *Immunobiology* **2014,** *219* (3), 172-8.

9. Ginhoux, F.; Schultze, J. L.; Murray, P. J.; Ochando, J.; Biswas, S. K., New insights into the multidimensional concept of macrophage ontogeny, activation and function. *Nat Immunol* **2016,** *17* (1), 34-40.

10. Martinez, F. O.; Gordon, S., The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Rep* **2014,** *6*, 13.

11. Fleming, B. D.; Mosser, D. M., Regulatory macrophages: setting the threshold for therapy. *Eur J Immunol* **2011,** *41* (9), 2498-502.

12. Gordon, S., Alternative activation of macrophages. *Nat Rev Immunol* **2003,** *3* (1), 23-35.

13. Mosser, Exploring the full spectrum of macrophage activation.

14. Benson, J. M.; Sachs, C. W.; Treacy, G.; Zhou, H.; Pendley, C. E.; Brodmerkel, C. M.; Shankar, G.; Mascelli, M. A., Therapeutic targeting of the IL-12/23 pathways: generation and characterization of ustekinumab. *Nat Biotechnol* **2011,** *29* (7), 615-24.

15. Hunter, C. A.; Jones, S. A., IL-6 as a keystone cytokine in health and disease. *Nat Immunol* **2015,** *16* (5), 448-57.

16. Kalliolias, G. D.; Ivashkiv, L. B., TNF biology, pathogenic mechanisms and emerging therapeutic strategies. *Nat Rev Rheumatol* **2016,** *12* (1), 49-62.

17. Arnold, C. E.; Gordon, P.; Barker, R. N.; Wilson, H. M., The activation status of human macrophages presenting antigen determines the efficiency of Th17 responses. *Immunobiology* **2015,** *220* (1), 10-9.

18. Dale, D. C.; Boxer, L.; Liles, W. C., The phagocytes: neutrophils and monocytes. *Blood* **2008,** *112* (4), 935-45.

19. Tarique, A. A.; Logan, J.; Thomas, E.; Holt, P. G.; Sly, P. D.; Fantino, E., Phenotypic, functional, and plasticity features of classical and alternatively activated human macrophages. *Am J Respir Cell Mol Biol* **2015,** *53* (5), 676-88.

20. Steiner, D. J.; Furuya, Y.; Jordan, M. B.; Metzger, D. W., Protective Role for Macrophages in Respiratory Francisella tularensis Infection. *Infect Immun* **2017,** *85* (6).

21. Kawane, K.; Ohtani, M.; Miwa, K.; Kizawa, T.; Kanbara, Y.; Yoshioka, Y.; Yoshikawa, H.; Nagata, S., Chronic polyarthritis caused by mammalian DNA that escapes from degradation in macrophages. *Nature* **2006,** *443* (7114), 998-1002.

22. Murphy, C. A.; Langrish, C. L.; Chen, Y.; Blumenschein, W.; McClanahan, T.; Kastelein, R. A.; Sedgwick, J. D.; Cua, D. J., Divergent pro- and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation. *J Exp Med* **2003,** *198* (12), 1951-7.

23. Brück, W.; Sommermeier, N.; Bergmann, M.; Zettl, U.; Goebel, H. H.; Kretzschmar, H. A.; Lassmann, H., Macrophages in multiple sclerosis. *Immunobiology* **1996,** *195* (4-5), 588-600.

24. Kamada, N.; Hisamatsu, T.; Okamoto, S.; Chinen, H.; Kobayashi, T.; Sato, T.; Sakuraba, A.; Kitazume, M. T.; Sugita, A.; Koganei, K.; Akagawa, K. S.; Hibi, T., Unique CD14 intestinal macrophages contribute to the pathogenesis of Crohn disease via IL-23/IFN-gamma axis. *J Clin Invest* **2008,** *118* (6), 2269-80.

25. Iyer, S. S.; Cheng, G., Role of interleukin 10 transcriptional regulation in inflammation and autoimmune disease. *Crit Rev Immunol* **2012,** *32* (1), 23-63.

26. Anderson, C. F.; Mosser, D. M., A novel phenotype for an activated macrophage: the type 2 activated macrophage. *J Leukoc Biol* **2002,** *72* (1), 101-6.

27. Sutterwala, F. S.; Noel, G. J.; Salgame, P.; Mosser, D. M., Reversal of proinflammatory responses by ligating the macrophage Fcgamma receptor type I. *J Exp Med* **1998,** *188* (1), 217-22.

28. Grazia Cappiello, M.; Sutterwala, F. S.; Trinchieri, G.; Mosser, D. M.; Ma, X., Suppression of Il-12 transcription in macrophages following Fc gamma receptor ligation. *J Immunol* **2001,** *166* (7), 4498-506.

29. Fleming, B. D.; Chandrasekaran, P.; Dillon, L. A.; Dalby, E.; Suresh, R.; Sarkar, A.; El-Sayed, N. M.; Mosser, D. M., The generation of macrophages with anti-inflammatory activity in the absence of STAT6 signaling. *J Leukoc Biol* **2015,** *98* (3), 395-407.

30. Ambarus, C. A.; Santegoets, K. C.; van Bon, L.; Wenink, M. H.; Tak, P. P.; Radstake, T. R.; Baeten, D. L., Soluble immune complexes shift the TLR-induced cytokine production of distinct polarized human macrophage subsets towards IL-10. *PLoS One* **2012,** *7* (4), e35994.

31. Erwig, L. P.; Henson, P. M., Immunological consequences of apoptotic cell phagocytosis. *Am J Pathol* **2007,** *171* (1), 2-8.

32. Strassmann, G.; Patil-Koota, V.; Finkelman, F.; Fong, M.; Kambayashi, T., Evidence for the involvement of interleukin 10 in the differential deactivation of murine peritoneal macrophages by prostaglandin E2. *J Exp Med* **1994,** *180* (6), 2365-70.

33. Sternberg, E. M., Neural regulation of innate immunity: a coordinated nonspecific host response to pathogens. *Nat Rev Immunol* **2006,** *6* (4), 318-28.

34. Martinez, F. O.; Sica, A.; Mantovani, A.; Locati, M., Macrophage activation and polarization. *Front Biosci* **2008,** *13*, 453-61.

35. Vos, A. C.; Wildenberg, M. E.; Duijvestein, M.; Verhaar, A. P.; van den Brink, G. R.; Hommes, D. W., Anti-tumor necrosis factor-α antibodies induce regulatory macrophages in an Fc region-dependent manner. *Gastroenterology* **2011,** *140* (1), 221-30.

36. Riquelme, P.; Tomiuk, S.; Kammler, A.; Fändrich, F.; Schlitt, H. J.; Geissler, E. K.; Hutchinson, J. A., IFN-γ-induced iNOS expression in mouse regulatory macrophages prolongs allograft survival in fully immunocompetent recipients. *Mol Ther* **2013,** *21* (2), 409-22.

37. Broichhausen, C.; Riquelme, P.; Geissler, E. K.; Hutchinson, J. A., Regulatory macrophages as therapeutic targets and therapeutic agents in solid organ transplantation. *Curr Opin Organ Transplant* **2012,** *17* (4), 332-42.

38. Lucas, M.; Zhang, X.; Prasanna, V.; Mosser, D. M., ERK activation following macrophage FcgammaR ligation leads to chromatin modifications at the IL-10 locus. *J Immunol* **2005,** *175* (1), 469-77.

39. Gallo, P.; Gonçalves, R.; Mosser, D. M., The influence of IgG density and macrophage Fc (gamma) receptor cross-linking on phagocytosis and IL-10 production. *Immunol Lett* **2010,** *133* (2), 70-7.

40. Li, Y. N.; Qin, X. J.; Kuang, F.; Wu, R.; Duan, X. L.; Ju, G.; Wang, B. R., Alterations of Fc gamma receptor I and Toll-like receptor 4 mediate the antiinflammatory actions of microglia and astrocytes after adrenaline-induced blood-brain barrier opening in rats. *J Neurosci Res* **2008,** *86* (16), 3556-65.

41. Yang, Z.; Mosser, D. M.; Zhang, X., Activation of the MAPK, ERK, following Leishmania amazonensis infection of macrophages. *J Immunol* **2007,** *178* (2), 1077-85.

42. Loke, P.; Gallagher, I.; Nair, M. G.; Zang, X.; Brombacher, F.; Mohrs, M.; Allison, J. P.; Allen, J. E., Alternative activation is an innate response to injury that requires CD4+ T cells to be sustained during chronic infection. *J Immunol* **2007,** *179* (6), 3926-36.

43. Brandt, E.; Woerly, G.; Younes, A. B.; Loiseau, S.; Capron, M., IL-4 production by human polymorphonuclear neutrophils. *J Leukoc Biol* **2000,** *68* (1), 125-30.

44. Reese, T. A.; Liang, H. E.; Tager, A. M.; Luster, A. D.; Van Rooijen, N.; Voehringer, D.; Locksley, R. M., Chitin induces accumulation in tissue of innate immune cells associated with allergy. *Nature* **2007,** *447* (7140), 92-6.

45. Schroeder, J. T.; Lichtenstein, L. M.; Roche, E. M.; Xiao, H.; Liu, M. C., IL-4 production by human basophils found in the lung following segmental allergen challenge. *J Allergy Clin Immunol* **2001,** *107* (2), 265-71.

46. Martinez, F. O.; Helming, L.; Gordon, S., Alternative activation of macrophages: an immunologic functional perspective. *Annu Rev Immunol* **2009,** *27*, 451-83.

47. Yang, Z.; Ming, X. F., Functions of arginase isoforms in macrophage inflammatory responses: impact on cardiovascular diseases and metabolic disorders. *Front Immunol* **2014,** *5*, 533.

48. Rath, M.; Müller, I.; Kropf, P.; Closs, E. I.; Munder, M., Metabolism via Arginase or Nitric Oxide Synthase: Two Competing Arginine Pathways in Macrophages. *Front Immunol* **2014,** *5*, 532.

49. Edwards, J. P.; Zhang, X.; Frauwirth, K. A.; Mosser, D. M., Biochemical and functional characterization of three activated macrophage populations. *J Leukoc Biol* **2006,** *80* (6), 1298-307.

50. Murray, P. J.; Allen, J. E.; Biswas, S. K.; Fisher, E. A.; Gilroy, D. W.; Goerdt, S.; Gordon, S.; Hamilton, J. A.; Ivashkiv, L. B.; Lawrence, T.; Locati, M.; Mantovani, A.; Martinez, F. O.; Mege, J. L.; Mosser, D. M.; Natoli, G.; Saeij, J. P.; Schultze, J. L.; Shirey, K. A.; Sica, A.; Suttles, J.; Udalova, I.; van Ginderachter, J. A.; Vogel, S. N.; Wynn, T. A., Macrophage activation and polarization: nomenclature and experimental guidelines. *Immunity* **2014,** *41* (1), 14-20.

51. Mia, S.; Warnecke, A.; Zhang, X. M.; Malmström, V.; Harris, R. A., An optimized protocol for human M2 macrophages using M-CSF and IL-4/IL-10/TGF-β yields a dominant immunosuppressive phenotype. *Scand J Immunol* **2014,** *79* (5), 305-14.

52. Lumeng, C. N.; Bodzin, J. L.; Saltiel, A. R., Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *J Clin Invest* **2007,** *117* (1), 175-84.

53. Mora, A. L.; Torres-González, E.; Rojas, M.; Corredor, C.; Ritzenthaler, J.; Xu, J.; Roman, J.; Brigham, K.; Stecenko, A., Activation of alveolar macrophages via the alternative pathway in herpesvirus-induced lung fibrosis. *Am J Respir Cell Mol Biol* **2006,** *35* (4), 466-73.

54. Gibbons, M. A.; MacKinnon, A. C.; Ramachandran, P.; Dhaliwal, K.; Duffin, R.; Phythian-Adams, A. T.; van Rooijen, N.; Haslett, C.; Howie, S. E.; Simpson, A. J.; Hirani, N.; Gauldie, J.; Iredale, J. P.; Sethi, T.; Forbes, S. J., Ly6Chi monocytes direct alternatively activated profibrotic macrophage regulation of lung fibrosis. *Am J Respir Crit Care Med* **2011,** *184* (5), 569-81.

55. Lang, R.; Patel, D.; Morris, J. J.; Rutschman, R. L.; Murray, P. J., Shaping gene expression in activated and resting primary macrophages by IL-10. *J Immunol* **2002,** *169* (5), 2253-63.

56. Mauer, J.; Chaurasia, B.; Goldau, J.; Vogt, M. C.; Ruud, J.; Nguyen, K. D.; Theurich, S.; Hausen, A. C.; Schmitz, J.; Brönneke, H. S.; Estevez, E.; Allen, T. L.; Mesaros, A.; Partridge, L.; Febbraio, M. A.; Chawla, A.; Wunderlich, F. T.; Brüning, J. C., Signaling by IL-6 promotes alternative activation of macrophages to limit endotoxemia and obesity-associated resistance to insulin. *Nat Immunol* **2014,** *15* (5), 423-30.

57. Fiorentino, D. F.; Bond, M. W.; Mosmann, T. R., Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J Exp Med* **1989,** *170* (6), 2081-95.

58. Fiorentino, D. F.; Zlotnik, A.; Mosmann, T. R.; Howard, M.; O'Garra, A., IL-10 inhibits cytokine production by activated macrophages. *J Immunol* **1991,** *147* (11), 3815-22.

59. Hedrich, C. M.; Bream, J. H., Cell type-specific regulation of IL-10 expression in inflammation and disease. *Immunol Res* **2010,** *47* (1-3), 185-206.

60. de Waal Malefyt, R.; Abrams, J.; Bennett, B.; Figdor, C. G.; de Vries, J. E., Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *J Exp Med* **1991,** *174* (5), 1209-20.

61. Mosser, D. M.; Zhang, X., Interleukin-10: new perspectives on an old cytokine. *Immunol Rev* **2008,** *226*, 205-18.

62. Ma, X.; Yan, W.; Zheng, H.; Du, Q.; Zhang, L.; Ban, Y.; Li, N.; Wei, F., Regulation of IL-10 and IL-12 production and function in macrophages and dendritic cells. *F1000Res* **2015,** *4*.

63. Corinti, S.; Albanesi, C.; la Sala, A.; Pastore, S.; Girolomoni, G., Regulatory activity of autocrine IL-10 on dendritic cell functions. *J Immunol* **2001,** *166* (7), 4312-8.

64. Denning, T. L.; Campbell, N. A.; Song, F.; Garofalo, R. P.; Klimpel, G. R.; Reyes, V. E.; Ernst, P. B., Expression of IL-10 receptors on epithelial cells from the murine small and large intestine. *Int Immunol* **2000,** *12* (2), 133-9.

65. Moroguchi, A.; Ishimura, K.; Okano, K.; Wakabayashi, H.; Maeba, T.; Maeta, H., Interleukin-10 suppresses proliferation and remodeling of extracellular matrix of cultured human skin fibroblasts. *Eur Surg Res* **2004,** *36* (1), 39-44.

66. Kominsky, D. J.; Campbell, E. L.; Ehrentraut, S. F.; Wilson, K. E.; Kelly, C. J.; Glover, L. E.; Collins, C. B.; Bayless, A. J.; Saeedi, B.; Dobrinskikh, E.; Bowers, B. E.; MacManus, C. F.; Müller, W.; Colgan, S. P.; Bruder, D., IFN-γ-mediated induction of an apical IL-10 receptor on polarized intestinal epithelia. *J Immunol* **2014,** *192* (3), 1267-76.

67. Itoh, K.; Hirohata, S., The role of IL-10 in human B cell activation, proliferation, and differentiation. *J Immunol* **1995,** *154* (9), 4341-50.

68. Groux, H.; Bigler, M.; de Vries, J. E.; Roncarolo, M. G., Inhibitory and stimulatory effects of IL-10 on human CD8+ T cells. *J Immunol* **1998,** *160* (7), 3188-93.

69. Jenkins, J. K.; Malyak, M.; Arend, W. P., The effects of interleukin-10 on interleukin-1 receptor antagonist and interleukin-1 beta production in human monocytes and neutrophils. *Lymphokine Cytokine Res* **1994,** *13* (1), 47-54.

70. O'Garra, A.; Vieira, P., T(H)1 cells control themselves by producing interleukin-10. *Nat Rev Immunol* **2007,** *7* (6), 425-8.

71. Ouyang, P.; Rakus, K.; van Beurden, S. J.; Westphal, A. H.; Davison, A. J.; Gatherer, D.; Vanderplasschen, A. F., IL-10 encoded by viruses: a remarkable example of independent acquisition of a cellular gene by viruses and its subsequent evolution in the viral genome. *J Gen Virol* **2014,** *95* (Pt 2), 245-62.

72. Wang, Y.; Liu, X. H.; Li, Y. H.; Li, O., The paradox of IL-10-mediated modulation in cervical cancer. *Biomed Rep* **2013,** *1* (3), 347-351.

73. Boonstra, A.; Rajsbaum, R.; Holman, M.; Marques, R.; Asselin-Paturel, C.; Pereira, J. P.; Bates, E. E.; Akira, S.; Vieira, P.; Liu, Y. J.; Trinchieri, G.; O'Garra, A., Macrophages and myeloid dendritic cells, but not plasmacytoid dendritic cells, produce IL-10 in response to MyD88- and TRIF-dependent TLR signals, and TLR-independent signals. *J Immunol* **2006,** *177* (11), 7551-8.

74. Shouval, D. S.; Ouahed, J.; Biswas, A.; Goettel, J. A.; Horwitz, B. H.; Klein, C.; Muise, A. M.; Snapper, S. B., Interleukin 10 receptor signaling: master regulator of intestinal mucosal homeostasis in mice and humans. *Adv Immunol* **2014,** *122*, 177-210.

75. Tan, J. C.; Indelicato, S. R.; Narula, S. K.; Zavodny, P. J.; Chou, C. C., Characterization of interleukin-10 receptors on human and mouse cells. *J Biol Chem* **1993,** *268* (28), 21053-9.

76. Ouyang, W.; Rutz, S.; Crellin, N. K.; Valdez, P. A.; Hymowitz, S. G., Regulation and functions of the IL-10 family of cytokines in inflammation and disease. *Annu Rev Immunol* **2011,** *29*, 71-109.

77. Yoon, S. I.; Jones, B. C.; Logsdon, N. J.; Harris, B. D.; Deshpande, A.; Radaeva, S.; Halloran, B. A.; Gao, B.; Walter, M. R., Structure and mechanism of receptor sharing by the IL-10R2 common chain. *Structure* **2010,** *18* (5), 638-48.

78. Williams, L.; Bradley, L.; Smith, A.; Foxwell, B., Signal transducer and activator of transcription 3 is the dominant mediator of the anti-inflammatory effects of IL-10 in human macrophages. *J Immunol* **2004,** *172* (1), 567-76.

79. Williams, L. M.; Ricchetti, G.; Sarma, U.; Smallie, T.; Foxwell, B. M., Interleukin-10 suppression of myeloid cell activation--a continuing puzzle. *Immunology* **2004,** *113* (3), 281-92.

80. Saraiva, M.; O'Garra, A., The regulation of IL-10 production by immune cells. *Nat Rev Immunol* **2010,** *10* (3), 170-81.

81. Akira, S.; Takeda, K., Toll-like receptor signalling. *Nat Rev Immunol* **2004,** *4* (7), 499-511.

82. Häcker, H.; Redecke, V.; Blagoev, B.; Kratchmarova, I.; Hsu, L. C.; Wang, G. G.; Kamps, M. P.; Raz, E.; Wagner, H.; Häcker, G.; Mann, M.; Karin, M., Specificity in Toll-like receptor signalling through distinct effector functions of TRAF3 and TRAF6. *Nature* **2006,** *439* (7073), 204-7.

83. Beinke, S.; Ley, S. C., Functions of NF-kappaB1 and NF-kappaB2 in immune cell biology. *Biochem J* **2004,** *382* (Pt 2), 393-409.

84. Chang, E. Y.; Guo, B.; Doyle, S. E.; Cheng, G., Cutting edge: involvement of the type I IFN production and signaling pathway in lipopolysaccharide-induced IL-10 production. *J Immunol* **2007,** *178* (11), 6705-9.

85. Iyer, S. S.; Ghaffari, A. A.; Cheng, G., Lipopolysaccharide-mediated IL-10 transcriptional regulation requires sequential induction of type I IFNs and IL-27 in macrophages. *J Immunol* **2010,** *185* (11), 6599-607.

86. Ziegler-Heitbrock, L.; Lötzerich, M.; Schaefer, A.; Werner, T.; Frankenberger, M.; Benkhart, E., IFN-alpha induces the human IL-10 gene by recruiting both IFN regulatory factor 1 and Stat3. *J Immunol* **2003,** *171* (1), 285-90.

87. VanDeusen, J. B.; Shah, M. H.; Becknell, B.; Blaser, B. W.; Ferketich, A. K.; Nuovo, G. J.; Ahmer, B. M.; Durbin, J.; Caligiuri, M. A., STAT-1-mediated repression of monocyte interleukin-10 gene expression in vivo. *Eur J Immunol* **2006,** *36* (3), 623-30.

88. Staples, K. J.; Smallie, T.; Williams, L. M.; Foey, A.; Burke, B.; Foxwell, B. M.; Ziegler-Heitbrock, L., IL-10 induces IL-10 in primary human monocyte-derived macrophages via the transcription factor Stat3. *J Immunol* **2007,** *178* (8), 4779-85.

89. Saraiva, M.; Christensen, J. R.; Tsytsykova, A. V.; Goldfeld, A. E.; Ley, S. C.; Kioussis, D.; O'Garra, A., Identification of a macrophage-specific chromatin signature in the IL-10 locus. *J Immunol* **2005,** *175* (2), 1041-6.

90. Hu, X.; Paik, P. K.; Chen, J.; Yarilina, A.; Kockeritz, L.; Lu, T. T.; Woodgett, J. R.; Ivashkiv, L. B., IFN-gamma suppresses IL-10 production and synergizes with TLR2 by regulating GSK3 and CREB/AP-1 proteins. *Immunity* **2006,** *24* (5), 563-74.

91. Hammer, M.; Mages, J.; Dietrich, H.; Schmitz, F.; Striebel, F.; Murray, P. J.; Wagner, H.; Lang, R., Control of dual-specificity phosphatase-1 expression in activated macrophages by IL-10. *Eur J Immunol* **2005,** *35* (10), 2991-3001.

92. Sharma, A.; Kumar, M.; Aich, J.; Hariharan, M.; Brahmachari, S. K.; Agrawal, A.; Ghosh, B., Posttranscriptional regulation of interleukin-10 expression by hsa-miR-106a. *Proc Natl Acad Sci U S A* **2009,** *106* (14), 5761-6.

93. Grimbaldeston, M. A.; Nakae, S.; Kalesnikoff, J.; Tsai, M.; Galli, S. J., Mast cell-derived interleukin 10 limits skin pathology in contact dermatitis and chronic irradiation with ultraviolet B. *Nat Immunol* **2007,** *8* (10), 1095-104.

94. Strle, K.; Zhou, J. H.; Shen, W. H.; Broussard, S. R.; Johnson, R. W.; Freund, G. G.; Dantzer, R.; Kelley, K. W., Interleukin-10 in the brain. *Crit Rev Immunol* **2001,** *21* (5), 427-49.

95. Couper, K. N.; Blount, D. G.; Riley, E. M., IL-10: the master regulator of immunity to infection. *J Immunol* **2008,** *180* (9), 5771-7.

96. Schroeder, H. W.; Cavacini, L., Structure and function of immunoglobulins. *J Allergy Clin Immunol* **2010,** *125* (2 Suppl 2), S41-52.

97. Li, X.; Ptacek, T. S.; Brown, E. E.; Edberg, J. C., Fcgamma receptors: structure, function and role as genetic risk factors in SLE. *Genes Immun* **2009,** *10* (5), 380-9.

98. Nimmerjahn, F.; Ravetch, J. V., Fcgamma receptors as regulators of immune responses. *Nat Rev Immunol* **2008,** *8* (1), 34-47.

99. Bruhns, P.; Jönsson, F., Mouse and human FcR effector functions. *Immunol Rev* **2015,** *268* (1), 25-51.

100. Billadeau, D. D.; Leibson, P. J., ITAMs versus ITIMs: striking a balance during cell regulation. *J Clin Invest* **2002,** *109* (2), 161-8.

101. Amigorena, S.; Bonnerot, C.; Drake, J. R.; Choquet, D.; Hunziker, W.; Guillet, J. G.; Webster, P.; Sautes, C.; Mellman, I.; Fridman, W. H., Cytoplasmic domain heterogeneity and functions of IgG Fc receptors in B lymphocytes. *Science* **1992,** *256* (5065), 1808-12.

102. Guilliams, M.; Bruhns, P.; Saeys, Y.; Hammad, H.; Lambrecht, B. N., The function of Fcγ receptors in dendritic cells and macrophages. *Nat Rev Immunol* **2014,** *14* (2), 94-108.

103. Gergely, J.; Sarmay, G., The two binding-site models of human IgG binding Fc gamma receptors. *FASEB J* **1990,** *4* (15), 3275-83.

104. Nimmerjahn, F.; Ravetch, J. V., Fcgamma receptors: old friends and new family members. *Immunity* **2006,** *24* (1), 19-28.

105. Bruhns, P., Properties of mouse and human IgG receptors and their contribution to disease models. *Blood* **2012,** *119* (24), 5640-9.

106. van Mirre, E.; Teeling, J. L.; van der Meer, J. W.; Bleeker, W. K.; Hack, C. E., Monomeric IgG in intravenous Ig preparations is a functional antagonist of FcgammaRII and FcgammaRIIIb. *J Immunol* **2004,** *173* (1), 332-9.

107. Mancardi, D. A.; Albanesi, M.; Jönsson, F.; Iannascoli, B.; Van Rooijen, N.; Kang, X.; England, P.; Daëron, M.; Bruhns, P., The high-affinity human IgG receptor FcγRI (CD64) promotes IgG-mediated inflammation, anaphylaxis, and antitumor immunotherapy. *Blood* **2013,** *121* (9), 1563-73.

108. Bournazos, S.; Woof, J. M.; Hart, S. P.; Dransfield, I., Functional and clinical consequences of Fc receptor polymorphic and copy number variants. *Clin Exp Immunol* **2009,** *157* (2), 244-54.

109. Hayes, J. M.; Frostell, A.; Cosgrave, E. F.; Struwe, W. B.; Potter, O.; Davey, G. P.; Karlsson, R.; Anneren, C.; Rudd, P. M., Fc gamma receptor glycosylation modulates the binding

of IgG glycoforms: a requirement for stable antibody interactions. *J Proteome Res* **2014,** *13* (12), 5471-85.

110. Chauhan, A. K., Human CD4(+) T-Cells: A Role for Low-Affinity Fc Receptors. *Front Immunol* **2016,** *7*, 215.

111. Selvaraj, P.; Fifadara, N.; Nagarajan, S.; Cimino, A.; Wang, G., Functional regulation of human neutrophil Fc gamma receptors. *Immunol Res* **2004,** *29* (1-3), 219-30.

112. Nagelkerke, S. Q.; Kuijpers, T. W., Immunomodulation by IVIg and the Role of Fc-Gamma Receptors: Classic Mechanisms of Action after all? *Front Immunol* **2014,** *5*, 674.

113. Hayes, J. M.; Wormald, M. R.; Rudd, P. M.; Davey, G. P., Fc gamma receptors: glycobiology and therapeutic prospects. *J Inflamm Res* **2016,** *9*, 209-219.

114. Starbeck-Miller, G. R.; Badovinac, V. P.; Barber, D. L.; Harty, J. T., Cutting edge: Expression of FcγRIIB tempers memory CD8 T cell function in vivo. *J Immunol* **2014,** *192* (1), 35-9.

115. Perussia, B.; Tutt, M. M.; Qiu, W. Q.; Kuziel, W. A.; Tucker, P. W.; Trinchieri, G.; Bennett, M.; Ravetch, J. V.; Kumar, V., Murine natural killer cells express functional Fc gamma receptor II encoded by the Fc gamma R alpha gene. *J Exp Med* **1989,** *170* (1), 73-86.

116. Dutertre, C. A.; Bonnin-Gélizé, E.; Pulford, K.; Bourel, D.; Fridman, W. H.; Teillaud, J. L., A novel subset of NK cells expressing high levels of inhibitory FcgammaRIIB modulating antibody-dependent function. *J Leukoc Biol* **2008,** *84* (6), 1511-20.

117. Zhi, H.; Dai, J.; Liu, J.; Zhu, J.; Newman, D. K.; Gao, C.; Newman, P. J., Platelet Activation and Thrombus Formation over IgG Immune Complexes Requires Integrin αIIbβ3 and Lyn Kinase. *PLoS One* **2015,** *10* (8), e0135738.

118. Isakov, N., Immunoreceptor tyrosine-based activation motif (ITAM), a unique module linking antigen and Fc receptors to their signaling cascades. *J Leukoc Biol* **1997,** *61* (1), 6-16.

119. Ghazizadeh, S.; Bolen, J. B.; Fleit, H. B., Physical and functional association of Src-related protein tyrosine kinases with Fc gamma RII in monocytic THP-1 cells. *J Biol Chem* **1994,** *269* (12), 8878-84.

120. Wang, A. V.; Scholl, P. R.; Geha, R. S., Physical and functional association of the high affinity immunoglobulin G receptor (Fc gamma RI) with the kinases Hck and Lyn. *J Exp Med* **1994,** *180* (3), 1165-70.

121. Kiener, P. A.; Rankin, B. M.; Burkhardt, A. L.; Schieven, G. L.; Gilliland, L. K.; Rowley, R. B.; Bolen, J. B.; Ledbetter, J. A., Cross-linking of Fc gamma receptor I (Fc gamma RI) and receptor II (Fc gamma RII) on monocytic cells activates a signal transduction pathway common to both Fc receptors that involves the stimulation of p72 Syk protein tyrosine kinase. *J Biol Chem* **1993,** *268* (32), 24442-8.

122. Takai, T., Roles of Fc receptors in autoimmunity. *Nat Rev Immunol* **2002,** *2* (8), 580-92.

123. Liao, F.; Shin, H. S.; Rhee, S. G., Tyrosine phosphorylation of phospholipase C-gamma 1 induced by cross-linking of the high-affinity or low-affinity Fc receptor for IgG in U937 cells. *Proc Natl Acad Sci U S A* **1992,** *89* (8), 3659-63.

124. Jun, J. E.; Yang, M.; Chen, H.; Chakraborty, A. K.; Roose, J. P., Activation of extracellular signal-regulated kinase but not of p38 mitogen-activated protein kinase pathways in lymphocytes requires allosteric activation of SOS. *Mol Cell Biol* **2013,** *33* (12), 2470-84.

125. Fuller, J. P.; Stavenhagen, J. B.; Teeling, J. L., New roles for Fc receptors in neurodegeneration-the impact on Immunotherapy for Alzheimer's Disease. *Front Neurosci* **2014,** *8*, 235.

126. Radstake, T. R.; van Lieshout, A. W.; van Riel, P. L.; van den Berg, W. B.; Adema, G. J., Dendritic cells, Fc{gamma} receptors, and Toll-like receptors: potential allies in the battle against rheumatoid arthritis. *Ann Rheum Dis* **2005,** *64* (11), 1532-8.

127. Getahun, A.; Cambier, J. C., Of ITIMs, ITAMs, and ITAMis: revisiting immunoglobulin Fc receptor signaling. *Immunol Rev* **2015,** *268* (1), 66-73.

128. Ono, M.; Bolland, S.; Tempst, P.; Ravetch, J. V., Role of the inositol phosphatase SHIP in negative regulation of the immune system by the receptor Fc(gamma)RIIB. *Nature* **1996,** *383* (6597), 263-6.

129. Ben Mkaddem, S.; Hayem, G.; Jönsson, F.; Rossato, E.; Boedec, E.; Boussetta, T.; El Benna, J.; Launay, P.; Goujon, J. M.; Benhamou, M.; Bruhns, P.; Monteiro, R. C., Shifting FcγRIIA-ITAM from activation to inhibitory configuration ameliorates arthritis. *J Clin Invest* **2014,** *124* (9), 3945-59.

130. Ben Mkaddem, S.; Aloulou, M.; Benhamou, M.; Monteiro, R. C., Role of FcγRIIIA (CD16) in IVIg-mediated anti-inflammatory function. *J Clin Immunol* **2014,** *34 Suppl 1*, S46-50.

131. Aloulou, M.; Ben Mkaddem, S.; Biarnes-Pelicot, M.; Boussetta, T.; Souchet, H.; Rossato, E.; Benhamou, M.; Crestani, B.; Zhu, Z.; Blank, U.; Launay, P.; Monteiro, R. C., IgG1 and IVIg induce inhibitory ITAM signaling through FcγRIII controlling inflammatory responses. *Blood* **2012,** *119* (13), 3084-96.

132. Ivashkiv, L. B., How ITAMs inhibit signaling. *Sci Signal* **2011,** *4* (169), pe20.

133. Pfirsch-Maisonnas, S.; Aloulou, M.; Xu, T.; Claver, J.; Kanamaru, Y.; Tiwari, M.; Launay, P.; Monteiro, R. C.; Blank, U., Inhibitory ITAM signaling traps activating receptors with the phosphatase SHP-1 to form polarized "inhibisome" clusters. *Sci Signal* **2011,** *4* (169), ra24.

134. Bournazos, S.; Wang, T. T.; Ravetch, J. V., The Role and Function of Fcγ Receptors on Myeloid Cells. *Microbiol Spectr* **2016,** *4* (6).

135. Sutterwala, F. S.; Noel, G. J.; Clynes, R.; Mosser, D. M., Selective suppression of interleukin-12 induction after macrophage receptor ligation. *J Exp Med* **1997,** *185* (11), 1977-85.

136. Yuasa, T.; Kubo, S.; Yoshino, T.; Ujike, A.; Matsumura, K.; Ono, M.; Ravetch, J. V.; Takai, T., Deletion of fcgamma receptor IIB renders H-2(b) mice susceptible to collagen-induced arthritis. *J Exp Med* **1999,** *189* (1), 187-94.

137. Joller, N.; Weber, S. S.; Müller, A. J.; Spörri, R.; Selchow, P.; Sander, P.; Hilbi, H.; Oxenius, A., Antibodies protect against intracellular bacteria by Fc receptor-mediated lysosomal targeting. *Proc Natl Acad Sci U S A* **2010,** *107* (47), 20441-6.

138. Gordon, S. B.; Irving, G. R.; Lawson, R. A.; Lee, M. E.; Read, R. C., Intracellular trafficking and killing of Streptococcus pneumoniae by human alveolar macrophages are influenced by opsonins. *Infect Immun* **2000,** *68* (4), 2286-93.

139. Maglione, P. J.; Xu, J.; Casadevall, A.; Chan, J., Fc gamma receptors regulate immune activation and susceptibility during Mycobacterium tuberculosis infection. *J Immunol* **2008,** *180* (5), 3329-38.

140. Yeap, W. H.; Wong, K. L.; Shimasaki, N.; Teo, E. C.; Quek, J. K.; Yong, H. X.; Diong, C. P.; Bertoletti, A.; Linn, Y. C.; Wong, S. C., CD16 is indispensable for antibody-dependent cellular cytotoxicity by human monocytes. *Sci Rep* **2016,** *6*, 34310.

141. Overdijk, M. B.; Verploegen, S.; Ortiz Buijsse, A.; Vink, T.; Leusen, J. H.; Bleeker, W. K.; Parren, P. W., Crosstalk between human IgG isotypes and murine effector cells. *J Immunol* **2012,** *189* (7), 3430-8.

142. Johnson, W. J.; Bolognesi, D. P.; Adams, D. O., Antibody-dependent cytolysis (ADCC) of tumor cells by activated murine macrophages is a two-step process: quantification of target binding and subsequent target lysis. *Cell Immunol* **1984,** *83* (1), 170-80.

143. Hirvinen, M.; Heiskanen, R.; Oksanen, M.; Pesonen, S.; Liikanen, I.; Joensuu, T.; Kanerva, A.; Cerullo, V.; Hemminki, A., Fc-gamma receptor polymorphisms as predictive and prognostic factors in patients receiving oncolytic adenovirus treatment. *J Transl Med* **2013,** *11*, 193.

144. Cañete, J. D.; Suárez, B.; Hernández, M. V.; Sanmartí, R.; Rego, I.; Celis, R.; Moll, C.; Pinto, J. A.; Blanco, F. J.; Lozano, F., Influence of variants of Fc gamma receptors IIA and IIIA on the American College of Rheumatology and European League Against Rheumatism responses to anti-tumour necrosis factor alpha therapy in rheumatoid arthritis. *Ann Rheum Dis* **2009,** *68* (10), 1547-52.

145. Clark, M. R.; Clarkson, S. B.; Ory, P. A.; Stollman, N.; Goldstein, I. M., Molecular basis for a polymorphism involving Fc receptor II on human monocytes. *J Immunol* **1989,** *143* (5), 1731-4.

146. Parren, P. W.; Warmerdam, P. A.; Boeije, L. C.; Arts, J.; Westerdaal, N. A.; Vlug, A.; Capel, P. J.; Aarden, L. A.; van de Winkel, J. G., On the interaction of IgG subclasses with the low affinity Fc gamma RIIa (CD32) on human monocytes, neutrophils, and platelets. Analysis of a functional polymorphism to human IgG2. *J Clin Invest* **1992,** *90* (4), 1537-46.

147. Denomme, G. A.; Warkentin, T. E.; Horsewood, P.; Sheppard, J. A.; Warner, M. N.; Kelton, J. G., Activation of platelets by sera containing IgG1 heparin-dependent antibodies: an explanation for the predominance of the Fc gammaRIIa "low responder" (his131) gene in patients with heparin-induced thrombocytopenia. *J Lab Clin Med* **1997,** *130* (3), 278-84.

148. Lehrnbecher, T.; Foster, C. B.; Zhu, S.; Leitman, S. F.; Goldin, L. R.; Huppi, K.; Chanock, S. J., Variant genotypes of the low-affinity Fcgamma receptors in two control populations and a review of low-affinity Fcgamma receptor polymorphisms in control and disease populations. *Blood* **1999,** *94* (12), 4220-32.

149. Asano, K.; Matsushita, T.; Umeno, J.; Hosono, N.; Takahashi, A.; Kawaguchi, T.; Matsumoto, T.; Matsui, T.; Kakuta, Y.; Kinouchi, Y.; Shimosegawa, T.; Hosokawa, M.; Arimura, Y.; Shinomura, Y.; Kiyohara, Y.; Tsunoda, T.; Kamatani, N.; Iida, M.; Nakamura, Y.; Kubo, M., A genome-wide association study identifies three new susceptibility loci for ulcerative colitis in the Japanese population. *Nat Genet* **2009,** *41* (12), 1325-9.

150. Weersma, R. K.; Crusius, J. B.; Roberts, R. L.; Koeleman, B. P.; Palomino-Morales, R.; Wolfkamp, S.; Hollis-Moffatt, J. E.; Festen, E. A.; Meisneris, S.; Heijmans, R.; Noble, C. L.; Gearry, R. B.; Barclay, M. L.; Gómez-Garcia, M.; Lopez-Nevot, M. A.; Nieto, A.; Rodrigo, L.; Radstake, T. R.; van Bodegraven, A. A.; Wijmenga, C.; Merriman, T. R.; Stokkers, P. C.; Peña, A. S.; Martín, J.; Alizadeh, B. Z., Association of FcgR2a, but not FcgR3a, with inflammatory bowel diseases across three Caucasian populations. *Inflamm Bowel Dis* **2010,** *16* (12), 2080-9.

151. Khor, C. C.; Davila, S.; Breunis, W. B.; Lee, Y. C.; Shimizu, C.; Wright, V. J.; Yeung, R. S.; Tan, D. E.; Sim, K. S.; Wang, J. J.; Wong, T. Y.; Pang, J.; Mitchell, P.; Cimaz, R.; Dahdah, N.; Cheung, Y. F.; Huang, G. Y.; Yang, W.; Park, I. S.; Lee, J. K.; Wu, J. Y.; Levin, M.; Burns, J. C.; Burgner, D.; Kuijpers, T. W.; Hibberd, M. L.; Consortium, H. K. S. K. D. G.; Consortium, K. K. D. G.; Consortium, T. K. D. G.; Consortium, I. K. D. G.; Consortium, U. K. D. G.; Study, B. M. E., Genome-wide association study identifies FCGR2A as a susceptibility locus for Kawasaki disease. *Nat Genet* **2011,** *43* (12), 1241-6.

152. van der Pol, W. L.; van den Berg, L. H.; Scheepers, R. H.; van der Bom, J. G.; van Doorn, P. A.; van Koningsveld, R.; van den Broek, M. C.; Wokke, J. H.; van de Winkel, J. G., IgG receptor IIa alleles determine susceptibility and severity of Guillain-Barré syndrome. *Neurology* **2000,** *54* (8), 1661-5.

153. Alizadeh, B. Z.; Valdigem, G.; Coenen, M. J.; Zhernakova, A.; Franke, B.; Monsuur, A.; van Riel, P. L.; Barrera, P.; Radstake, T. R.; Roep, B. O.; Wijmenga, C.; Koeleman, B. P., Association analysis of functional variants of the FcgRIIa and FcgRIIIa genes with type 1 diabetes, celiac disease and rheumatoid arthritis. *Hum Mol Genet* **2007,** *16* (21), 2552-9.

154. van Sorge, N. M.; van der Pol, W. L.; van de Winkel, J. G., FcgammaR polymorphisms: Implications for function, disease susceptibility and immunotherapy. *Tissue Antigens* **2003,** *61* (3), 189-202.

155. Beppler, J.; Koehler-Santos, P.; Pasqualim, G.; Matte, U.; Alho, C. S.; Dias, F. S.; Kowalski, T. W.; Velasco, I. T.; Monteiro, R. C.; Pinheiro da Silva, F., Fc Gamma Receptor IIA (CD32A) R131 Polymorphism as a Marker of Genetic Susceptibility to Sepsis. *Inflammation* **2016,** *39* (2), 518-25.

156. Endeman, H.; Cornips, M. C.; Grutters, J. C.; van den Bosch, J. M.; Ruven, H. J.; van Velzen-Blad, H.; Rijkers, G. T.; Biesma, D. H., The Fcgamma receptor IIA-R/R131 genotype is associated with severe sepsis in community-acquired pneumonia. *Clin Vaccine Immunol* **2009,** *16* (7), 1087-90.

157. Platonov, A. E.; Shipulin, G. A.; Vershinina, I. V.; Dankert, J.; van de Winkel, J. G.; Kuijper, E. J., Association of human Fc gamma RIIa (CD32) polymorphism with susceptibility to and severity of meningococcal disease. *Clin Infect Dis* **1998,** *27* (4), 746-50.

158. Dávila-Fajardo, C. L.; van der Straaten, T.; Baak-Pablo, R.; Medarde Caballero, C.; Cabeza Barrera, J.; Huizinga, T. W.; Guchelaar, H. J.; Swen, J. J., FcGR genetic polymorphisms and the response to adalimumab in patients with rheumatoid arthritis. *Pharmacogenomics* **2015,** *16* (4), 373-81.

159. Yamamoto, K.; Kobayashi, T.; Sugita, N.; Tai, H.; Yoshie, H., The FcgammaRIIa polymorphism influences production of interleukin-1 by mononuclear cells. *Int J Immunogenet* **2007,** *34* (5), 369-72.

160. Nicu, E. A.; Van der Velden, U.; Everts, V.; Van Winkelhoff, A. J.; Roos, D.; Loos, B. G., Hyper-reactive PMNs in FcgammaRIIa 131 H/H genotype periodontitis patients. *J Clin Periodontol* **2007,** *34* (11), 938-45.

161. Gelfand, E. W., Intravenous immune globulin in autoimmune and inflammatory diseases. *N Engl J Med* **2013,** *368* (8), 777.

162. Miller, J. L.; Petteway, S. R.; Lee, D. C., Ensuring the pathogen safety of intravenous immunoglobulin and other human plasma-derived therapeutic proteins. *J Allergy Clin Immunol* **2001,** *108* (4 Suppl), S91-4.

163. Lemm, G., Composition and properties of IVIg preparations that affect tolerability and therapeutic efficacy. *Neurology* **2002,** *59* (12 Suppl 6), S28-32.

164. Duhem, C.; Dicato, M. A.; Ries, F., Side-effects of intravenous immune globulins. *Clin Exp Immunol* **1994,** *97 Suppl 1*, 79-83.

165. Perez, E. E.; Orange, J. S.; Bonilla, F.; Chinen, J.; Chinn, I. K.; Dorsey, M.; El-Gamal, Y.; Harville, T. O.; Hossny, E.; Mazer, B.; Nelson, R.; Secord, E.; Jordan, S. C.; Stiehm, E. R.; Vo, A. A.; Ballow, M., Update on the use of immunoglobulin in human disease: A review of evidence. *J Allergy Clin Immunol* **2017,** *139* (3S), S1-S46.

166. Anderson, D.; Ali, K.; Blanchette, V.; Brouwers, M.; Couban, S.; Radmoor, P.; Huebsch, L.; Hume, H.; McLeod, A.; Meyer, R.; Moltzan, C.; Nahirniak, S.; Nantel, S.; Pineo, G.; Rock, G., Guidelines on the use of intravenous immune globulin for hematologic conditions. *Transfus Med Rev* **2007,** *21* (2 Suppl 1), S9-56.

167. Galeotti, C.; Kaveri, S. V.; Bayry, J., IVIG-mediated effector functions in autoimmune and inflammatory diseases. *Int Immunol* **2017,** *29* (11), 491-498.

168. Prins, C.; Gelfand, E. W.; French, L. E., Intravenous immunoglobulin: properties, mode of action and practical use in dermatology. *Acta Derm Venereol* **2007,** *87* (3), 206-18.

169. Nimmerjahn, F.; Ravetch, J. V., Anti-inflammatory actions of intravenous immunoglobulin. *Annu Rev Immunol* **2008,** *26*, 513-33.

170. Nimmerjahn, F.; Ravetch, J. V., The antiinflammatory activity of IgG: the intravenous IgG paradox. *J Exp Med* **2007,** *204* (1), 11-5.

171. Abe, Y.; Horiuchi, A.; Miyake, M.; Kimura, S., Anti-cytokine nature of natural human immunoglobulin: one possible mechanism of the clinical effect of intravenous immunoglobulin therapy. *Immunol Rev* **1994,** *139*, 5-19.

172. Debré, M.; Bonnet, M. C.; Fridman, W. H.; Carosella, E.; Philippe, N.; Reinert, P.; Vilmer, E.; Kaplan, C.; Teillaud, J. L.; Griscelli, C., Infusion of Fc gamma fragments for treatment of children with acute immune thrombocytopenic purpura. *Lancet* **1993,** *342* (8877), 945-9.

173. Vogelpoel, L. T.; Baeten, D. L.; de Jong, E. C.; den Dunnen, J., Control of cytokine production by human fc gamma receptors: implications for pathogen defense and autoimmunity. *Front Immunol* **2015,** *6*, 79.

174. Rath, T.; Baker, K.; Pyzik, M.; Blumberg, R. S., Regulation of immune responses by the neonatal fc receptor and its therapeutic implications. *Front Immunol* **2014,** *5*, 664.

175. Hansen, R. J.; Balthasar, J. P., Intravenous immunoglobulin mediates an increase in anti-platelet antibody clearance via the FcRn receptor. *Thromb Haemost* **2002,** *88* (6), 898-9.

176. Crow, A. R.; Suppa, S. J.; Chen, X.; Mott, P. J.; Lazarus, A. H., The neonatal Fc receptor (FcRn) is not required for IVIg or anti-CD44 monoclonal antibody-mediated amelioration of murine immune thrombocytopenia. *Blood* **2011,** *118* (24), 6403-6.

177. Schwab, I.; Lux, A.; Nimmerjahn, F., Pathways Responsible for Human Autoantibody and Therapeutic Intravenous IgG Activity in Humanized Mice. *Cell Rep* **2015,** *13* (3), 610-620.

178. Anthony, R. M.; Kobayashi, T.; Wermeling, F.; Ravetch, J. V., Intravenous gammaglobulin suppresses inflammation through a novel T(H)2 pathway. *Nature* **2011,** *475* (7354), 110-3.

179. Campbell, I. K.; Miescher, S.; Branch, D. R.; Mott, P. J.; Lazarus, A. H.; Han, D.; Maraskovsky, E.; Zuercher, A. W.; Neschadim, A.; Leontyev, D.; McKenzie, B. S.; Käsermann, F., Therapeutic effect of IVIG on inflammatory arthritis in mice is dependent on the Fc portion and independent of sialylation or basophils. *J Immunol* **2014,** *192* (11), 5031-8.

180. Anthony, R. M.; Wermeling, F.; Karlsson, M. C.; Ravetch, J. V., Identification of a receptor required for the anti-inflammatory activity of IVIG. *Proc Natl Acad Sci U S A* **2008,** *105* (50), 19571-8.

181. Leontyev, D.; Katsman, Y.; Ma, X. Z.; Miescher, S.; Käsermann, F.; Branch, D. R., Sialylation-independent mechanism involved in the amelioration of murine immune thrombocytopenia using intravenous gammaglobulin. *Transfusion* **2012,** *52* (8), 1799-805.

182. Schwab, I.; Biburger, M.; Krönke, G.; Schett, G.; Nimmerjahn, F., IVIg-mediated amelioration of ITP in mice is dependent on sialic acid and SIGNR1. *Eur J Immunol* **2012,** *42* (4), 826-30.

183. Audia, S.; Santegoets, K.; Laarhoven, A. G.; Vidarsson, G.; Facy, O.; Ortega-Deballon, P.; Samson, M.; Janikashvili, N.; Saas, P.; Bonnotte, B.; Radstake, T. R., Fcγ receptor expression on splenic macrophages in adult immune thrombocytopenia. *Clin Exp Immunol* **2017,** *188* (2), 275-282.

184. Ogata, S.; Shimizu, C.; Franco, A.; Touma, R.; Kanegaye, J. T.; Choudhury, B. P.; Naidu, N. N.; Kanda, Y.; Hoang, L. T.; Hibberd, M. L.; Tremoulet, A. H.; Varki, A.; Burns, J. C., Treatment response in Kawasaki disease is associated with sialylation levels of endogenous but not therapeutic intravenous immunoglobulin G. *PLoS One* **2013,** *8* (12), e81448.

185. Sehgal, K.; Guo, X.; Koduru, S.; Shah, A.; Lin, A.; Yan, X.; Dhodapkar, K. M., Plasmacytoid dendritic cells, interferon signaling, and FcγR contribute to pathogenesis and therapeutic response in childhood immune thrombocytopenia. *Sci Transl Med* **2013,** *5* (193), 193ra89.

186. Neurath, M., Current and emerging therapeutic targets for IBD. *Nat Rev Gastroenterol Hepatol* **2017,** *14* (11), 688.

187. Abraham, C.; Cho, J. H., Inflammatory bowel disease. *N Engl J Med* **2009,** *361* (21), 2066-78.

188. Loftus, E. V.; Sandborn, W. J., Epidemiology of inflammatory bowel disease. *Gastroenterol Clin North Am* **2002,** *31* (1), 1-20.

189. Baumgart, D. C.; Sandborn, W. J., Crohn's disease. *Lancet* **2012,** *380* (9853), 1590-605.

190. Ungaro, R.; Mehandru, S.; Allen, P. B.; Peyrin-Biroulet, L.; Colombel, J. F., Ulcerative colitis. *Lancet* **2017,** *389* (10080), 1756-1770.

191. Danese, S.; Fiocchi, C., Ulcerative colitis. *N Engl J Med* **2011,** *365* (18), 1713-25.

192. Bernstein, C. N.; Wajda, A.; Blanchard, J. F., The clustering of other chronic inflammatory diseases in inflammatory bowel disease: a population-based study. *Gastroenterology* **2005,** *129* (3), 827-36.

193. Rocchi, A.; Benchimol, E. I.; Bernstein, C. N.; Bitton, A.; Feagan, B.; Panaccione, R.; Glasgow, K. W.; Fernandes, A.; Ghosh, S., Inflammatory bowel disease: a Canadian burden of illness review. *Can J Gastroenterol* **2012,** *26* (11), 811-7.

194. Kaplan, G. G., The global burden of IBD: from 2015 to 2025. *Nat Rev Gastroenterol Hepatol* **2015,** *12* (12), 720-7.

195. Ng, S. C.; Shi, H. Y.; Hamidi, N.; Underwood, F. E.; Tang, W.; Benchimol, E. I.; Panaccione, R.; Ghosh, S.; Wu, J. C. Y.; Chan, F. K. L.; Sung, J. J. Y.; Kaplan, G. G., Worldwide incidence and prevalence of inflammatory bowel disease in the 21st century: a systematic review of population-based studies. *Lancet* **2018,** *390* (10114), 2769-2778.

196. Zelinkova, Z.; Bultman, E.; Vogelaar, L.; Bouziane, C.; Kuipers, E. J.; van der Woude, C. J., Sex-dimorphic adverse drug reactions to immune suppressive agents in inflammatory bowel disease. *World J Gastroenterol* **2012,** *18* (47), 6967-73.

197. Nguyen, G. C.; Chong, C. A.; Chong, R. Y., National estimates of the burden of inflammatory bowel disease among racial and ethnic groups in the United States. *J Crohns Colitis* **2014,** *8* (4), 288-95.

198. Afzali, A.; Cross, R. K., Racial and Ethnic Minorities with Inflammatory Bowel Disease in the United States: A Systematic Review of Disease Characteristics and Differences. *Inflamm Bowel Dis* **2016,** *22* (8), 2023-40.

199. Ahluwalia, B.; Moraes, L.; Magnusson, M. K.; Öhman, L., Immunopathogenesis of inflammatory bowel disease and mechanisms of biological therapies. *Scand J Gastroenterol* **2018,** *53* (4), 379-389.

200. Neurath, M. F., Cytokines in inflammatory bowel disease. *Nat Rev Immunol* **2014,** *14* (5), 329-42.

201. Mawdsley, J. E.; Rampton, D. S., The role of psychological stress in inflammatory bowel disease. *Neuroimmunomodulation* **2006,** *13* (5-6), 327-36.

202. Maunder, R. G., Evidence that stress contributes to inflammatory bowel disease: evaluation, synthesis, and future directions. *Inflamm Bowel Dis* **2005,** *11* (6), 600-8.

203. Kaplan, G. G.; Hubbard, J.; Korzenik, J.; Sands, B. E.; Panaccione, R.; Ghosh, S.; Wheeler, A. J.; Villeneuve, P. J., The inflammatory bowel diseases and ambient air pollution: a novel association. *Am J Gastroenterol* **2010,** *105* (11), 2412-9.

204. van Eeden, S. F.; Tan, W. C.; Suwa, T.; Mukae, H.; Terashima, T.; Fujii, T.; Qui, D.; Vincent, R.; Hogg, J. C., Cytokines involved in the systemic inflammatory response induced by exposure to particulate matter air pollutants (PM(10)). *Am J Respir Crit Care Med* **2001,** *164* (5), 826-30.

205. Thia, K. T.; Loftus, E. V.; Sandborn, W. J.; Yang, S. K., An update on the epidemiology of inflammatory bowel disease in Asia. *Am J Gastroenterol* **2008,** *103* (12), 3167-82.

206. Knight-Sepulveda, K.; Kais, S.; Santaolalla, R.; Abreu, M. T., Diet and Inflammatory Bowel Disease. *Gastroenterol Hepatol (N Y)* **2015,** *11* (8), 511-20.

207. Amre, D. K.; D'Souza, S.; Morgan, K.; Seidman, G.; Lambrette, P.; Grimard, G.; Israel, D.; Mack, D.; Ghadirian, P.; Deslandres, C.; Chotard, V.; Budai, B.; Law, L.; Levy, E.; Seidman, E. G., Imbalances in dietary consumption of fatty acids, vegetables, and fruits are associated with risk for Crohn's disease in children. *Am J Gastroenterol* **2007,** *102* (9), 2016-25.

208. Leslie, W. D.; Miller, N.; Rogala, L.; Bernstein, C. N., Vitamin D status and bone density in recently diagnosed inflammatory bowel disease: the Manitoba IBD Cohort Study. *Am J Gastroenterol* **2008,** *103* (6), 1451-9.

209. Nicholson, I.; Dalzell, A. M.; El-Matary, W., Vitamin D as a therapy for colitis: a systematic review. *J Crohns Colitis* **2012,** *6* (4), 405-11.

210. Yang, H.; McElree, C.; Roth, M. P.; Shanahan, F.; Targan, S. R.; Rotter, J. I., Familial empirical risks for inflammatory bowel disease: differences between Jews and non-Jews. *Gut* **1993,** *34* (4), 517-24.

211. Jostins, L.; Ripke, S.; Weersma, R. K.; Duerr, R. H.; McGovern, D. P.; Hui, K. Y.; Lee, J. C.; Schumm, L. P.; Sharma, Y.; Anderson, C. A.; Essers, J.; Mitrovic, M.; Ning, K.; Cleynen, I.; Theatre, E.; Spain, S. L.; Raychaudhuri, S.; Goyette, P.; Wei, Z.; Abraham, C.; Achkar, J. P.; Ahmad, T.; Amininejad, L.; Ananthakrishnan, A. N.; Andersen, V.; Andrews, J. M.; Baidoo, L.; Balschun, T.; Bampton, P. A.; Bitton, A.; Boucher, G.; Brand, S.; Büning, C.; Cohain, A.; Cichon, S.; D'Amato, M.; De Jong, D.; Devaney, K. L.; Dubinsky, M.; Edwards, C.; Ellinghaus, D.; Ferguson, L. R.; Franchimont, D.; Fransen, K.; Gearry, R.; Georges, M.; Gieger, C.; Glas, J.; Haritunians, T.; Hart, A.; Hawkey, C.; Hedl, M.; Hu, X.; Karlsen, T. H.; Kupcinskas, L.; Kugathasan, S.; Latiano, A.; Laukens, D.; Lawrance, I. C.; Lees, C. W.; Louis, E.; Mahy, G.; Mansfield, J.; Morgan, A. R.; Mowat, C.; Newman, W.; Palmieri, O.; Ponsioen, C. Y.; Potocnik, U.; Prescott, N. J.; Regueiro, M.; Rotter, J. I.;

Russell, R. K.; Sanderson, J. D.; Sans, M.; Satsangi, J.; Schreiber, S.; Simms, L. A.; Sventoraityte, J.; Targan, S. R.; Taylor, K. D.; Tremelling, M.; Verspaget, H. W.; De Vos, M.; Wijmenga, C.; Wilson, D. C.; Winkelmann, J.; Xavier, R. J.; Zeissig, S.; Zhang, B.; Zhang, C. K.; Zhao, H.; Silverberg, M. S.; Annese, V.; Hakonarson, H.; Brant, S. R.; Radford-Smith, G.; Mathew, C. G.; Rioux, J. D.; Schadt, E. E.; Daly, M. J.; Franke, A.; Parkes, M.; Vermeire, S.; Barrett, J. C.; Cho, J. H.; (IIBDGC), I. I. G. C., Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. *Nature* **2012,** *491* (7422), 119-24.

212. de Lange, K. M.; Moutsianas, L.; Lee, J. C.; Lamb, C. A.; Luo, Y.; Kennedy, N. A.; Jostins, L.; Rice, D. L.; Gutierrez-Achury, J.; Ji, S. G.; Heap, G.; Nimmo, E. R.; Edwards, C.; Henderson, P.; Mowat, C.; Sanderson, J.; Satsangi, J.; Simmons, A.; Wilson, D. C.; Tremelling, M.; Hart, A.; Mathew, C. G.; Newman, W. G.; Parkes, M.; Lees, C. W.; Uhlig, H.; Hawkey, C.; Prescott, N. J.; Ahmad, T.; Mansfield, J. C.; Anderson, C. A.; Barrett, J. C., Genome-wide association study implicates immune activation of multiple integrin genes in inflammatory bowel disease. *Nat Genet* **2017,** *49* (2), 256-261.

213. Ogura, Y.; Bonen, D. K.; Inohara, N.; Nicolae, D. L.; Chen, F. F.; Ramos, R.; Britton, H.; Moran, T.; Karaliuskas, R.; Duerr, R. H.; Achkar, J. P.; Brant, S. R.; Bayless, T. M.; Kirschner, B. S.; Hanauer, S. B.; Nuñez, G.; Cho, J. H., A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature* **2001,** *411* (6837), 603-6.

214. Zhang, Y. Z.; Li, Y. Y., Inflammatory bowel disease: pathogenesis. *World J Gastroenterol* **2014,** *20* (1), 91-9.

215. Feagan, B. G.; Sandborn, W. J.; Gasink, C.; Jacobstein, D.; Lang, Y.; Friedman, J. R.; Blank, M. A.; Johanns, J.; Gao, L. L.; Miao, Y.; Adedokun, O. J.; Sands, B. E.; Hanauer, S.

B.; Vermeire, S.; Targan, S.; Ghosh, S.; de Villiers, W. J.; Colombel, J. F.; Tulassay, Z.; Seidler, U.; Salzberg, B. A.; Desreumaux, P.; Lee, S. D.; Loftus, E. V.; Dieleman, L. A.; Katz, S.; Rutgeerts, P.; Group, U. I.-U. S., Ustekinumab as Induction and Maintenance Therapy for Crohn's Disease. *N Engl J Med* **2016,** *375* (20), 1946-1960.

216. Natividad, J. M.; Verdu, E. F., Modulation of intestinal barrier by intestinal microbiota: pathological and therapeutic implications. *Pharmacol Res* **2013,** *69* (1), 42-51.

217. Joossens, M.; Huys, G.; Cnockaert, M.; De Preter, V.; Verbeke, K.; Rutgeerts, P.; Vandamme, P.; Vermeire, S., Dysbiosis of the faecal microbiota in patients with Crohn's disease and their unaffected relatives. *Gut* **2011,** *60* (5), 631-7.

218. Bäckhed, F.; Ley, R. E.; Sonnenburg, J. L.; Peterson, D. A.; Gordon, J. I., Host-bacterial mutualism in the human intestine. *Science* **2005,** *307* (5717), 1915-20.

219. Gill, S. R.; Pop, M.; Deboy, R. T.; Eckburg, P. B.; Turnbaugh, P. J.; Samuel, B. S.; Gordon, J. I.; Relman, D. A.; Fraser-Liggett, C. M.; Nelson, K. E., Metagenomic analysis of the human distal gut microbiome. *Science* **2006,** *312* (5778), 1355-9.

220. Jandhyala, S. M.; Talukdar, R.; Subramanyam, C.; Vuyyuru, H.; Sasikala, M.; Nageshwar Reddy, D., Role of the normal gut microbiota. *World J Gastroenterol* **2015,** *21* (29), 8787-803.

221. Martinez, C.; Antolin, M.; Santos, J.; Torrejon, A.; Casellas, F.; Borruel, N.; Guarner, F.; Malagelada, J. R., Unstable composition of the fecal microbiota in ulcerative colitis during clinical remission. *Am J Gastroenterol* **2008,** *103* (3), 643-8.

222. Frank, D. N.; St Amand, A. L.; Feldman, R. A.; Boedeker, E. C.; Harpaz, N.; Pace, N. R., Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proc Natl Acad Sci U S A* **2007,** *104* (34), 13780-5.

223. Meconi, S.; Vercellone, A.; Levillain, F.; Payré, B.; Al Saati, T.; Capilla, F.; Desreumaux, P.; Darfeuille-Michaud, A.; Altare, F., Adherent-invasive Escherichia coli isolated from Crohn's disease patients induce granulomas in vitro. *Cell Microbiol* **2007,** *9* (5), 1252-61.

224. Martinez-Medina, M.; Aldeguer, X.; Gonzalez-Huix, F.; Acero, D.; Garcia-Gil, L. J., Abnormal microbiota composition in the ileocolonic mucosa of Crohn's disease patients as revealed by polymerase chain reaction-denaturing gradient gel electrophoresis. *Inflamm Bowel Dis* **2006,** *12* (12), 1136-45.

225. Ni, J.; Wu, G. D.; Albenberg, L.; Tomov, V. T., Gut microbiota and IBD: causation or correlation? *Nat Rev Gastroenterol Hepatol* **2017,** *14* (10), 573-584.

226. Michielan, A.; D'Incà, R., Intestinal Permeability in Inflammatory Bowel Disease: Pathogenesis, Clinical Evaluation, and Therapy of Leaky Gut. *Mediators Inflamm* **2015,** *2015*, 628157.

227. Groschwitz, K. R.; Hogan, S. P., Intestinal barrier function: molecular regulation and disease pathogenesis. *J Allergy Clin Immunol* **2009,** *124* (1), 3-20; quiz 21-2.

228. Wehkamp, J.; Harder, J.; Weichenthal, M.; Mueller, O.; Herrlinger, K. R.; Fellermann, K.; Schroeder, J. M.; Stange, E. F., Inducible and constitutive beta-defensins are differentially expressed in Crohn's disease and ulcerative colitis. *Inflamm Bowel Dis* **2003,** *9* (4), 215-23.

229. Zeissig, S.; Bürgel, N.; Günzel, D.; Richter, J.; Mankertz, J.; Wahnschaffe, U.; Kroesen, A. J.; Zeitz, M.; Fromm, M.; Schulzke, J. D., Changes in expression and distribution of claudin 2, 5 and 8 lead to discontinuous tight junctions and barrier dysfunction in active Crohn's disease. *Gut* **2007,** *56* (1), 61-72.

230. Wapenaar, M. C.; Monsuur, A. J.; van Bodegraven, A. A.; Weersma, R. K.; Bevova, M. R.; Linskens, R. K.; Howdle, P.; Holmes, G.; Mulder, C. J.; Dijkstra, G.; van Heel, D. A.;

Wijmenga, C., Associations with tight junction genes PARD3 and MAGI2 in Dutch patients point to a common barrier defect for coeliac disease and ulcerative colitis. *Gut* **2008,** *57* (4), 463-7.

231. Johnston, D. G.; Corr, S. C., Toll-Like Receptor Signalling and the Control of Intestinal Barrier Function. *Methods Mol Biol* **2016,** *1390*, 287-300.

232. de Souza, H. S.; Fiocchi, C., Immunopathogenesis of IBD: current state of the art. *Nat Rev Gastroenterol Hepatol* **2016,** *13* (1), 13-27.

233. Brazil, J. C.; Louis, N. A.; Parkos, C. A., The role of polymorphonuclear leukocyte trafficking in the perpetuation of inflammation during inflammatory bowel disease. *Inflamm Bowel Dis* **2013,** *19* (7), 1556-65.

234. Geremia, A.; Arancibia-Cárcamo, C. V.; Fleming, M. P.; Rust, N.; Singh, B.; Mortensen, N. J.; Travis, S. P.; Powrie, F., IL-23-responsive innate lymphoid cells are increased in inflammatory bowel disease. *J Exp Med* **2011,** *208* (6), 1127-33.

235. Tait Wojno, E. D.; Artis, D., Innate lymphoid cells: balancing immunity, inflammation, and tissue repair in the intestine. *Cell Host Microbe* **2012,** *12* (4), 445-57.

236. Rimoldi, M.; Chieppa, M.; Salucci, V.; Avogadri, F.; Sonzogni, A.; Sampietro, G. M.; Nespoli, A.; Viale, G.; Allavena, P.; Rescigno, M., Intestinal immune homeostasis is regulated by the crosstalk between epithelial cells and dendritic cells. *Nat Immunol* **2005,** *6* (5), 507-14.

237. Hart, A. L.; Al-Hassi, H. O.; Rigby, R. J.; Bell, S. J.; Emmanuel, A. V.; Knight, S. C.; Kamm, M. A.; Stagg, A. J., Characteristics of intestinal dendritic cells in inflammatory bowel diseases. *Gastroenterology* **2005,** *129* (1), 50-65.

238. Zigmond, E.; Bernshtein, B.; Friedlander, G.; Walker, C. R.; Yona, S.; Kim, K. W.; Brenner, O.; Krauthgamer, R.; Varol, C.; Müller, W.; Jung, S., Macrophage-restricted

interleukin-10 receptor deficiency, but not IL-10 deficiency, causes severe spontaneous colitis. *Immunity* **2014,** *40* (5), 720-33.

239. Shouval, D. S.; Biswas, A.; Goettel, J. A.; McCann, K.; Conaway, E.; Redhu, N. S.; Mascanfroni, I. D.; Al Adham, Z.; Lavoie, S.; Ibourk, M.; Nguyen, D. D.; Samsom, J. N.; Escher, J. C.; Somech, R.; Weiss, B.; Beier, R.; Conklin, L. S.; Ebens, C. L.; Santos, F. G.; Ferreira, A. R.; Sherlock, M.; Bhan, A. K.; Müller, W.; Mora, J. R.; Quintana, F. J.; Klein, C.; Muise, A. M.; Horwitz, B. H.; Snapper, S. B., Interleukin-10 receptor signaling in innate immune cells regulates mucosal immune tolerance and anti-inflammatory macrophage function. *Immunity* **2014,** *40* (5), 706-19.

240. Smythies, L. E.; Sellers, M.; Clements, R. H.; Mosteller-Barnum, M.; Meng, G.; Benjamin, W. H.; Orenstein, J. M.; Smith, P. D., Human intestinal macrophages display profound inflammatory anergy despite avid phagocytic and bacteriocidal activity. *J Clin Invest* **2005,** *115* (1), 66-75.

241. Baillie, J. K.; Arner, E.; Daub, C.; De Hoon, M.; Itoh, M.; Kawaji, H.; Lassmann, T.; Carninci, P.; Forrest, A. R.; Hayashizaki, Y.; Faulkner, G. J.; Wells, C. A.; Rehli, M.; Pavli, P.; Summers, K. M.; Hume, D. A.; Consortium, F., Analysis of the human monocyte-derived macrophage transcriptome and response to lipopolysaccharide provides new insights into genetic aetiology of inflammatory bowel disease. *PLoS Genet* **2017,** *13* (3), e1006641.

242. Kamada, N.; Hisamatsu, T.; Honda, H.; Kobayashi, T.; Chinen, H.; Kitazume, M. T.; Takayama, T.; Okamoto, S.; Koganei, K.; Sugita, A.; Kanai, T.; Hibi, T., Human CD14+ macrophages in intestinal lamina propria exhibit potent antigen-presenting ability. *J Immunol* **2009,** *183* (3), 1724-31.

243. Rugtveit, J.; Nilsen, E. M.; Bakka, A.; Carlsen, H.; Brandtzaeg, P.; Scott, H., Cytokine profiles differ in newly recruited and resident subsets of mucosal macrophages from inflammatory bowel disease. *Gastroenterology* **1997,** *112* (5), 1493-505.

244. Cosín-Roger, J.; Ortiz-Masiá, D.; Calatayud, S.; Hernández, C.; Alvarez, A.; Hinojosa, J.; Esplugues, J. V.; Barrachina, M. D., M2 macrophages activate WNT signaling pathway in epithelial cells: relevance in ulcerative colitis. *PLoS One* **2013,** *8* (10), e78128.

245. Scharl, M.; Huber, N.; Lang, S.; Fürst, A.; Jehle, E.; Rogler, G., Hallmarks of epithelial to mesenchymal transition are detectable in Crohn's disease associated intestinal fibrosis. *Clin Transl Med* **2015,** *4*, 1.

246. Kühl, A. A.; Erben, U.; Kredel, L. I.; Siegmund, B., Diversity of Intestinal Macrophages in Inflammatory Bowel Diseases. *Front Immunol* **2015,** *6*, 613.

247. Zhu, J.; Paul, W. E., CD4 T cells: fates, functions, and faults. *Blood* **2008,** *112* (5), 1557-69.

248. Breese, E.; Braegger, C. P.; Corrigan, C. J.; Walker-Smith, J. A.; MacDonald, T. T., Interleukin-2- and interferon-gamma-secreting T cells in normal and diseased human intestinal mucosa. *Immunology* **1993,** *78* (1), 127-31.

249. Monteleone, G.; Biancone, L.; Marasco, R.; Morrone, G.; Marasco, O.; Luzza, F.; Pallone, F., Interleukin 12 is expressed and actively released by Crohn's disease intestinal lamina propria mononuclear cells. *Gastroenterology* **1997,** *112* (4), 1169-78.

250. Fuss, I. J.; Neurath, M.; Boirivant, M.; Klein, J. S.; de la Motte, C.; Strong, S. A.; Fiocchi, C.; Strober, W., Disparate CD4+ lamina propria (LP) lymphokine secretion profiles in inflammatory bowel disease. Crohn's disease LP cells manifest increased secretion of IFN-

gamma, whereas ulcerative colitis LP cells manifest increased secretion of IL-5. *J Immunol* **1996,** *157* (3), 1261-70.

251. Miossec, P.; Kolls, J. K., Targeting IL-17 and TH17 cells in chronic inflammation. *Nat Rev Drug Discov* **2012,** *11* (10), 763-76.

252. Rovedatti, L.; Kudo, T.; Biancheri, P.; Sarra, M.; Knowles, C. H.; Rampton, D. S.; Corazza, G. R.; Monteleone, G.; Di Sabatino, A.; Macdonald, T. T., Differential regulation of interleukin 17 and interferon gamma production in inflammatory bowel disease. *Gut* **2009,** *58* (12), 1629-36.

253. Fujino, S.; Andoh, A.; Bamba, S.; Ogawa, A.; Hata, K.; Araki, Y.; Bamba, T.; Fujiyama, Y., Increased expression of interleukin 17 in inflammatory bowel disease. *Gut* **2003,** *52* (1), 65-70.

254. Gerlach, K.; Hwang, Y.; Nikolaev, A.; Atreya, R.; Dornhoff, H.; Steiner, S.; Lehr, H. A.; Wirtz, S.; Vieth, M.; Waisman, A.; Rosenbauer, F.; McKenzie, A. N.; Weigmann, B.; Neurath, M. F., TH9 cells that express the transcription factor PU.1 drive T cell-mediated colitis via IL-9 receptor signaling in intestinal epithelial cells. *Nat Immunol* **2014,** *15* (7), 676-86.

255. Hufford, M. M.; Kaplan, M. H., A gut reaction to IL-9. *Nat Immunol* **2014,** *15* (7), 599-600.

256. Lord, J. D., Promises and paradoxes of regulatory T cells in inflammatory bowel disease. *World J Gastroenterol* **2015,** *21* (40), 11236-45.

257. Sakaguchi, S.; Sakaguchi, N.; Asano, M.; Itoh, M.; Toda, M., Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* **1995,** *155* (3), 1151-64.

258. Maul, J.; Loddenkemper, C.; Mundt, P.; Berg, E.; Giese, T.; Stallmach, A.; Zeitz, M.; Duchmann, R., Peripheral and intestinal regulatory CD4+ CD25(high) T cells in inflammatory bowel disease. *Gastroenterology* **2005,** *128* (7), 1868-78.

259. Fantini, M. C.; Rizzo, A.; Fina, D.; Caruso, R.; Sarra, M.; Stolfi, C.; Becker, C.; Macdonald, T. T.; Pallone, F.; Neurath, M. F.; Monteleone, G., Smad7 controls resistance of colitogenic T cells to regulatory T cell-mediated suppression. *Gastroenterology* **2009,** *136* (4), 1308-16, e1-3.

260. Noronha, A. M.; Liang, Y.; Hetzel, J. T.; Hasturk, H.; Kantarci, A.; Stucchi, A.; Zhang, Y.; Nikolajczyk, B. S.; Farraye, F. A.; Ganley-Leal, L. M., Hyperactivated B cells in human inflammatory bowel disease. *J Leukoc Biol* **2009,** *86* (4), 1007-16.

261. Macpherson, A.; Khoo, U. Y.; Forgacs, I.; Philpott-Howard, J.; Bjarnason, I., Mucosal antibodies in inflammatory bowel disease are directed against intestinal bacteria. *Gut* **1996,** *38* (3), 365-75.

262. Grimm, M. C.; Elsbury, S. K.; Pavli, P.; Doe, W. F., Interleukin 8: cells of origin in inflammatory bowel disease. *Gut* **1996,** *38* (1), 90-8.

263. Kühn, R.; Löhler, J.; Rennick, D.; Rajewsky, K.; Müller, W., Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* **1993,** *75* (2), 263-74.

264. Sellon, R. K.; Tonkonogy, S.; Schultz, M.; Dieleman, L. A.; Grenther, W.; Balish, E.; Rennick, D. M.; Sartor, R. B., Resident enteric bacteria are necessary for development of spontaneous colitis and immune system activation in interleukin-10-deficient mice. *Infect Immun* **1998,** *66* (11), 5224-31.

265. Spencer, S. D.; Di Marco, F.; Hooley, J.; Pitts-Meek, S.; Bauer, M.; Ryan, A. M.; Sordat, B.; Gibbs, V. C.; Aguet, M., The orphan receptor CRF2-4 is an essential subunit of the interleukin 10 receptor. *J Exp Med* **1998,** *187* (4), 571-8.

266. Franke, A.; Balschun, T.; Karlsen, T. H.; Sventoraityte, J.; Nikolaus, S.; Mayr, G.; Domingues, F. S.; Albrecht, M.; Nothnagel, M.; Ellinghaus, D.; Sina, C.; Onnie, C. M.; Weersma, R. K.; Stokkers, P. C.; Wijmenga, C.; Gazouli, M.; Strachan, D.; McArdle, W. L.; Vermeire, S.; Rutgeerts, P.; Rosenstiel, P.; Krawczak, M.; Vatn, M. H.; Mathew, C. G.; Schreiber, S.; group, I. s., Sequence variants in IL10, ARPC2 and multiple other loci contribute to ulcerative colitis susceptibility. *Nat Genet* **2008,** *40* (11), 1319-23.

267. Franke, A.; McGovern, D. P.; Barrett, J. C.; Wang, K.; Radford-Smith, G. L.; Ahmad, T.; Lees, C. W.; Balschun, T.; Lee, J.; Roberts, R.; Anderson, C. A.; Bis, J. C.; Bumpstead, S.; Ellinghaus, D.; Festen, E. M.; Georges, M.; Green, T.; Haritunians, T.; Jostins, L.; Latiano, A.; Mathew, C. G.; Montgomery, G. W.; Prescott, N. J.; Raychaudhuri, S.; Rotter, J. I.; Schumm, P.; Sharma, Y.; Simms, L. A.; Taylor, K. D.; Whiteman, D.; Wijmenga, C.; Baldassano, R. N.; Barclay, M.; Bayless, T. M.; Brand, S.; Büning, C.; Cohen, A.; Colombel, J. F.; Cottone, M.; Stronati, L.; Denson, T.; De Vos, M.; D'Inca, R.; Dubinsky, M.; Edwards, C.; Florin, T.; Franchimont, D.; Gearry, R.; Glas, J.; Van Gossum, A.; Guthery, S. L.; Halfvarson, J.; Verspaget, H. W.; Hugot, J. P.; Karban, A.; Laukens, D.; Lawrance, I.; Lemann, M.; Levine, A.; Libioulle, C.; Louis, E.; Mowat, C.; Newman, W.; Panés, J.; Phillips, A.; Proctor, D. D.; Regueiro, M.; Russell, R.; Rutgeerts, P.; Sanderson, J.; Sans, M.; Seibold, F.; Steinhart, A. H.; Stokkers, P. C.; Torkvist, L.; Kullak-Ublick, G.; Wilson, D.; Walters, T.; Targan, S. R.; Brant, S. R.; Rioux, J. D.; D'Amato, M.; Weersma, R. K.; Kugathasan, S.; Griffiths, A. M.; Mansfield, J. C.; Vermeire, S.; Duerr, R. H.; Silverberg, M.

S.; Satsangi, J.; Schreiber, S.; Cho, J. H.; Annese, V.; Hakonarson, H.; Daly, M. J.; Parkes, M., Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. *Nat Genet* **2010,** *42* (12), 1118-25.

268. Glocker, E. O.; Kotlarz, D.; Boztug, K.; Gertz, E. M.; Schäffer, A. A.; Noyan, F.; Perro, M.; Diestelhorst, J.; Allroth, A.; Murugan, D.; Hätscher, N.; Pfeifer, D.; Sykora, K. W.; Sauer, M.; Kreipe, H.; Lacher, M.; Nustede, R.; Woellner, C.; Baumann, U.; Salzer, U.; Koletzko, S.; Shah, N.; Segal, A. W.; Sauerbrey, A.; Buderus, S.; Snapper, S. B.; Grimbacher, B.; Klein, C., Inflammatory bowel disease and mutations affecting the interleukin-10 receptor. *N Engl J Med* **2009,** *361* (21), 2033-45.

269. Kotlarz, D.; Beier, R.; Murugan, D.; Diestelhorst, J.; Jensen, O.; Boztug, K.; Pfeifer, D.; Kreipe, H.; Pfister, E. D.; Baumann, U.; Puchalka, J.; Bohne, J.; Egritas, O.; Dalgic, B.; Kolho, K. L.; Sauerbrey, A.; Buderus, S.; Güngör, T.; Enninger, A.; Koda, Y. K.; Guariso, G.; Weiss, B.; Corbacioglu, S.; Socha, P.; Uslu, N.; Metin, A.; Wahbeh, G. T.; Husain, K.; Ramadan, D.; Al-Herz, W.; Grimbacher, B.; Sauer, M.; Sykora, K. W.; Koletzko, S.; Klein, C., Loss of interleukin-10 signaling and infantile inflammatory bowel disease: implications for diagnosis and therapy. *Gastroenterology* **2012,** *143* (2), 347-55.

270. Moran, C. J.; Walters, T. D.; Guo, C. H.; Kugathasan, S.; Klein, C.; Turner, D.; Wolters, V. M.; Bandsma, R. H.; Mouzaki, M.; Zachos, M.; Langer, J. C.; Cutz, E.; Benseler, S. M.; Roifman, C. M.; Silverberg, M. S.; Griffiths, A. M.; Snapper, S. B.; Muise, A. M., IL-10R polymorphisms are associated with very-early-onset ulcerative colitis. *Inflamm Bowel Dis* **2013,** *19* (1), 115-23.

271. Wang, A. H.; Lam, W. J.; Han, D. Y.; Ding, Y.; Hu, R.; Fraser, A. G.; Ferguson, L. R.; Morgan, A. R., The effect of IL-10 genetic variation and interleukin 10 serum levels on Crohn's disease susceptibility in a New Zealand population. *Hum Immunol* **2011,** *72* (5), 431-5.

272. Kucharzik, T.; Stoll, R.; Lügering, N.; Domschke, W., Circulating antiinflammatory cytokine IL-10 in patients with inflammatory bowel disease (IBD). *Clin Exp Immunol* **1995,** *100* (3), 452-6.

273. Mitsuyama, K.; Tomiyasu, N.; Takaki, K.; Masuda, J.; Yamasaki, H.; Kuwaki, K.; Takeda, T.; Kitazaki, S.; Tsuruta, O.; Sata, M., Interleukin-10 in the pathophysiology of inflammatory bowel disease: increased serum concentrations during the recovery phase. *Mediators Inflamm* **2006,** *2006* (6), 26875.

274. Ljuca, F.; Gegic, A.; Salkic, N. N.; Pavlovic-Calic, N., Circulating cytokines reflect mucosal inflammatory status in patients with Crohn's disease. *Dig Dis Sci* **2010,** *55* (8), 2316-26.

275. Meresse, B.; Rutgeerts, P.; Malchow, H.; Dubucquoi, S.; Dessaint, J. P.; Cohard, M.; Colombel, J. F.; Desreumaux, P., Low ileal interleukin 10 concentrations are predictive of endoscopic recurrence in patients with Crohn's disease. *Gut* **2002,** *50* (1), 25-8.

276. Asadullah, K.; Sterry, W.; Volk, H. D., Interleukin-10 therapy--review of a new approach. *Pharmacol Rev* **2003,** *55* (2), 241-69.

277. Marlow, G. J.; van Gent, D.; Ferguson, L. R., Why interleukin-10 supplementation does not work in Crohn's disease patients. *World J Gastroenterol* **2013,** *19* (25), 3931-41.

278. Orlando, A.; Guglielmi, F. W.; Cottone, M.; Orlando, E.; Romano, C.; Sinagra, E., Clinical implications of mucosal healing in the management of patients with inflammatory bowel disease. *Dig Liver Dis* **2013,** *45* (12), 986-91.

279. Engel, M. A.; Neurath, M. F., New pathophysiological insights and modern treatment of IBD. *J Gastroenterol* **2010,** *45* (6), 571-83.

280. Waljee, A. K.; Wiitala, W. L.; Govani, S.; Stidham, R.; Saini, S.; Hou, J.; Feagins, L. A.; Khan, N.; Good, C. B.; Vijan, S.; Higgins, P. D., Corticosteroid Use and Complications in a US Inflammatory Bowel Disease Cohort. *PLoS One* **2016,** *11* (6), e0158017.

281. Kozuch, P. L.; Hanauer, S. B., Treatment of inflammatory bowel disease: a review of medical therapy. *World J Gastroenterol* **2008,** *14* (3), 354-77.

282. Shah, S. C.; Colombel, J. F.; Sands, B. E.; Narula, N., Systematic review with meta-analysis: mucosal healing is associated with improved long-term outcomes in Crohn's disease. *Aliment Pharmacol Ther* **2016,** *43* (3), 317-33.

283. Chan, H. C.; Ng, S. C., Emerging biologics in inflammatory bowel disease. *J Gastroenterol* **2017,** *52* (2), 141-150.

284. Danese, S.; Vuitton, L.; Peyrin-Biroulet, L., Biologic agents for IBD: practical insights. *Nat Rev Gastroenterol Hepatol* **2015,** *12* (9), 537-45.

285. Ben-Horin, S.; Vande Casteele, N.; Schreiber, S.; Lakatos, P. L., Biosimilars in Inflammatory Bowel Disease: Facts and Fears of Extrapolation. *Clin Gastroenterol Hepatol* **2016,** *14* (12), 1685-1696.

286. Wils, P.; Bouhnik, Y.; Michetti, P.; Flourie, B.; Brixi, H.; Bourrier, A.; Allez, M.; Duclos, B.; Serrero, M.; Buisson, A.; Amiot, A.; Fumery, M.; Roblin, X.; Peyrin-Biroulet, L.; Filippi, J.; Bouguen, G.; Abitbol, V.; Coffin, B.; Simon, M.; Laharie, D.; Pariente, B.; (GETAID), G. d. E. T. d. A. I. d. T. D., Long-term efficacy and safety of ustekinumab in 122 refractory Crohn's disease patients: a multicentre experience. *Aliment Pharmacol Ther* **2018,** *47* (5), 588-595.

287. Stallmach, A.; Hagel, S.; Bruns, T., Adverse effects of biologics used for treating IBD. *Best Pract Res Clin Gastroenterol* **2010,** *24* (2), 167-82.

288. Kaser, A.; Zeissig, S.; Blumberg, R. S., Inflammatory bowel disease. *Annu Rev Immunol* **2010,** *28*, 573-621.

289. Hwang, J. M.; Varma, M. G., Surgery for inflammatory bowel disease. *World J Gastroenterol* **2008,** *14* (17), 2678-90.

290. Schwab, I.; Nimmerjahn, F., Intravenous immunoglobulin therapy: how does IgG modulate the immune system? *Nat Rev Immunol* **2013,** *13* (3), 176-89.

291. McGovern, D. P.; Gardet, A.; Törkvist, L.; Goyette, P.; Essers, J.; Taylor, K. D.; Neale, B. M.; Ong, R. T.; Lagacé, C.; Li, C.; Green, T.; Stevens, C. R.; Beauchamp, C.; Fleshner, P. R.; Carlson, M.; D'Amato, M.; Halfvarson, J.; Hibberd, M. L.; Lördal, M.; Padyukov, L.; Andriulli, A.; Colombo, E.; Latiano, A.; Palmieri, O.; Bernard, E. J.; Deslandres, C.; Hommes, D. W.; de Jong, D. J.; Stokkers, P. C.; Weersma, R. K.; Sharma, Y.; Silverberg, M. S.; Cho, J. H.; Wu, J.; Roeder, K.; Brant, S. R.; Schumm, L. P.; Duerr, R. H.; Dubinsky, M. C.; Glazer, N. L.; Haritunians, T.; Ippoliti, A.; Melmed, G. Y.; Siscovick, D. S.; Vasiliauskas, E. A.; Targan, S. R.; Annese, V.; Wijmenga, C.; Pettersson, S.; Rotter, J. I.; Xavier, R. J.; Daly, M. J.; Rioux, J. D.; Seielstad, M.; Consortium, N. I. G., Genome-wide association identifies multiple ulcerative colitis susceptibility loci. *Nat Genet* **2010,** *42* (4), 332-7.

292. Avila-Pedretti, G.; Tornero, J.; Fernández-Nebro, A.; Blanco, F.; González-Alvaro, I.; Cañete, J. D.; Maymó, J.; Alperiz, M.; Fernández-Gutiérrez, B.; Olivé, A.; Corominas, H.; Erra, A.; Aterido, A.; López Lasanta, M.; Tortosa, R.; Julià, A.; Marsal, S., Variation at

FCGR2A and functionally related genes is associated with the response to anti-TNF therapy in rheumatoid arthritis. *PLoS One* **2015,** *10* (4), e0122088.

293. Fadok, V. A.; Bratton, D. L.; Konowal, A.; Freed, P. W.; Westcott, J. Y.; Henson, P. M., Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. *J Clin Invest* **1998,** *101* (4), 890-8.

294. Anderson, C. F.; Gerber, J. S.; Mosser, D. M., Modulating macrophage function with IgG immune complexes. *J Endotoxin Res* **2002,** *8* (6), 477-81.

295. Mosser, D. M.; Zhang, X., Activation of murine macrophages. *Curr Protoc Immunol* **2008,** *Chapter 14*, Unit 14.2.

296. Hutchinson, J. A.; Riquelme, P.; Geissler, E. K., Human regulatory macrophages as a cell-based medicinal product. *Curr Opin Organ Transplant* **2012,** *17* (1), 48-54.

297. Hutchinson, J. A.; Riquelme, P.; Sawitzki, B.; Tomiuk, S.; Miqueu, P.; Zuhayra, M.; Oberg, H. H.; Pascher, A.; Lützen, U.; Janssen, U.; Broichhausen, C.; Renders, L.; Thaiss, F.; Scheuermann, E.; Henze, E.; Volk, H. D.; Chatenoud, L.; Lechler, R. I.; Wood, K. J.; Kabelitz, D.; Schlitt, H. J.; Geissler, E. K.; Fändrich, F., Cutting Edge: Immunological consequences and trafficking of human regulatory macrophages administered to renal transplant recipients. *J Immunol* **2011,** *187* (5), 2072-8.

298. Nimmerjahn, F.; Ravetch, J. V., Antibody-mediated modulation of immune responses. *Immunol Rev* **2010,** *236*, 265-75.

299. Negi, V. S.; Elluru, S.; Sibéril, S.; Graff-Dubois, S.; Mouthon, L.; Kazatchkine, M. D.; Lacroix-Desmazes, S.; Bayry, J.; Kaveri, S. V., Intravenous immunoglobulin: an update on the clinical use and mechanisms of action. *J Clin Immunol* **2007,** *27* (3), 233-45.

300. Weisser, S. B.; McLarren, K. W.; Voglmaier, N.; van Netten-Thomas, C. J.; Antov, A.; Flavell, R. A.; Sly, L. M., Alternative activation of macrophages by IL-4 requires SHIP degradation. *European journal of immunology* **2011,** *41* (6), 1742-53.

301. Polumuri, S. K.; Toshchakov, V. Y.; Vogel, S. N., Role of phosphatidylinositol-3 kinase in transcriptional regulation of TLR-induced IL-12 and IL-10 by Fc gamma receptor ligation in murine macrophages. *J Immunol* **2007,** *179* (1), 236-46.

302. Siragam, V.; Crow, A. R.; Brinc, D.; Song, S.; Freedman, J.; Lazarus, A. H., Intravenous immunoglobulin ameliorates ITP via activating Fc gamma receptors on dendritic cells. *Nat Med* **2006,** *12* (6), 688-92.

303. Anthony, R. M.; Ravetch, J. V., A novel role for the IgG Fc glycan: the anti-inflammatory activity of sialylated IgG Fcs. *J Clin Immunol* **2010,** *30 Suppl 1*, S9-14.

304. Chaikuad, A.; Tacconi, E. M.; Zimmer, J.; Liang, Y.; Gray, N. S.; Tarsounas, M.; Knapp, S., A unique inhibitor binding site in ERK1/2 is associated with slow binding kinetics. *Nat Chem Biol* **2014,** *10* (10), 853-60.

305. Nishimoto, S.; Nishida, E., MAPK signalling: ERK5 versus ERK1/2. *EMBO Rep* **2006,** *7* (8), 782-6.

306. Kalesnikoff, J.; Huber, M.; Lam, V.; Damen, J. E.; Zhang, J.; Siraganian, R. P.; Krystal, G., Monomeric IgE stimulates signaling pathways in mast cells that lead to cytokine production and cell survival. *Immunity* **2001,** *14* (6), 801-11.

307. Bayry, J.; Lacroix-Desmazes, S.; Delignat, S.; Mouthon, L.; Weill, B.; Kazatchkine, M. D.; Kaveri, S. V., Intravenous immunoglobulin abrogates dendritic cell differentiation induced by interferon-alpha present in serum from patients with systemic lupus erythematosus. *Arthritis Rheum* **2003,** *48* (12), 3497-502.

308. Durandy, A.; Kaveri, S. V.; Kuijpers, T. W.; Basta, M.; Miescher, S.; Ravetch, J. V.; Rieben, R., Intravenous immunoglobulins--understanding properties and mechanisms. *Clin Exp Immunol* **2009,** *158 Suppl 1*, 2-13.

309. Tatake, R. J.; O'Neill, M. M.; Kennedy, C. A.; Wayne, A. L.; Jakes, S.; Wu, D.; Kugler, S. Z.; Kashem, M. A.; Kaplita, P.; Snow, R. J., Identification of pharmacological inhibitors of the MEK5/ERK5 pathway. *Biochem Biophys Res Commun* **2008,** *377* (1), 120-5.

310. Zhu, W.; Downey, J. S.; Gu, J.; Di Padova, F.; Gram, H.; Han, J., Regulation of TNF expression by multiple mitogen-activated protein kinase pathways. *J Immunol* **2000,** *164* (12), 6349-58.

311. Alejandria, M. M.; Lansang, M. A.; Dans, L. F.; Mantaring, J. B., Intravenous immunoglobulin for treating sepsis and septic shock. *Cochrane Database Syst Rev* **2002,** (1), CD001090.

312. Alejandria, M. M.; Lansang, M. A.; Dans, L. F.; Mantaring, J. B., Intravenous immunoglobulin for treating sepsis, severe sepsis and septic shock. *Cochrane Database Syst Rev* **2013,** *9*, CD001090.

313. Ben-Horin, S.; Chowers, Y., Tailoring anti-TNF therapy in IBD: drug levels and disease activity. *Nat Rev Gastroenterol Hepatol* **2014,** *11* (4), 243-55.

314. Heinsbroek, S. E.; Gordon, S., The role of macrophages in inflammatory bowel diseases. *Expert Rev Mol Med* **2009,** *11*, e14.

315. Sica, A.; Mantovani, A., Macrophage plasticity and polarization: in vivo veritas. *J Clin Invest* **2012,** *122* (3), 787-95.

316. Weisser, S. B.; Brugger, H. K.; Voglmaier, N. S.; McLarren, K. W.; van Rooijen, N.; Sly, L. M., SHIP-deficient, alternatively activated macrophages protect mice during DSS-induced colitis. *J Leukoc Biol* **2011,** *90* (3), 483-92.

317. Hunter, M. M.; Wang, A.; Parhar, K. S.; Johnston, M. J.; Van Rooijen, N.; Beck, P. L.; McKay, D. M., In vitro-derived alternatively activated macrophages reduce colonic inflammation in mice. *Gastroenterology* **2010,** *138* (4), 1395-405.

318. Yen, D.; Cheung, J.; Scheerens, H.; Poulet, F.; McClanahan, T.; McKenzie, B.; Kleinschek, M. A.; Owyang, A.; Mattson, J.; Blumenschein, W.; Murphy, E.; Sathe, M.; Cua, D. J.; Kastelein, R. A.; Rennick, D., IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6. *J Clin Invest* **2006,** *116* (5), 1310-6.

319. Krause, P.; Morris, V.; Greenbaum, J. A.; Park, Y.; Bjoerheden, U.; Mikulski, Z.; Muffley, T.; Shui, J. W.; Kim, G.; Cheroutre, H.; Liu, Y. C.; Peters, B.; Kronenberg, M.; Murai, M., IL-10-producing intestinal macrophages prevent excessive antibacterial innate immunity by limiting IL-23 synthesis. *Nat Commun* **2015,** *6*, 7055.

320. Aithal, G. P.; Craggs, A.; Day, C. P.; Welfare, M.; Daly, A. K.; Mansfield, J. C.; Hudson, M., Role of polymorphisms in the interleukin-10 gene in determining disease susceptibility and phenotype in inflamatory bowel disease. *Dig Dis Sci* **2001,** *46* (7), 1520-5.

321. von Gunten, S.; Shoenfeld, Y.; Blank, M.; Branch, D. R.; Vassilev, T.; Käsermann, F.; Bayry, J.; Kaveri, S.; Simon, H. U., IVIG pluripotency and the concept of Fc-sialylation: challenges to the scientist. *Nat Rev Immunol* **2014,** *14* (5), 349.

322. Kozicky, L. K.; Zhao, Z. Y.; Menzies, S. C.; Fidanza, M.; Reid, G. S.; Wilhelmsen, K.; Hellman, J.; Hotte, N.; Madsen, K. L.; Sly, L. M., Intravenous immunoglobulin skews

macrophages to an anti-inflammatory, IL-10-producing activation state. *J Leukoc Biol* **2015,** *98* (6), 983-94.

323. Weisser, S. B.; Kozicky, L. K.; Brugger, H. K.; Ngoh, E. N.; Cheung, B.; Jen, R.; Menzies, S. C.; Samarakoon, A.; Murray, P. J.; Lim, C. J.; Johnson, P.; Boucher, J. L.; van Rooijen, N.; Sly, L. M., Arginase activity in alternatively activated macrophages protects PI3Kp110δ deficient mice from dextran sodium sulfate induced intestinal inflammation. *Eur J Immunol* **2014,** *44* (11), 3353-67.

324. Ligumsky, M.; Simon, P. L.; Karmeli, F.; Rachmilewitz, D., Role of interleukin 1 in inflammatory bowel disease--enhanced production during active disease. *Gut* **1990,** *31* (6), 686-9.

325. Vounotrypidis, P.; Kouklakis, G.; Anagnostopoulos, K.; Zezos, P.; Polychronidis, A.; Maltezos, E.; Efremidou, E.; Pitiakoudis, M.; Lyratzopoulos, N., Interleukin-1 associations in inflammatory bowel disease and the enteropathic seronegative spondylarthritis. *Auto Immun Highlights* **2013,** *4* (3), 87-94.

326. Okazaki, K.; Nakase, H.; Watanabe, N.; Tabata, Y.; Ikada, Y.; Chiba, T., Intestinal drug delivery systems with biodegradable microspheres targeting mucosal immune-regulating cells for chronic inflammatory colitis. *J Gastroenterol* **2002,** *37 Suppl 14*, 44-52.

327. Jarry, A.; Bossard, C.; Bou-Hanna, C.; Masson, D.; Espaze, E.; Denis, M. G.; Laboisse, C. L., Mucosal IL-10 and TGF-beta play crucial roles in preventing LPS-driven, IFN-gamma-mediated epithelial damage in human colon explants. *J Clin Invest* **2008,** *118* (3), 1132-42.

328. Schmidt, C.; Giese, T.; Ludwig, B.; Mueller-Molaian, I.; Marth, T.; Zeuzem, S.; Meuer, S. C.; Stallmach, A., Expression of interleukin-12-related cytokine transcripts in

inflammatory bowel disease: elevated interleukin-23p19 and interleukin-27p28 in Crohn's disease but not in ulcerative colitis. *Inflamm Bowel Dis* **2005,** *11* (1), 16-23.

329. Guan, Q.; Zhang, J., Recent Advances: The Imbalance of Cytokines in the Pathogenesis of Inflammatory Bowel Disease. *Mediators Inflamm* **2017,** *2017*, 4810258.

330. Siqueira Mietto, B.; Kroner, A.; Girolami, E. I.; Santos-Nogueira, E.; Zhang, J.; David, S., Role of IL-10 in Resolution of Inflammation and Functional Recovery after Peripheral Nerve Injury. *J Neurosci* **2015,** *35* (50), 16431-42.

331. Quiros, M.; Nishio, H.; Neumann, P. A.; Siuda, D.; Brazil, J. C.; Azcutia, V.; Hilgarth, R.; O'Leary, M. N.; Garcia-Hernandez, V.; Leoni, G.; Feng, M.; Bernal, G.; Williams, H.; Dedhia, P. H.; Gerner-Smidt, C.; Spence, J.; Parkos, C. A.; Denning, T. L.; Nusrat, A., Macrophage-derived IL-10 mediates mucosal repair by epithelial WISP-1 signaling. *J Clin Invest* **2017,** *127* (9), 3510-3520.

332. Vos, A. C.; Wildenberg, M. E.; Arijs, I.; Duijvestein, M.; Verhaar, A. P.; de Hertogh, G.; Vermeire, S.; Rutgeerts, P.; van den Brink, G. R.; Hommes, D. W., Regulatory macrophages induced by infliximab are involved in healing in vivo and in vitro. *Inflamm Bowel Dis* **2012,** *18* (3), 401-8.

333. Gerber, J. S.; Mosser, D. M., Reversing lipopolysaccharide toxicity by ligating the macrophage Fc gamma receptors. *J Immunol* **2001,** *166* (11), 6861-8.

334. Park, M. J.; Lee, S. H.; Kim, E. K.; Lee, E. J.; Baek, J. A.; Park, S. H.; Kwok, S. K.; Cho, M. L., Interleukin-10 produced by myeloid-derived suppressor cells is critical for the induction of Tregs and attenuation of rheumatoid inflammation in mice. *Sci Rep* **2018,** *8* (1), 3753.

335. Akyol, G. Y.; Manaenko, A.; Akyol, O.; Solaroglu, I.; Ho, W. M.; Ding, Y.; Flores, J.; Zhang, J. H.; Tang, J., IVIG activates FcγRIIB-SHIP1-PIP3 Pathway to stabilize mast cells and suppress inflammation after ICH in mice. *Sci Rep* **2017,** *7* (1), 15583.

336. Shintani, N.; Nakajima, T.; Nakakubo, H.; Nagai, H.; Kagitani, Y.; Takizawa, H.; Asakura, H., Intravenous immunoglobulin (IVIG) treatment of experimental colitis induced by dextran sulfate sodium in rats. *Clin Exp Immunol* **1997,** *108* (2), 340-5.

337. Bayry, J.; Lacroix-Desmazes, S.; Carbonneil, C.; Misra, N.; Donkova, V.; Pashov, A.; Chevailler, A.; Mouthon, L.; Weill, B.; Bruneval, P.; Kazatchkine, M. D.; Kaveri, S. V., Inhibition of maturation and function of dendritic cells by intravenous immunoglobulin. *Blood* **2003,** *101* (2), 758-65.

338. Lories, R. J.; Casteels-Van Daele, M.; Ceuppens, J. L.; Van Gool, S. W., Polyclonal immunoglobulins for intravenous use induce interleukin 10 release in vivo and in vitro. *Ann Rheum Dis* **2004,** *63* (6), 747-8.

339. Cooper, N.; Heddle, N. M.; Haas, M.; Reid, M. E.; Lesser, M. L.; Fleit, H. B.; Woloski, B. M.; Bussel, J. B., Intravenous (IV) anti-D and IV immunoglobulin achieve acute platelet increases by different mechanisms: modulation of cytokine and platelet responses to IV anti-D by FcgammaRIIa and FcgammaRIIIa polymorphisms. *Br J Haematol* **2004,** *124* (4), 511-8.

340. Horton, N.; Kochhar, G.; Patel, K.; Lopez, R.; Shen, B., Efficacy and Factors Associated with Treatment Response of Intravenous Immunoglobulin in Inpatients with Refractory Inflammatory Bowel Diseases. *Inflamm Bowel Dis* **2017,** *23* (7), 1080-1087.

341. Canete, J. D.; Suarez, B.; Hernandez, M. V.; Sanmarti, R.; Rego, I.; Celis, R.; Moll, C.; Pinto, J. A.; Blanco, F. J.; Lozano, F., Influence of variants of Fc gamma receptors IIA and

IIIA on the American College of Rheumatology and European League Against Rheumatism responses to anti-tumour necrosis factor alpha therapy in rheumatoid arthritis. *Ann Rheum Dis* **2009,** *68* (10), 1547-52.

342. Domínguez-Soto, Á.; Simón-Fuentes, M.; de Las Casas-Engel, M.; Cuevas, V. D.; López-Bravo, M.; Domínguez-Andrés, J.; Saz-Leal, P.; Sancho, D.; Ardavín, C.; Ochoa-Grullón, J.; Sánchez-Ramón, S.; Vega, M. A.; Corbí, A. L., IVIg Promote Cross-Tolerance against Inflammatory Stimuli In Vitro and In Vivo. *J Immunol* **2018,** *201* (1), 41-52.

343. Kuo, H. C.; Hsu, Y. W.; Wu, M. S.; Chien, S. C.; Liu, S. F.; Chang, W. C., Intravenous immunoglobulin, pharmacogenomics, and Kawasaki disease. *J Microbiol Immunol Infect* **2016,** *49* (1), 1-7.

344. Gupta, M.; Noel, G. J.; Schaefer, M.; Friedman, D.; Bussel, J.; Johann-Liang, R., Cytokine modulation with immune gamma-globulin in peripheral blood of normal children and its implications in Kawasaki disease treatment. *J Clin Immunol* **2001,** *21* (3), 193-9.

345. Sharief, M. K.; Ingram, D. A.; Swash, M.; Thompson, E. J., I.v. immunoglobulin reduces circulating proinflammatory cytokines in Guillain-Barré syndrome. *Neurology* **1999,** *52* (9), 1833-8.

346. Kalliolias, G. D.; Ivashkiv, L. B., IL-27 activates human monocytes via STAT1 and suppresses IL-10 production but the inflammatory functions of IL-27 are abrogated by TLRs and p38. *J Immunol* **2008,** *180* (9), 6325-33.

347. Wojtal, K. A.; Rogler, G.; Scharl, M.; Biedermann, L.; Frei, P.; Fried, M.; Weber, A.; Eloranta, J. J.; Kullak-Ublick, G. A.; Vavricka, S. R., Fc gamma receptor CD64 modulates the inhibitory activity of infliximab. *PLoS One* **2012,** *7* (8), e43361.

348. Vogelpoel, L. T.; Hansen, I. S.; Rispens, T.; Muller, F. J.; van Capel, T. M.; Turina, M. C.; Vos, J. B.; Baeten, D. L.; Kapsenberg, M. L.; de Jong, E. C.; den Dunnen, J., Fc gamma receptor-TLR cross-talk elicits pro-inflammatory cytokine production by human M2 macrophages. *Nat Commun* **2014,** *5*, 5444.

349. Blank, U.; Launay, P.; Benhamou, M.; Monteiro, R. C., Inhibitory ITAMs as novel regulators of immunity. *Immunol Rev* **2009,** *232* (1), 59-71.

350. Drechsler, Y.; Dolganiuc, A.; Norkina, O.; Romics, L.; Li, W.; Kodys, K.; Bach, F. H.; Mandrekar, P.; Szabo, G., Heme oxygenase-1 mediates the anti-inflammatory effects of acute alcohol on IL-10 induction involving p38 MAPK activation in monocytes. *J Immunol* **2006,** *177* (4), 2592-600.

351. Merkley, S. A.; Beaulieu, D. B.; Horst, S.; Duley, C.; Annis, K.; Nohl, A.; Schwartz, D. A., Use of Intravenous Immunoglobulin for Patients with Inflammatory Bowel Disease with Contraindications or Who Are Unresponsive to Conventional Treatments. *Inflamm Bowel Dis* **2015,** *21* (8), 1854-9.

352. Levine, D. S.; Fischer, S. H.; Christie, D. L.; Haggitt, R. C.; Ochs, H. D., Intravenous immunoglobulin therapy for active, extensive, and medically refractory idiopathic ulcerative or Crohn's colitis. *Am J Gastroenterol* **1992,** *87* (1), 91-100.

353. Rogosnitzky, M.; Danks, R.; Holt, D., Intravenous immunoglobulin for the treatment of Crohn's disease. *Autoimmun Rev* **2012,** *12* (2), 275-80.

354. Li, M. C.; He, S. H., IL-10 and its related cytokines for treatment of inflammatory bowel disease. *World J Gastroenterol* **2004,** *10* (5), 620-5.

355. Schreiber, S.; Fedorak, R. N.; Nielsen, O. H.; Wild, G.; Williams, C. N.; Nikolaus, S.; Jacyna, M.; Lashner, B. A.; Gangl, A.; Rutgeerts, P.; Isaacs, K.; van Deventer, S. J.;

Koningsberger, J. C.; Cohard, M.; LeBeaut, A.; Hanauer, S. B., Safety and efficacy of recombinant human interleukin 10 in chronic active Crohn's disease. Crohn's Disease IL-10 Cooperative Study Group. *Gastroenterology* **2000,** *119* (6), 1461-72.

356. Anguille, S.; Smits, E. L.; Lion, E.; van Tendeloo, V. F.; Berneman, Z. N., Clinical use of dendritic cells for cancer therapy. *Lancet Oncol* **2014,** *15* (7), e257-67.

357. Romero, D., Haematological cancer: Favourable outcomes with CAR T cells. *Nat Rev Clin Oncol* **2018,** *15* (2), 65.

358. Desreumaux, P.; Foussat, A.; Allez, M.; Beaugerie, L.; Hébuterne, X.; Bouhnik, Y.; Nachury, M.; Brun, V.; Bastian, H.; Belmonte, N.; Ticchioni, M.; Duchange, A.; Morel-Mandrino, P.; Neveu, V.; Clerget-Chossat, N.; Forte, M.; Colombel, J. F., Safety and efficacy of antigen-specific regulatory T-cell therapy for patients with refractory Crohn's disease. *Gastroenterology* **2012,** *143* (5), 1207-1217.e2.

359. Streitz, M.; Miloud, T.; Kapinsky, M.; Reed, M. R.; Magari, R.; Geissler, E. K.; Hutchinson, J. A.; Vogt, K.; Schlickeiser, S.; Kverneland, A. H.; Meisel, C.; Volk, H. D.; Sawitzki, B., Standardization of whole blood immune phenotype monitoring for clinical trials: panels and methods from the ONE study. *Transplant Res* **2013,** *2* (1), 17.

360. Lee, S.; Kivimäe, S.; Dolor, A.; Szoka, F. C., Macrophage-based cell therapies: The long and winding road. *J Control Release* **2016,** *240*, 527-540.

361. Barrera, P.; Blom, A.; van Lent, P. L.; van Bloois, L.; Beijnen, J. H.; van Rooijen, N.; de Waal Malefijt, M. C.; van de Putte, L. B.; Storm, G.; van den Berg, W. B., Synovial macrophage depletion with clodronate-containing liposomes in rheumatoid arthritis. *Arthritis Rheum* **2000,** *43* (9), 1951-9.

362. Leung, G.; Petri, B.; Reyes, J. L.; Wang, A.; Iannuzzi, J.; Mckay, D. M., Cryopreserved IL-4-treated macrophages attenuate murine colitis in an integrin β7-dependent manner. *Mol Med* **2015**.

363. Raja, J.; Nihtyanova, S. I.; Murray, C. D.; Denton, C. P.; Ong, V. H., Sustained benefit from intravenous immunoglobulin therapy for gastrointestinal involvement in systemic sclerosis. *Rheumatology (Oxford)* **2016,** *55* (1), 115-9.

364. Corbí, A. L.; Sánchez-Ramón, S.; Domínguez-Soto, A., The potential of intravenous immunoglobulins for cancer therapy: a road that is worth taking? *Immunotherapy* **2016,** *8* (5), 601-12.

365. Meyer, T.; Robles-Carrillo, L.; Davila, M.; Brodie, M.; Desai, H.; Rivera-Amaya, M.; Francis, J. L.; Amirkhosravi, A., CD32a antibodies induce thrombocytopenia and type II hypersensitivity reactions in FCGR2A mice. *Blood* **2015,** *126* (19), 2230-8.

366. Pietersz, G. A.; Mottram, P. L.; van de Velde, N. C.; Sardjono, C. T.; Esparon, S.; Ramsland, P. A.; Moloney, G.; Baell, J. B.; McCarthy, T. D.; Matthews, B. R.; Powell, M. S.; Hogarth, P. M., Inhibition of destructive autoimmune arthritis in FcgammaRIIa transgenic mice by small chemical entities. *Immunol Cell Biol* **2009,** *87* (1), 3-12.

367. Orange, J. S., Considering the demand for and prioritization of intravenous immunoglobulin. *Clin Exp Immunol* **2014,** *178 Suppl 1*, 78-82.

368. Veri, M. C.; Gorlatov, S.; Li, H.; Burke, S.; Johnson, S.; Stavenhagen, J.; Stein, K. E.; Bonvini, E.; Koenig, S., Monoclonal antibodies capable of discriminating the human inhibitory Fcgamma-receptor IIB (CD32B) from the activating Fcgamma-receptor IIA (CD32A): biochemical, biological and functional characterization. *Immunology* **2007,** *121* (3), 392-404.

369. Li, X.; Kimberly, R. P., Targeting the Fc receptor in autoimmune disease. *Expert Opin Ther Targets* **2014,** *18* (3), 335-50.

370. Mimoto, F.; Katada, H.; Kadono, S.; Igawa, T.; Kuramochi, T.; Muraoka, M.; Wada, Y.; Haraya, K.; Miyazaki, T.; Hattori, K., Engineered antibody Fc variant with selectively enhanced FcγRIIb binding over both FcγRIIa(R131) and FcγRIIa(H131). *Protein Eng Des Sel* **2013,** *26* (10), 589-98.