Mechanisms of interferon-α inhibition by intravenous immunoglobulin

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Abstract

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Several lines of evidence implicate type I interferons (IFN-I, including IFN-α and IFN-β) in the pathogenesis of systemic lupus erythematosus (SLE). Plasmacytoid dendritic cells (pDCs) are specialized in the production of IFN-α and produce high concentrations of this cytokine following exposure to immune complexes (ICs) containing nucleic acids such as those that are found in the serum and tissues of patients with SLE. We previously reported that normal human serum inhibits IFN-α production by pDCs in response to SLE ICs and that inhibition is mediated, in part, by immunoglobulin G (IgG). IgG is the major component of intravenous Ig (IVIg), a therapeutic that is well known to exert anti-inflammatory properties and is used to treat several diseases associated with increased IFN-I expression. Although the sialylated subfraction of IVIg has been implicated as the key anti-inflammatory component in murine models of arthritis and

thrombocytopenia, the mechanism of inhibition of IFN-α by IgG and the importance of sialylation have not been studied.

To address these questions we stimulated human primary cells with immune complexes or Toll-like receptor (TLR) agonists and then evaluated their IFN-α production after addition of total IgG, its proteolytic fragments or differentially glycosylated subfractions. We discovered two very different mechanisms of inhibition by IgG. In the first, IgG potently inhibited IC-induced IFN-α by blocking binding of ICs to FcγRIIa on pDCs, which required the Fc portion of IgG but not sialylation. We also elucidated a novel second mechanism by which IgG inhibited TLR agonist-induced IFN-α that was independent of FcγR interaction. F(ab')2 fragments from the sialylated subfraction of IgG induced monocytes to produce prostaglandin E2 (PGE2) which potently inhibited pDC production of IFN-α. We found that PGE2 could inhibit IFN-α by direct activity on pDCs, but the signaling pathways involved in the inhibition of IFN-α by PGE2 are not well understood. We demonstrated that an activator of PKA (dibutyryl-cAMP) or a suppressor of mTOR (rapamycin) also inhibited IFN-α production, implicating the PKA and mTOR pathways as key regulators. Future studies will address the identification of the target molecule(s) on monocytes that are important for PGE2 production and the signal transduction pathways responsible for PGE2 suppression of IFN-α production by pDCs. These findings could lead to more efficient therapies for immune modulation in SLE and other diseases in which IFN-I is thought to play an important role.

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List of Abbreviations

4E-BP	4E binding protein
APC	antigen presenting cell
BAFF	B-cell activating factor
BDCA2	blood dendritic cell antigen 2
BLNK/BCAP	B-cell linker
Ca2+	calcium
cAMP	cyclic adenosine monophosphate
CDS	cytoplasmic DNA sensor
cGAS	cyclic-GMP-AMP (cGAMP) synthase
DAI/ZBP-1	DNA-dependent activator of IRFs
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
DHX	DEAH box polypeptide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxribonuclease
ds	double-stranded
EBNA-1	Epstein-Barr nuclear antigen 1
EBV	Epstein–Barr virus
eIF4E	eukaryotic translation initiation factor 4E
ELISA	enzyme-linked immunosorbent assay
EP	prostaglandin E2 receptor
ER	endoplasmic reticulum
Fab	fragment, antigen binding
Fc	fragment, crystallizable
FcR	Fc receptor
GAS6	growth arrest-specific 6
Gase	galactosidase
GlcNAc	*N*-acetylglucosamine
HPLC	high-performance liquid chromatography
HRP	horseradish peroxidase
HS	hypersialylated
IC	immune complex
IFI	IFN-induced with helicase C domain
IFN	interferon

List of Abbreviations (continued)

IFNaR	interferon-α/β receptor
Ig	immunoglobulin
IKK	IκB kinase
IL	interleukin
ILT7	immunoglobulin-like transcript 7
IPC	interferon producing cell
IRAK	interleukin receptor associated kinase
IRF	interferon regulatory factor
ISG	interferon response gene
ISRE	interferon-stimulated response element
ITAM	immunoreceptor-based tyrosine activation motif
ITP	immune thrombocytopenic purpura
IVIg	intravenous immunoglobulin
JAK	Janus kinase
La/SS-B	Sjögren's syndrome-associated autoantigen B
Lox	loxoribine
LPS	ipopolysaccharide
LRRFIP1	leucine-rich repeat flightless-interacting protein 1
Mac	macrophage
MAVS/IPS-1	mitochondrial antiviral signaling
MDA5/IFIH1	melanoma-differentiation antigen 5
Med	medium
MFG-E8	milk fat globule-EGF factor 8 protein
MFI	mean fluorescence intensity
Mo	monocyte
mTOR	mammalian target of rapamycin
MxA	myoxvirus resistance A
MyD88	myeloid differentiation factor 88
Nase	neuraminidase
NCE	necrotic cell extract
NET	neutrophil extracellular trap
NK	natural killer
NLR	NOD-like receptor
NO	nitric oxide
NOD	nucleotide oligomerization domain
Pase/PNGase	peptide-N4-(N-acetyl-beta-glucosaminyl)asparagine amidase
PBMC	peripheral blood mononuclear cells

List of Abbreviations (continued)

pDC	plasmacytoid dendritic cell
PGE2	prostaglandin E2
PI3K	phosphatidylinositide 3-kinase
PKA	protein kinase A
PKR	protein kinase
PRR	pattern recognition receptor
RIG-I	retinoic acid inducible gene-I
RLR	RIG-I-like receptor
RNA	ribonucleic acid
RNAse	ribonuclease
RNP	ribonucleoprotein
Ro/SS-A	Sjögren's syndrome-associated autoantigen A
S6K	S6 kinase
Siglec	sialic acid-binding immunoglobulin-type lectin
SIGN-R1	CD209
SLE	systemic lupus erythematosus
Sm	Smith antigen
SNA	*Sambucus nigra*
SNP	single nucelotide polymorphism
ss	single-stranded
STAT	signal transducer and activator of transcription
STING	stimulator of IFN genes
Syk	spleen tyrosine kinase
TAM	TYRO3, AXL and MER
TBK	TANK-binding kinase
Tc	cytotoxic T cell
Th	helper T cell
TLR	Toll-like receptor
TMB	3,3’,5,5’-Tetramethylbenzidin
TNF	tumor necrosis factor
TRAF	tumor necrosis factor receptor-associated factor
Treg	regulatory T cell
TRIF	TIR-containing adaptor molecule
TYK	tyrosine kinase
UV	ultraviolet
WT	wildtype
Zym	zymosan

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Dedication

To Grandpa Camel and Uncle David who continue to watch over me.

Chapter 1

Introduction

Systemic lupus erythematosus (SLE). SLE or lupus is a systemic autoimmune disease with inflammation in many organs, and is considered a prototype disease for systemic autoimmunity. SLE is characterized by production of pathogenic autoantibodies directed against nuclear components including nucleic acids (DNA and RNA) and their binding proteins. Autoantibodies in combination with autoantigen form immune complexes (ICs). These ICs deposit in tissues where they can engage Fc receptors (FcRs) on immune cells and also fix complement (1). Whereas SLE can affect any part of the body including the heart, joints, skin, lungs, blood vessels, liver, and nervous system, in both human lupus and mouse models of this disease, ICs almost invariably deposit in the kidney. Effector mechanisms driven by IC deposition initiate infiltration and activation of tissue-infiltrating macrophages that promotes inflammatory responses with resultant tissue injury and nephritis (2). There are two major effector mechanisms: FcγR engagement and complement activation (1).

SLE affects approximately one in 1,000 people in the United States, although the prevalence of SLE varies depending on the location and ethnicity of the population studied (3). Strikingly, ~90% of SLE patients are women of child-bearing age, which may indicate a role for female sex hormones in the development of disease (4). The course of the disease is unpredictable, with periods of illness (called flares) alternating with remissions. There is no cure for SLE, and it can be fatal. Therapies for SLE largely include anti-inflammatory agents and immunosuppressive drugs such as cyclophosphamide, azathioprine, mycophenolic acid, and methotrexate (5), methods which have been utilized for decades. Unfortunately, some of these

agents become toxic, and others may leave patients more susceptible to infection. Thus, newer biological therapeutics that target specific pathways involved in immune pathogenesis have emerged and are now being tested in clinical trials. Examples of biologics in clinical trials are antibodies or receptors specific for B cells (anti-CD20, anti-CD22, anti-BAFF), costimulatory molecules (anti-CD40L, CTLA4-Ig), and cytokines (anti-IFN-α, anti-IL-6 receptor, TNF receptor, and anti-IFN-γ) (5, 6). However, to date only one biologic, belimumab, which targets the B cell survival factor BAFF, has been approved by the FDA for the treatment of lupus.

The etiology of SLE is not completely understood, but is considered to be a combination of multiple genetic and environmental factors which in combination result in a breach of immune tolerance. The complex genetic role is evidenced by the finding that siblings of SLE patients are approximately 30 times more likely to develop SLE compared with individuals without and affected sibling, and the concordance rate for SLE is 24-69% among monozygotic twins and 2-9% among dizygotic twins (7, 8), suggesting that it is the contribution of susceptibility genes as well as environmental factors that likely cause disease. In addition, it has been known for more than 25 years that a proportion of SLE patients have increased serum interferon (IFN) (9, 10), a family of cytokines named after their capacity to 'interfere' with viral replication. More recently it has been demonstrated that ~60% of patients display a gene expression "signature" indicative of exposure to type I IFN (IFN-I, primarily IFN-α and IFN-β), and which correlates with SLE disease activity (11-13). Studies of in both humans and mice provide evidence that persistent type I IFN contributes a break in immune tolerance. Numerous case reports reveal that some patients with IFN-I treatment for malignancy or chronic viral infections exhibit characteristic features of SLE including rash, nephritis, autoantibodies (dsDNA, Sm antigen) (14-16), which are reversible when IFN treatment is removed (15). Additionally, in mouse

models with increased type I IFN, there is exacerbation of autoimmunity (17-20). Conversely, mice that are deficient in the type 1 IFN receptor (IFNaR) and thus lack the ability to respond to type I IFN, and are protected in several murine lupus models—exhibiting reduced severity of disease and delayed onset (17, 21). The next few sections will therefore focus on type I IFN induction, activity on the immune system, and regulation, as well as the genetic and environmental factors that may contribute to its upregulation and contribution to SLE.

The type I IFN system—sensors and signaling. Interferons are named for their ability to "interfere" with viral infection, but type 1 IFNs can also be produced in response to bacteria ligands. Inducers of type 1 IFN, typically nucleic acids, can be sensed by several families of innate pattern recognition receptors (PRRs) including Toll-like receptors (TLRs), retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs), and nucleotide oligomerization domain (NOD)-like receptors (NLRs) (22), as well as new families of cytoplasmic DNA sensors (CDSs) (23) (Figure 1.1). The type I IFN genes are tightly regulated and normally almost no constitutive IFN-α or -β is detected in healthy individuals in the absence of viruses, bacteria, or microbial nucleic acids. Most types of cells can produce small amounts of type I IFN when stimulated with certain RNA viruses. However, the principal type I IFN producer in vivo is the plasmacytoid dendritic cell (pDC) (24), previously designated natural IFN producing cell (IPC). Though rare, pDCs are particularly attuned for type I IFN production, making up to 10^9 IFN-α molecules per cell within 12 hours (24, 25).

The pDCs preferentially express TLR7/9 in their endosomes which, upon ligation with RNA and DNA respectively, causes recruitment and assemblage of a complex consisting of myeloid differentiation factor 88 (MyD88), tumor necrosis factor receptor-associated factor 6

(TRAF6), and interleukin receptor associated kinase (IRAK) 1 and 4 (22). This complex leads to the phosphorylation of interferon regulatory factor 3, 5 and 7 (IRF3, 5, and 7) which translocate to the nucleus and facilitate type I IFN gene transcription. Constitutive expression of IRF5 and 7 in pDCs enhances their capacity to produce type I IFN, and particularly IFN-α. In addition to TLR7 being located on the X-chromosome which may explain some of the female predominance of lupus, a SNP in TLR7 is associated to both lupus and a more pronounced IFN signature (26). Multiple SNPs within IRAK1 were associated with both adult-onset and childhood-onset SLE (27). Also, IRF5 is one of the most strongly and consistently SLE-associated loci outside the MHC region, and increased risk haplotypes are associated with functional changes in IRF5-mediated signaling, including increased expression of IRF5 mRNA and IFN-inducible chemokines, as well as elevated IFN-α activity (28, 29).

TLR3 is also expressed in the endosome and senses viral nucleic acids (dsRNA), resulting in production of type I IFN (30). TLR3 is expressed by several cell types, especially by myeloid DCs, but not pDCs or monocytes (31). Though TLR3 is also expressed in the endosome, signaling differs from that of TLR7/9. Upon ligation of TLR3, signal is transduced by TIR-containing adaptor molecule (TRIF) which acts via a TRAF3-containing complex to activate the transcription factors IRF3 and 7 (22).

In contrast to selective TLR expression in specialized cells, most cell types express RNA helicases belonging to the RLR family—RIG-I and melanoma-differentiation antigen 5 (MDA5/IFIH1) (32) which recognize intracellular viral nucleic acids, long and 5'-triphosphated RNA, respectively. Upon recognition of their ligand, these RNA helicases interact with the mitochondrial antiviral signaling (MAVS or IPS-1) adaptor protein which assembles with a signaling complex including TRAF3, TBK1, and IKKε. The complex promotes the

phosphorylation and activation of IRF3 and 7 which translocate to the nucleus and promote transcription of type I IFN genes (22). Recently, IKKε was identified as a risk locus in SLE (33).

The growing family of CDSs, upon ligation by cytosolic DNA, can also induce type I IFN production. DNA-dependent activator of IRFs (DAI or ZBP-1), IFN-induced with helicase C domain 16 (IFI16), RNA polymerase III, and newly discovered cyclic-GMP-AMP (cGAMP) synthase (cGAS) utilize many of the same signaling components as RLRs. Type I IFN induction is dependent upon stimulator of IFN genes (STING) which translocates from the endoplasmic reticulum (ER) to the Golgi and finally in the cytoplasm assembles with TBK1, promoting IRF3 and 7 activation (reviewed in (23)). In contrast, DHX9 and DHX36, members of the DExD/H box family of helicases, recognize CpG-DNA and mediate type I IFN production through a pathway involving myeloid differentiation factor 88 (MyD88). LRRFIP1 recognizes both dsRNA and dsDNA and, by activating β-catenin, facilitates the recruitment of the acetyltransferase p300 to the IFN enhanceosome, which potentiates IFNB gene transcription.

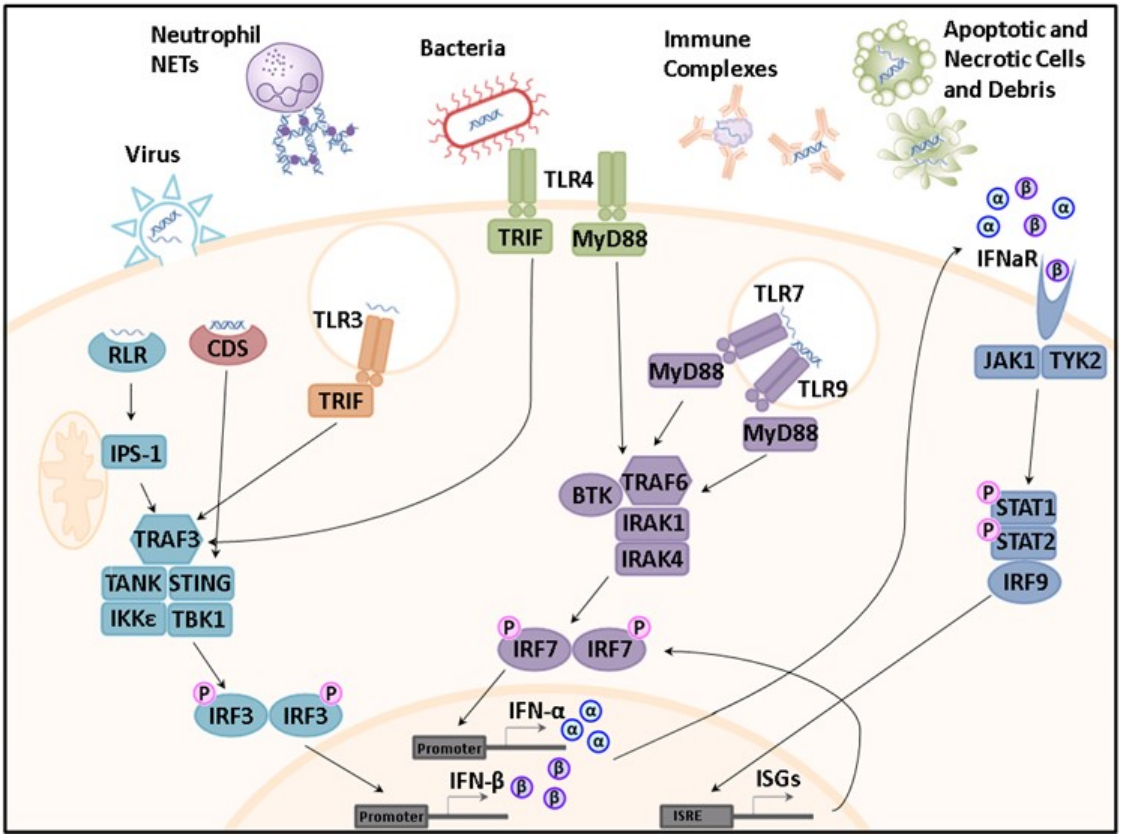

Figure 1.1. The type I IFN system. Innate sensor of the immune system recognize microbial ligands, primarily nucleic acids (RNA and DNA). Engagement with ligand initiates a signaling cascade culminating the in the phosphorylation of IRFs that then translocate to the nucleus and promote type I IFN production. Type I IFN then engages its receptor (IFNaR), and promotes JAK-STAT signaling that results in the expression of hundreds of interferon response genes which collectively set up an antiviral state, protecting the cell from viral infection. In addition to viral and bacterial pathogens, ligands that stimulate type I IFN production can also be provided by NETosing neutrophils, uncleared apoptotic cells, and immune complexes containing nuclear components.

Type I IFN effects. Type I IFNs are sensed by a ubiquitously expressed receptor, IFNaR, and initiates signaling which culminates in the expression of IFN response genes (ISGs) (reviewed in (34)). Engagement of IFNaR turns on the JAK-STAT signaling pathway which involves the Janus family kinases (JAK), tyrosine kinase 2 (TYK2) and JAK1. The activated kinases recruit and phosphorylate the transcription factors STAT1 and STAT2, which associate with IRF9 to form a complex that translocates to the nucleus and binds to IFN-stimulated response elements (ISREs) and activates the transcription of hundreds of ISGs. Polymorphisms in TYK2 are associated with SLE (35, 36), as are polymorphisms in STAT4 (37), another signaling molecule that interacts with the cytoplasmic part of IFNaR (38).

Though the exact functions of the majority of the ISGs are unclear, it is known that they collectively contribute to an antiviral state as mice deficient in IFNaR are highly susceptible to viral infection (39). Among ISGs with known function are enzymes which inhibit viral transcription and translation and promote degradation of viral RNA including myoxvirus resistance A (MxA), 2'5' oligoadenylate synthetase, and protein kinase (PKR) 16 (40). IRF7 is also an ISG, and thus IFN-β production primes cells for IFN-α production.

In addition to its antiviral effects on all cells, type I IFN also affects key functions of both innate and adaptive immunity. IFN-α has stimulatory effects on many cell types that could contribute to the autoimmune process in SLE (and for other IFN-associated autoimmune diseases such as autoimmune thyroid disease, type 1 diabetes, and rheumatoid arthritis (41)). Effects on immune cells include the activation of DCs with increased cross-presentation of antigens, the differentiation of monocytes into APCs and enhancement of their BAFF production, the stimulation of Th1 cells and prevention of the apoptosis of activated cytotoxic T cells, the suppression of regulatory T cells, the differentiation and antibody production of B cells, and

enhancement of NK cell cytotoxic activity (reviewed in (42)). In light of the extensive effects stimulatory effects of type I IFN on a broad range of immune cells, it is perhaps not surprising that defects in the regulation of type I IFN can cause a loss of tolerance and development of autoimmunity.

Type I IFN inducers. Normally, type I IFN synthesis is triggered by microorganisms and the production is tightly regulated and limited in time. However, the majority of lupus patients have a type I IFN signature, even in the absence of infection. Many of the sensors leading to type 1 IFN production recognize nucleic acids. Therefore, much work has been done to identify the interferon stimulus in SLE.

Viral infections induce type 1 IFN in healthy individuals. Many different viruses, including endogenous retrovirus, have been connected to the development of lupus, and cross-reactivity between viral antigens and lupus autoantigens have been reported (43-45). For instance, pediatric lupus patients have a high prevalence of Epstein Barr virus (EBV) seropositivity (46) and autoantibodies to the EBV antigen EBNA-1 cross-react with the SSA autoantigen (47), which is a common target in lupus. Additionally, the development of lupus as well as disease flares has been reported in connection with viral infections (48, 49).

There are also endogenous sources of nucleic acids that can drive type I IFN production. In healthy individuals, apoptotic cells are rapidly removed by macrophages—a process that is inherently anti-inflammatory in nature. In many mouse models and in human SLE, however, there is evidence of defective clearance of apoptotic cells (50), resulting in a transition to a necrotic form of cell death. Release of nuclear antigen, including nucleic acids, drives production of inflammatory cytokines, including IFN-α (51). Both genetic and environmental

factors can contribute to increased apoptosis as well as defective clearance of apoptotic cells. Though the incidence is rare, genetic mutations resulting in deficient C1q (which opsonizes apoptotic cells) has the highest penetrance for SLE (52). Mice deficient for molecules that aid in the clearance of apoptotic cells, such as the TAM subfamily of receptors (including Mer) which bind ligands GAS6 or protein S which are opsonins for apoptotic cells, or C1q or MFG-E8 that also bind to apoptotic cells, also develop signs of autoimmunity, including presence of anti-DNA autoantibodies (50). Ultraviolet (UV) radiation can induce apoptosis of keratinocytes (53) with redistribution of nuclear antigens to the cell surface and production of novel forms of autoantigens (54). Multiple studies have implicated UV light exposure to flares in disease activity of SLE patients, including increased risk of skin lesions following UV exposure (55-59) and increased incidence of flare during summer (60). A recent study also revealed an association among SLE patients with outdoor work in the 12 months preceding diagnosis (59), suggesting that UV exposure may not only exacerbate disease, but also contribute to its development.

Upregulation of granulopoesis-related genes was also observed in arrays obtained from SLE patients with active disease (12) and SLE patients have increased numbers of apoptotic neutrophils in blood that correlated with anti-DNA antibodies and disease activity (61). As part of their antimicrobial functions, activated neutrophils release web-like structures called neutrophils extracellular traps (NETs), composted of large amounts of nuclear DNA as part of a death program called NETosis (62). Neutrophils from SLE patients undergo accelerated cell death in vitro and release more NET DNA than healthy donors (63, 64), and thus may represent a potent source of interferonogenic material.

In addition to increased autoantigen, SLE patients also have autoantibodies that recognize nuclear components and nucleic acids. Sera from lupus patients contain immune complexes with

the capacity to specifically activate pDCs and promote IFN-α production (65, 66). Further studies revealed that these ICs contain nucleic acids and are internalized via the FcγRIIa expressed on pDCs (67) which reach the endosome and stimulate the relevant TLR with subsequent IFN-α production (68).

Type I IFN regulation. Because pDCs are the main producers of type I IFN *in vivo*, much of the research on IFN regulation has been focused on this cell subset. IFN-α production by pDCs can be inhibited both by cell-bound ligands, as well as by soluble factors. There is also active research to create drugs to regulate IFN-α.

pDCs express on their cell surface several molecules that when triggered, inhibit IFN-α by activation of immunoreceptor-based tyrosine activation motif (ITAM). Such molecules include blood dendritic cell antigen 2 (BDCA2), immunoglobulin-like transcript 7 (ILT7), and the high-affinity Fc receptor for IgE (FcεR1α) that associate with the ITAM-containing γ-chain of FcεR1, as well as human NKp44 and mouse sialic-acid-binding immunoglobulin-like lectin H (Siglec-H) which associate with the ITAM-containing adaptor protein DAP12 (69). After being crosslinked with ligand, ITAM signaling activates SRC family protein kinases which inhibit type I IFN production through SYK and BLNK/BCAP.

Soluble factors that are inhibitory include IL-10 (70), TNF-α (71, 72), and PGE2 (72-74). However, the mechanism of inhibition of IFN-α by these factors is not well understood. Additionally, the female sex hormone progesterone can inhibit IFN-α (75). In support of a role in lupus, administration progesterone to pre-autoimmune lupus-prone mice retarded the development of autoimmune disease and death (76). Accordingly, women with SLE produce abnormally low levels of progesterone (77, 78).

A number of IFN blocking agents for treating SLE are now in phase I and II clinical trials (thoroughly reviewed in (79) and (80)) including: three IFN-α neutralizing antibodies (sifalimumab by Medimmune, rontalizumab by Genentech, and AGS-009 by Argos Therapeutics), one blocking antibody to the type I IFN receptor (MEDI-546 by Medimmune), one IFN-α kinoid to immunize against IFN-α (IFN-α-K by Neovacs), and four TLR7/9 antagonists (CPG-52364 by Pfizer, IMO-8400 and IMO-3100 by Idera Pharmaceuticals, and DV 11179 by Dynavax/GlaxoSmithKline). Many of these therapies have proven effective at neutralizing IFN-α, as measured by expression of interferon signature genes, and sifalimumab has demonstrated a trend towards improved disease activity with less frequent flares (81, 82). Larger, phase II/III clinical trials will tell how effective IFN-α neutralizing therapies are compared to standard treatments. Thus far, however the IFN-based therapies have not been reported to be especially effective, and therefore the need for more effective therapies remains.

Most recently, our own lab has made the surprising discovery that *in vitro* SLE IC-induced IFN-α can be inhibited by human serum obtained from healthy controls, and identified immunoglobulin G (IgG) as one of the inhibitory factors (83).

Intravenous IgG (IVIg). IVIg is highly purified human IgG pooled from thousands of donors. It was originally developed more than 50 years ago to restore IgG levels in patients with hypogammaglobulinemia, but has been used more recently at high doses as an anti-inflammatory agent in a variety of acute and chronic inflammatory and autoimmune diseases, including some cases of SLE (84). Despite its broad use clinically, the mechanism of action within particular disease settings is still not clear. Many mechanisms have been proposed for the anti-inflammatory activity of IVIg (85), including activities associated with its antibody specificity

(cytokine and autoantibody neutralization, receptor blockade, and target cell depletion) but also with the Fc region (FcRn saturation leading to autoantibody catabolism, blockade of activating FcR, modulation of activating and inhibitory FcR expression on APCs and B cells, modulation of DC activation via FcγRIII, and expansion of regulatory T cells).

IgG has a conserved N-linked glycosylation site in the Fc region at Asn 297 of the heavy chain. This biantennary sugar moiety consists of a heptameric core of three mannose and four N-acetylglucosamine (GlcNAc) residues with variable additions of terminal galactose and sialic acid residues or branching GlcNAc and fucose residues. Recent evidence in mice has supported the theory that it is the rare subset of human IgG containing a glycan terminating in α-2,6-linked sialic acid that is the crucial glycoform necessary to exert IVIg's inhibitory action. In the K/BxN model of arthritis, Ravetch and colleagues showed that the sialylated subset acted via engagement of the receptor SIGN-R1 on myeloid cells (86). Subsequently, using a mouse expressing human DC-SIGN (a homologue to murine SIGN-R1), they demonstrated that sialylated Fc induced IL-33 by SIGN-R1/DC-SIGN expressing cells, culminating in a Th2 mediated reduction of inflammation (87). However, the requirement for sialylation of IVIg in reducing inflammation has recently been questioned in mouse models of immune thrombocytopenic purpura (ITP), as sialylation-enriched IVIg, neuraminidase-treated (desialylated) IVIg and normal IVIg did not differ in their efficacy to alleviate ITP (88, 89). Also, Fc was shown to be dispensable for expansion of regulatory T cells (90). These studies suggest that sialylated Fc may not represent the anti-inflammatory component of IVIg in all disease models.

Despite the extensive work done to identify the inhibitory subset of IVIg in this mouse model of arthritis, no research has been conducted to address its relevance in SLE. Also, despite

use of IVIg in diseases in which IFN-I has been implicated, there have been no studies looking at the mechanism of inhibition of IFN-α inhibition by IVIg.

Objective

The goals of this dissertation were: a) to determine the mechanism(s) by which IVIg attenuated IFN-α production in response to SLE ICs, and b) to elucidate the importance of IgG sialylation in this process. Answers to these questions could provide insight into basic pathogenic mechanisms in SLE, and also suggest novel therapeutic strategies for autoimmunity. We found that IVIg Fc fragments—independent of sialylation—were able to inhibit IC-induced IFN-α by blocking their binding to pDCs. In contrast, we discovered that IFN-α induced by TLR agonists, which circumvents the need for FcR interaction, was more robustly inhibited by sialylated IVIg F(ab')2 fragments. This inhibitory pathway resulted from induction of monocyte production of prostaglandin E2 (PGE2), a potent pDC inhibitor. This novel finding offers an alternate method of immune modulation in SLE and other autoimmune diseases involving IFN-I. Further elucidation of how this subset of IVIg regulates monocytes and the mechanism of PGE2 inhibition of pDCs could reveal novel therapeutic targets in the future.

Chapter 2

Materials and Methods

Reagents

Serum was collected from lupus patients that fulfilled the American College of Rheumatology 1982 revised criteria for the classification of SLE. Necrotic cell extract (NCE) was made by freeze thaw of U937 cells as described (83). Affinity-purified SmRNP antigen (Arotec Diagnostics) was labeled with Alexa Fluor 647 (Invitrogen). RNase A and DNase I were purchased from Thermo Fisher Scientific. TLR agonists Loxoribine CL097, and type A CpG (ODN 2216) were obtained from Invivogen, and LPS, Flagellin FliC, and zymosan were obtained from Sigma. Rapamycin and dibutyryl-cAMP were from Enzo Life Sciences. Blocking or neutralizing antibodies included anti-FcγRII (clone IV.3, StemCell Technologies), anti-DC-SIGN (clone 120507, R&D Systems), anti-PGE2 (clone 2B5, Cayman Chemical), and Ms IgG1,κ isotype control (clone MOPC-21/P3, eBioscience). Bovine fetuin, human transferrin, and fibrinogen were from Sigma. In certain experiments, Universal type I IFN (PBL Interferon Source), PGE2 (Cayman Chemical), TNF, IL-10, IL-8, IL-6 (Biolegend), or Polymyxin B (Invivogen) were used.

Cell purification

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood obtained from healthy human donors following consent (IRB approval #39712) using Ficoll-Paque (GE Healthcare). In certain experiments, PBMCs were depleted of monocytes, B cells, or NK/NKT cells using anti-CD14, anti-CD19, or anti-CD56 magnetic beads (Miltenyi Biotec), respectively

(<0.5% of the depleted cell type remained). pDCs were isolated by negative selection with a pDC enrichment kit (StemCell Technologies) to purities of 92-99% $CD123^{+}BDCA2^{+}$. Monocytes were purified using CD14 magnetic beads (Miltenyi Biotec), with purities of >95% $CD14^{+}$ cells. Using mAbs purchased from BioLegend and eBioscience, monocyte subsets ($CD14^{dim}CD16^{+}$, $CD14^{+}CD16^{+}$, and $CD14^{+}CD16^{-}$) were identified as HLA-DR^{+} but $CD2^{-}CD19^{-}CD56^{-}NKp46^{-}CD15^{-}$ and sorted base on expression of CD14 and CD16 using a BD FACS Aria to >90% purity.

Flow cytometry

Fluorescence-assisted cell sorting (FACS) was performed on a BD FACS Canto and analyzed using FlowJo software (TreeStar, Inc.). Monocytes ($CD14^{+}$), pDCs ($CD123^{+}BDCA2^{+}$), T cells ($CD3^{+}$), NK cells ($CD56^{+}$), and B cells ($CD19^{+}$) were identified using mAbs from eBioscience, Miltenyi Biotec, or BioLegend and analyzed on a BD FACS Canto. SLE IC binding experiments were performed by generating fluorescent ICs with diluted lupus serum (1:1000) and 1 μg/mL of labeled SmRNP. Expression of other surface proteins were analyzed by fluorescently-labeled monoclonal antibodies from Biolegend unless otherwise noted and included mAbs specific for: FcγRI (CD64), FcγRIIa (CD23A, StemCell Technologies), FcγRIIB (CD32B, clone 2B6, a gift), FcγRII (CD16), DC-SIGN (CD209, R&D Sytems, labeled with Alexa Fluor 647 from Invitrogen), CD86, CD40, Annexin V, and propidium iodide.

Intravenous immunoglobulin G (IVIg)

IVIg (Privigen®, CSL Behring, Bern, Switzerland) was fractionated by lectin affinity chromatography using the sialic acid-specific *Sambucus nigra* agglutinin (SNA), according to

the manufacturer's description (Vector Laboratories), as previously reported (91). Both the flow-through fraction (SNA- IgG) and the fraction bound to the SNA column (SNA+ IgG) were collected. All preparations had endotoxin levels <2 EU/mg, and most had <0.2 EU/mg, with no effect on activity (data not shown). By HPLC (91), unfractionated IVIg had 1.4 mg of total sialic acid per g of IgG. This was increased to 7.1 mg in the SNA+ fraction and reduced to <0.5 mg in the SNA- fraction (Figure 3.3A). All preparations contained 8-13% dimers and 0.4% or less aggregates, as is typical for IVIg.

F(ab')2 or Fc fragments were produced from IVIg (or SNA+ and SNA- fractions) by digestion with pepsin or papain respectively as described (91). Fc was polished by EndoTrapHD (Hyglos) resulting in endotoxin levels below 0.05 EU/mg. IgG and fragments were treated with neuramindase to cleave sialic acid, galactosidase to cleave terminal galactose, or PNGase to cleave the entire N-linked glycan (7U/mg protein, all from New England Biolabs) to cleave sialic acid for 24-48h at 37^{o}C, following manufacturers' protocols.

Both wild type (WT) and the hypersialylated (HS) variants of recombinant human IgG1 Fc were generated by transient transfection of 293-6E cells using mammalian expression vector pTT5. The HS variant contains two mutations in the CH2 region of Fc (F241A and F243A) that lead to increase of terminal α-2,6 linked sialylation species compared to the WT Fc (from 4% to 43%).

Lectin blots

Duplicated 2.5 µL of 1mg/mL samples were blotted onto nitrocellulose (Whatman) and blocked with 1x PBS + 1% BSA. Blots were probed using biotinylated lectins against terminal sialic acid (Sambucus nigra, Vector labs), galactose (RCA120, Vector labs) or with anti-IgG antibody

(Jackson labs) and developed using Streptavidin-HRP (Biolegend) and TMB substrate for membranes (KPL).

Cell culture, stimulation and cytokine detection

Cell cultures (3-4x10^6/mL PBMCs, 0.5-1.0x10^6/mL monocytes, or 1.5-2.0x10^4/mL pDCs) in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (Hyclone) were stimulated with either SLE ICs formed by combining a diluted (1:1000) serum from one of a set of donors with SLE with necrotic cell extract (1%) as described (83), TLR7 agonist Loxoribine (100-200 μM), TLR7/8 agonist CL097 (1 μg/mL), TLR9 agonist CpG-A (ODN 2216, 200-250 nM), TLR2 agonist zymosan (1-10 μg/mL), TLR5 agonist Flagellin FliC (1-10 ng/mL), or TLR4 agonist LPS (1-100 ng/mL). In some experiments, 50-5000 μg/mL IgG or molar equivalents of the F(ab')2 or Fc fragments were added to the cultures. To generate monocyte supernatants, monocytes (0.5-1.0x10^6/mL) were cultured with a TLR agonist and IgG for 4h, washed to remove unbound IgG, and then resuspended in fresh medium for the remaining 14-18 h. For certain experiments, monocyte supernatants were incubated with anti-PGE2 antibody or an isotype control, and then applied to a buffer exchange column (Amicon, 100 kDa) to deplete the supernatants of PGE2. The monocyte supernatants were then used in cultures of PBMCs or pDCs at 50% v/v. In certain experiments, increasing concentrations of sera from healthy human donors was added. In other experiments, Rapamycin (a suppressor of mTOR), dibutyryl-cAMP (a cell-permeable cAMP analog), PGE2, or DMSO (carrier control) was added to cultures. IFN-α levels in supernatants were quantified by ELISA as described (83), and detection of IL-6, IL-8, IL-10, TNF-α, IL-1β (Biolegend) and PGE2 (Cayman Chemical) by commercial ELISAs.

Microscopy

IRF7 detection was performed as follows: after 1h in culture, pDCs were fixed to glass slides with 2% paraformaldehyde and then permeabilized with 100% ice-cold methanol for 10 min at −20°C. Samples were labeled with rabbit polyclonal anti–human IRF-7 (Santa Cruz Biotechnology, Inc.) and anti–rabbit IgG Alexa Fluor 555 (Invitrogen) was used as a secondary antibody. Cells were counterstained with DAPI (Invitrogen) and preserved in mounting medium (Dako). Images were acquired using a fluorescence microscope (Nikon Eclipse E4000, with 40X/0.75) or a confocal microscope (LSM 510 META; Carl Zeiss, Inc. with 63×/1.4 NA objective). Images were analyzed using CellProfiler.

Data presentation and analysis

In view of donor-to-donor variation in circulating % pDCs and IFN-α production, for most experiments the results are expressed as the % of inhibition of IFN-α relative to cells that did not receive the inhibitor being tested. Only cultures producing at least 200 pg/mL IFN-α in the absence of inhibitor were included for analysis. Differences between groups were compared by the paired t test, unless otherwise indicated. Graphs and statistical analyses were performed using Prism software (version 4, Graphpad Software). A *p*-value of <0.05 was considered significant. Bars represent mean + SEM.

Chapter 3

IVIg inhibits SLE immune complex (IC)-induced IFN-α by preventing IC binding to FcγRIIa on pDCs

Brief introduction

Type 1 IFN (IFN-I, including IFN-α and -β) is implicated in the pathogenesis of several autoimmune diseases including dermatomyositis, scleroderma and SLE (41, 92, 93). The principal IFN-α producing cell is the pDC that expresses relatively high concentrations of endosomal TLR7 and TLR9. Sera from patients with systemic autoimmune diseases contain ICs with the capacity to specifically activate pDCs (94). These interferogenic ICs contain nucleic acids which, following internalization via FcγRIIa on pDCs, reach the endosome and stimulate the relevant TLRs with subsequent activation of transcription factors and production of IFN-α (95). We previously observed that serum from healthy donors inhibits SLE IC-induced IFN-α production and identified two serum proteins, C1q and immunoglobulin G (IgG), that exerted this effect (83). Our previous studies have in part elucidated the mechanism whereby C1q exerts its effect on attenuation of IFN-α production (96, 97). Therefore, in the current study, we explore the mechanism by which IgG inhibits IC-induced IFN-α.

High-dose IVIg (intravenous immunoglobulin, pooled IgG from thousands of donors) exerts an anti-inflammatory effect and has been used to treat many inflammatory and autoimmune disorders, including those described above, though studies to date have not examined the effect of IVIg treatment on the production of IFN-I. IgG molecules are heterogeneous by amino acid sequence and by differential glycosylation, both of which can alter its effector functions (98). The rare subset of human IgG that contains a glycan terminating in

sialic acid (~5% of serum IgG) has been shown to exert a more powerful anti-inflammatory effect in certain mouse models of inflammatory disease (99, 100). This led us to ask whether sialylated IgG was a more potent inhibitor of IC-induced IFN-α.

Immune complex-induced IFN-α is inhibited by IVIg Fc fragments

We generated ICs by combining a diluted (1:1000) serum from one of a set of donors with SLE with necrotic cell extract (1%) at a 1:10 ratio. These conditions were based on a study showing that such ICs can induce IFN-α in PBMC cultures after 16-24 hours (94). We found that when these SLE ICs were incubated with PBMCs, high concentrations of IFN-α were produced in the culture supernatants whereas other inflammatory cytokines tested were either not induced or produced at relatively low levels (Figure 3.1A). Induction of IFN-α was dependent on the presence of pDCs, as it was lost with depletion of the subset from PBMC (data not shown), and was dependent on the presence of RNA, as treatment of ICs with RNase but not DNase diminished IFN-α induction (Figure 3.1B and (83, 94, 101)).

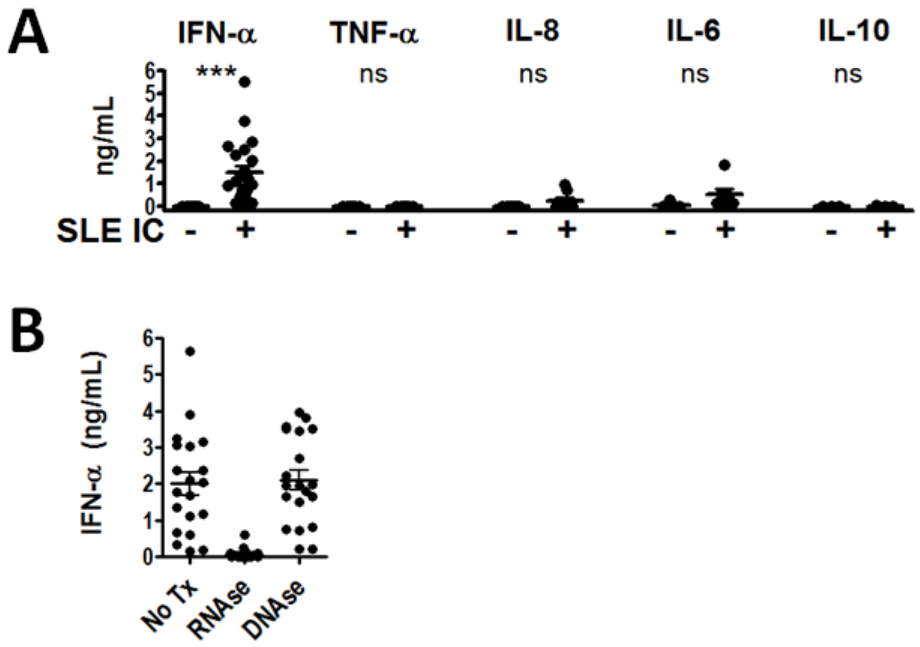

Figure 3.1. Immune complexes induce IFN-α dependent on RNA. (A) Human PBMCs were stimulated with immune complexes and after 20h the supernatants were assayed by ELISA for IFN-α, TNF-α, IL-8, IL-6 and IL-10. (B) Immune complexes were treated with RNase or DNase before stimulation of PBMC cultures. After 20h, the supernatants were assayed by ELISA for IFN-α. Results represent at least four experiments in each group. *, **, *** represent p-values of <0.05, <0.01, and <0.001, respectively; ns, not significant.

We had previously shown that normal human serum and IgG could inhibit IFN-α production in this bioassay (83). Here we show both serum (Figure 3.2A) and IVIg (Figure 3.2B) are able to robustly inhibit immune-complex induced IFN-α in a dose dependent manner. For further studies of the mechanism of IgG inhibition of SLE ICs, we wanted to use a concentration of IgG that was within the dynamic range of IFN-α inhibition. We observed that a concentration of 500 μg/mL IVIg consistently inhibited ~50% of IFN-α production so we used this concentration in all future experiments involving SLE ICs. We found that IVIg did not simply increase pDC death (data not shown). To address which portion of IgG contained inhibitory activity, we digested IgG with pepsin, isolated the fragments and compared the inhibitory activities of the Fc and F(ab')2 fragments. Addition of 500 μg/mL IgG or the molar equivalent of the Fc inhibited ~50% of IFN-α stimulation whereas F(ab')2 fragments had no effect (Figure 3.2C) implicating the Fc region as containing the inhibitory activity.

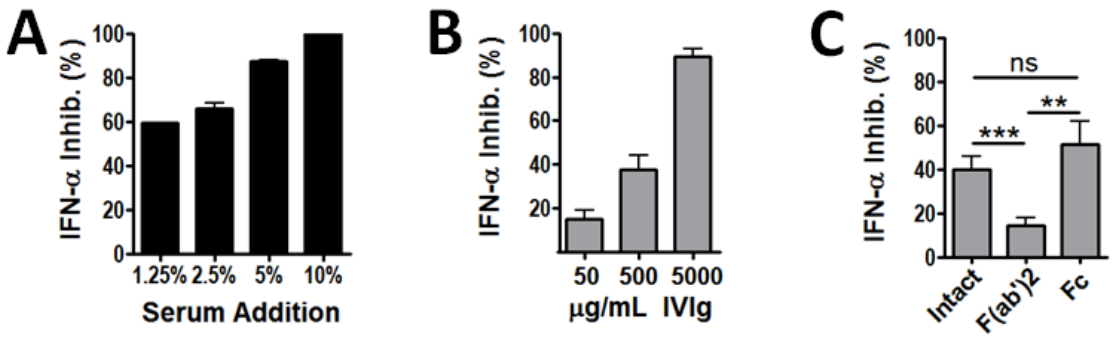

Figure 3.2. IVIg Fc is a potent inhibitor of immune complex-induced IFN-α. Human PBMC were stimulated with immune complexes and were collected at 20h. The following were tested for their ability to inhibit IFN-α production (results expressed as % inhibition compared to no serum or IVIg treatment): (A) normal human serum, (B) IVIg or (C) intact IVIg (500 µg/mL), F(ab')2 fragments (333 µg/mL), or Fc fragments (167 µg/mL). The results are expressed as % inhibition of IFN-α compared to cultures incubated with IC alone. Results represent at least three experiments in each group. Bars represent mean + SEM. *, **, *** represent p-values of <0.05, <0.01, and <0.001, respectively; ns, not significant.

IC-induced IFN-α inhibition by IVIg is independent of sialylation

The Fc region of IgG contains a N-linked glycosylation site (Asn 297) that is required for binding to Fc receptors and subsequent effector function. The rare subset (~5%) of Fcs that contains a glycan terminating in sialic acid was proposed to be critical for inhibition of inflammation in the K/BxN arthritis model by engaging the receptor SIGN-R1 (mouse) or DC-SIGN (human) (99). To determine whether sialylation was required for inhibition of IFN-α by SLE ICs, we compared the inhibitory activity of 500 μg/mL sialylation-enriched IgG (SNA+, ~5-fold enrichment in total sialic acid content) or sialylation-depleted IgG (SNA-, ~5-fold reduction in total sialic acid content)(91) (Figure 3.3A) but observed no difference in the inhibitory effect of SNA+ and SNA- IgG in the IFN bioassay (Figure 3.3C). Since the SNA lectin column used for enrichment may preferentially retain IgG which is sialylated in the Fab region (91, 98, 102, 103) (Figure 3.3A), we specifically examined the effect of Fc sialylation. To this end, we tested the IFN-α inhibitory activity of an equimolar concentration (167 μg/mL) of a recombinant IgG Fc that was hypersialylated (HS, >40% sialylated), but this preparation was no more effective at inhibiting IFN production than wildtype IgG (WT IgG, 3-4% sialylated) (Figure 3.3D). Furthermore, neuraminidase treatment to cleave sialic acid from the glycan on IgG Fc fragments did not reduce its inhibitory activity (Figure 3.3E). Consistent with these findings, blockade of DC-SIGN by using specific antibody did not alter inhibition (data not shown). Therefore, inhibition of IC stimulation of IFN-α by IVIg Fc is independent of sialylation.

Changes in the Fc glycan can have profound effects on effector function such as the increased affinity for FcγRIII and consequently antibody-dependent cell mediated cytotoxycity (ADCC) if the glycan contains a bisecting N-acetylglucosamine residue or lacks either fucose or sialic acid (98), and alterations in glycans such as reduced presence of galactose are described in

patients suffering from various inflammatory diseases (99). Therefore, we also addressed whether galactose was required for inhibition. We used galactosidase to cleave the terminal galactose residue of the Fc (Figure 3.3B), but observed no change in inhibition of the treated IgG compared to untreated IgG (Figure 3.3F). However, when the entire glycan was cleaved (Figure 3.3B), we saw a marked reduction in IFN-α inhibition (Figure 3.3G). Since removal of the entire glycan markedly impairs Fc affinity for FcγR (98), this suggested that IVIg interaction with FcγR plays a role in the inhibition of SLE IC-induced IFN-α.

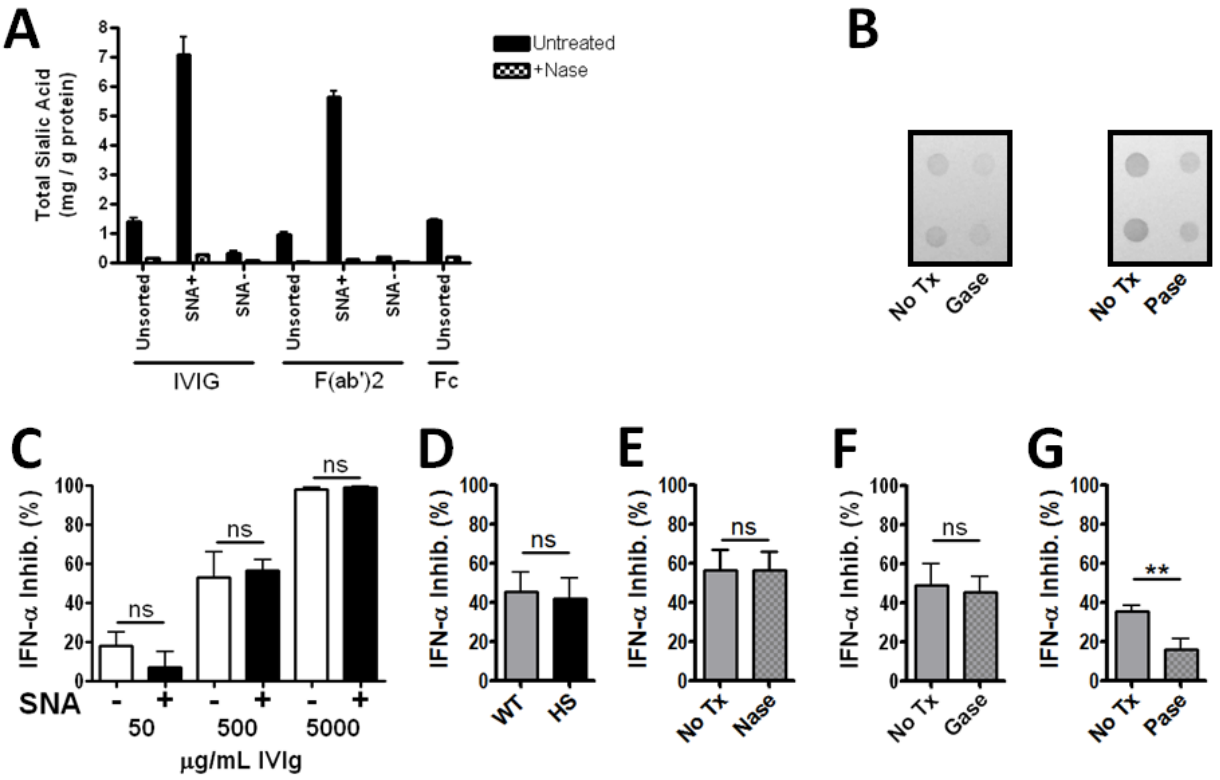

Figure 3.3. IVIg inhibition of immune complex-induced IFN-α does not require sialylation. (A) Sialic acid content determined by HPLC for IVIg and fragments that have been sorted for sialylation via a SNA lectin column with or without subsequent neuramindase treatment to cleave sialic acid. (B) Lectin blot (RCA120) analysis of IVIg galactosylation with or without galactosidase (Gase) treatment to cleave galactose (left) or PNGase (Pase) to cleave the N-glycan (right). (C-G) Human PBMCs were stimulated with immune complexes and supernatants collected at 20h and assayed for IFN-α by ELISA (results expressed as % inhibition compared to no IVIg treatment) after addition of (C) sialylation enriched (SNA+) and depleted (SNA-) IVIg, (D) recombinant Fc fragments (167 µg/mL) that is wildtype (WT) or engineered to be hypersialylated (HS), (E) IVIg Fc (167 µg/mL) with or without treatment with neuramindase (Nase) to cleave sialic acid, (F) IVIg Fc (167 µg/mL) with or without treatment with galactosidase (Gase) to cleave the terminal galactose residue, and (G) IVIg (500 µg/mL) with or without treatment with PNGase (Pase) to the N-glycan. In C-G, results represent at least four experiments in each group. Bars represent mean + SEM. *, **, *** represent p-values of <0.05, <0.01, and <0.001, respectively; ns, not significant.

IVIg inhibits IC-induced IFN-α by preventing IC binding to FcγRIIa on pDCs

To address whether IVIg attenuation of IC stimulated IFN-α from PBMCs was a direct effect on pDCs, we isolated pDCs and performed inhibition studies with IVIg. As shown in Figure 3.4A, we found that IVIg significantly inhibited SLE IC-induced IFN-α by both PBMCs and by purified pDCs from different donors.

The low affinity receptor, FcγRIIa, is the only FcγR expressed on human pDCs (67, 104) (Figure 3.4B). Inhibition of a low affinity receptor by monomeric IgG was surprising. However, it has been shown that monomeric IgG can antagonize the low-affinity receptor FcγRIIa (105) and compete with ICs or oligomeric IgG for binding to FcγRII or FcγRIII (106-108). To address the mechanism of inhibition directly, we tested whether IgG Fc inhibited SLE IC-induced IFN-α production by interacting with FcγRIIa to block ICs binding to pDCs. As expected and also shown in Figure 3.4C, all binding of ICs to pDCs was mediated by FcγRII as binding was abrogated by a blocking antibody to the receptor. IC binding to pDCs was significantly reduced (~50%) by IVIg or Fc fragments but not by F(ab')2 fragments, in agreement with the pattern of IFN-α inhibition seen with the same conditions in Figure 3.1B. Together, these findings demonstrate that intact IgG and the IgG Fc fragments inhibit SLE IC-induced IFN-α production independent of sialylation and also that IgG Fc fragments inhibit SLE ICs binding to FcγRIIa on pDCs.

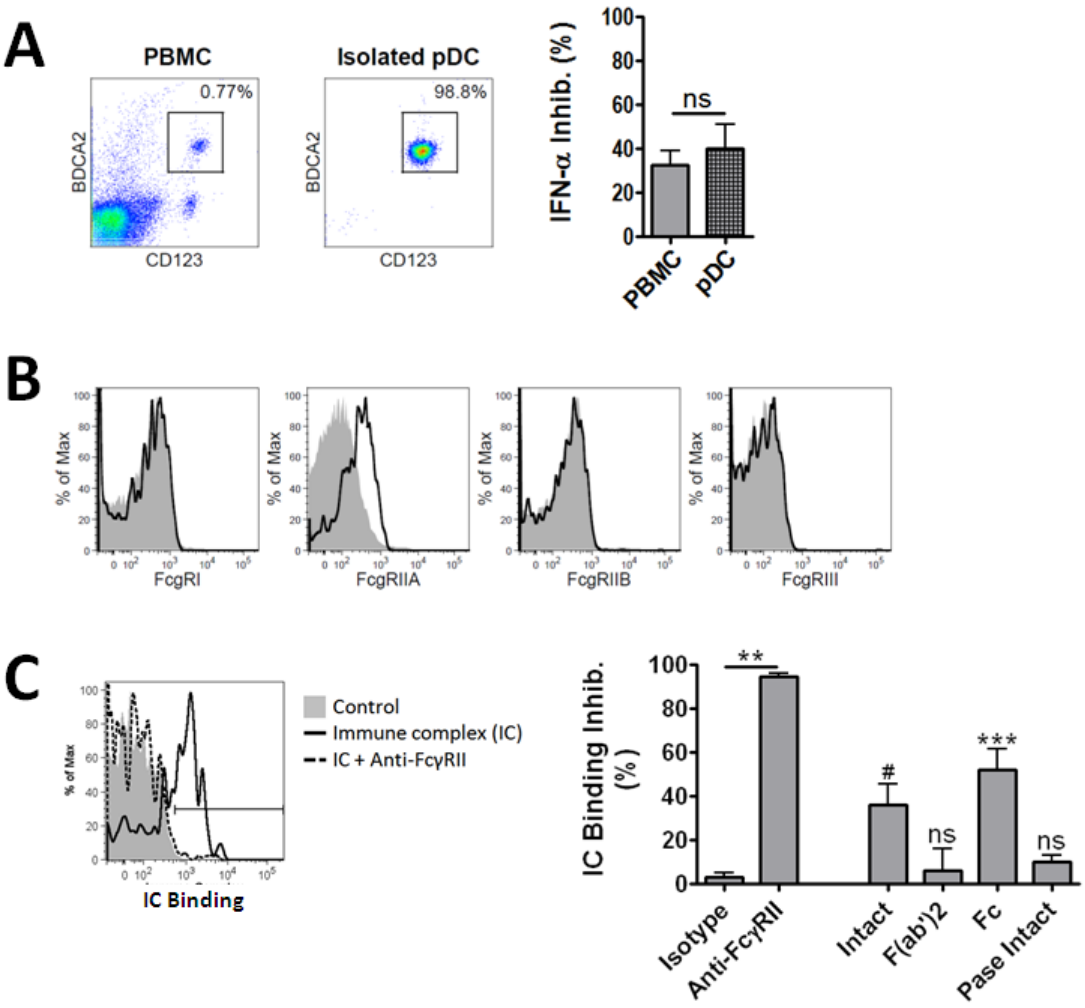

Figure 3.4. IVIg directly inhibits immune complex stimulation of pDCs by blocking binding of ICs to FcγRIIa. (A) Human PBMCs and isolated pDCs were treated cultured in the presence of IVIg (500 μg/mL), and supernatants assayed for IFN-α after 20h (results presented as % inhibition compared to cultures without IVIg treatment). (B) Flow cytometric analysis of FcγR on pDCs. Solid grey = isotype, black line = FcγR expression. (C) Immune complexes were formed with fluorescently labeled antigen and allowed to bind pDCs (identified by flow cytometry as $CD123^{+}BDCA2^{+}$) in PBMC with or without treatment by FcγRII blocking antibody or isotype (3 μg/mL), intact (whole, undigested into fragments) IVIg, (500 μg/mL), F(ab')2 fragments (333 μg/mL), Fc fragments (167 μg/mL), or intact IgG treated with PNGase (Pase) to remove the N-linked glycans, and binding to pDCs was assessed by flow cytometry and represented as % inhibition compared to ICs binding in the absence of treatment. Results represent at least four experiments in each group. Bars represent mean + SEM. *, **, *** represent p-values of <0.05, <0.01, and <0.001, respectively; ns, not significant.

Brief discussion

Here, we have shown that IVIg Fc fragments inhibit IC-induced IFN-α by direct interaction with pDCs and, specifically, block binding of ICs to pDCs which occurs through FcγRIIa.

The affinity of binding of monomeric IgG to FcγRIIa is estimated to be ~$2x10^6$ M^{-1} as compared to $1x10^8$ M^{-1} for FcγRI, and therefore monomeric IgG is considered a poor competitor for IC binding to FcγRIIa (109). However, following isolation by gel filtration monomeric IgG without dimers was able to bind, but not activate human FcγRIIa (105), and monomeric IgG can inhibit binding of dimeric or oligomeric IgG to FcγRIII on neutrophils (107). In addition, IC binding to low-affinity FcγR in mice can be blocked by IVIg at high concentrations (thousands of times greater than the concentration of IC) (106). We observed that IgG, and Fc fragments specifically, inhibited ICs binding to pDCs and IFN-α production in response to these ICs. Since pDCs express only FcγRIIa (67, 104) and IgG Fc inhibited SLE IC-induced IFN-α by acting directly on pDCs, other FcγR cannot be implicated in this inhibitory process.

In the K/BxN model of arthritis in mice, Ravetch and colleagues showed that the Fc fragment with glycans terminating in 2,6-linked sialic acid is the crucial glycoform necessary to exert its inhibitory action via engagement of the receptor SIGN-R1 on myeloid cells (86). Subsequently, using a mouse expressing human DC-SIGN (a homologue to murine SIGN-R1), they demonstrated that sialylated Fc induced IL-33 by SIGN-R1/DC-SIGN expressing cells, culminating in a Th2 mediated prevention of inflammation (87). Since human pDCs do not express DC-SIGN (110), and we observed that Fc fragments—independent of sialylation—reduced IC stimulated IFN-α production by pDC, the mechanism of prevention of inflammation in the mouse model of arthritis is different to that described here. The requirement for sialylation of IVIg in reducing inflammation has also recently been questioned in mouse models of immune

thrombocytopenic purpura (ITP), as sialylation-enriched IVIg, neuraminidase-treated (desialylated) IVIg and normal IVIg did not differ in their efficacy to alleviate ITP (88, 89). The data presented here demonstrate that IVIg inhibition of IFN-α induced by SLE ICs also does not follow the model reported by Ravetch et al.—the inhibitory effect was independent of the sialylation of IgG, was direct on pDCs, and did not require DC-SIGN.

Glycosylation of IgG at the conserved site in the Fc (Asn 297) affects affinity for FcγR. A complete lack of the glycan is known to abrogate its effector function (98), but subtler changes such as the absence of a fucose residue or the presence of a bisecting GlcNAc both increase affinity for activating FcγR (111-114), while the presence of a sialic acid residue reduces the affinity (115). Patients with chronic inflammatory diseases are reported to have reduced galactosylation (116, 117) and sialylation (118) on serum IgG Fc, but other alterations in glycans have not been so thoroughly studied. Though our data suggest that neither sialic acid nor galactose is essential for inhibition of IC-induced IFN-α production, it is possible that differential glycosylation or subclass distribution of normal versus 'immune IgG' in the ICs allows for preferential binding of IVIg to FcγRIIa. This topic is discussed further in Chapter 7.

Chapter 4

IFN-α induced by TLR agonists is inhibited by sialylation-enriched IVIg via induction of PGE2 production by monocytes

Brief introduction

After uptake via FcγRIIa, SLE ICs stimulate IFN-α production by nucleic acid activation of intracellular TLR7 or TLR9 receptors in pDCs (68, 95). Therefore, pDCs may receive activating stimuli from both FcγR engagement as well as TLR7 activation by nucleic acids. We and others previously observed that SLE IC stimulation of IFN-α depends primarily on the presence of RNA and stimulation of TLR7 (83, 94); but anti-dsDNA complexes that stimulate TLR9 can also induce IFN-α (66). Although we demonstrated that IVIg reduced IFN-α stimulation by blocking FcγRII engagement, these experiments could not address whether IVIg also affects TLR activation. We therefore addressed whether IVIg had inhibitory effects on IFN-α production independent of blockade of IC binding to FcR.

IVIg F(ab')2 fragments inhibit TLR agonist-induced IFN-α

To induce IFN-α independent of FcR interaction, we used synthetic analogs of TLR ligands to directly activate TLR7 (Loxoribine, CL097) or TLR9 (CpG-A). As was seen with SLE IC stimulation, these agonists robustly induced IFN-α. Interestingly, while Loxoribine and CpG induced little or no production of other inflammatory cytokines with the exception a small amount of IL-6 following Loxoribine but not CpG-A stimulation, CL097 induced robust quantities of IL-6 and IL-8, as well as the cytokines, TNF and IL-10 (Figure 4.1), possibly due to its ability to also simulate TLR8 (119).

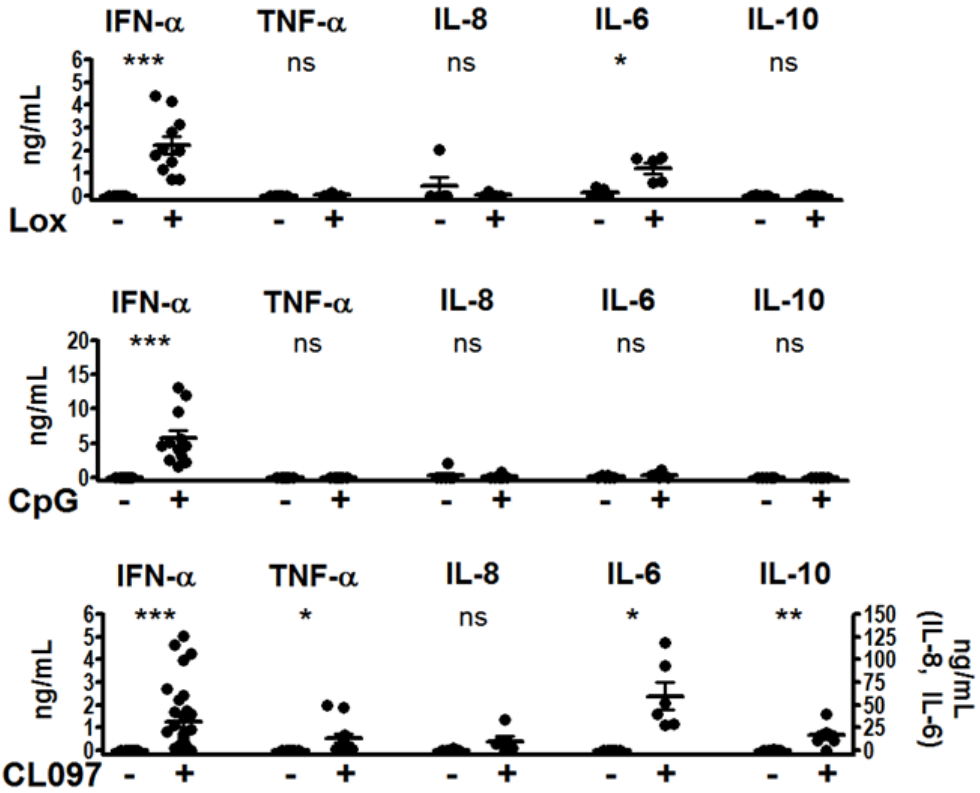

Figure 4.1. TLR7 and 9 agonists induce IFN-α. Human PBMCs were stimulated with agonists of TLR7 (Loxoribine, CL097) or TLR9 (CpG) and after 20h the supernatants were assayed by ELISA for IFN-α, TNF-α, IL-8, IL-6 and IL-10. Note that for CL097 stimulation, IL-6 and IL-8 are shown on a different scale (right y axis). Results represent at least four experiments in each group. *, **, *** represent p-values of <0.05, <0.01, and <0.001, respectively; ns, not significant.

Like SLE IC-stimulated IFN-α, TLR agonist-induced IFN-α was also inhibited by normal serum (Figure 4.2A). However, we observed that at the lower concentration of IVIg that inhibited SLE IC-stimulated IFN-α production by ~50% (500 μg/ml), inhibition of TLR agonist-induced IFN-α was insignificant (Figure 4.2B), even though a similar range of IFN-α was induced by either stimulus (compare Figures 4.1A and 3.1A). These results suggest that for SLE IC stimulation, IgG was interfering with IFN-α induction upstream of these TLRs, consistent with the findings described in the previous chapter which demonstrated a blockade of FcγRIIa by IVIg. However, we did observe a modest but reproducible inhibition of TLR agonist-stimulated IFN-α at the higher dose of 5,000 μg/mL IVIg (Figure 4.2B), demonstrating that IgG also has inhibitory activity downstream of TLR activation. Interestingly, the inhibition by IVIg appears to be specific for IFN-α, as the other proinflammatory cytokines that were induced by CL097 stimulation were not inhibited (Figure 4.2C).

We then wished to address which part of the IgG molecules contained the inhibitory activity. Unexpectedly, we found that in contrast to the Fc-dependence of IgG inhibition of SLE IC-stimulated IFN-α, for TLR agonist-stimulated IFN-α the inhibitory activity of IgG resided in the F(ab')2 fragment. Whole (intact) IVIg and F(ab')2 fragments inhibited Loxoribine-induced IFN-α while Fc fragments did not (Figure 4.2D).

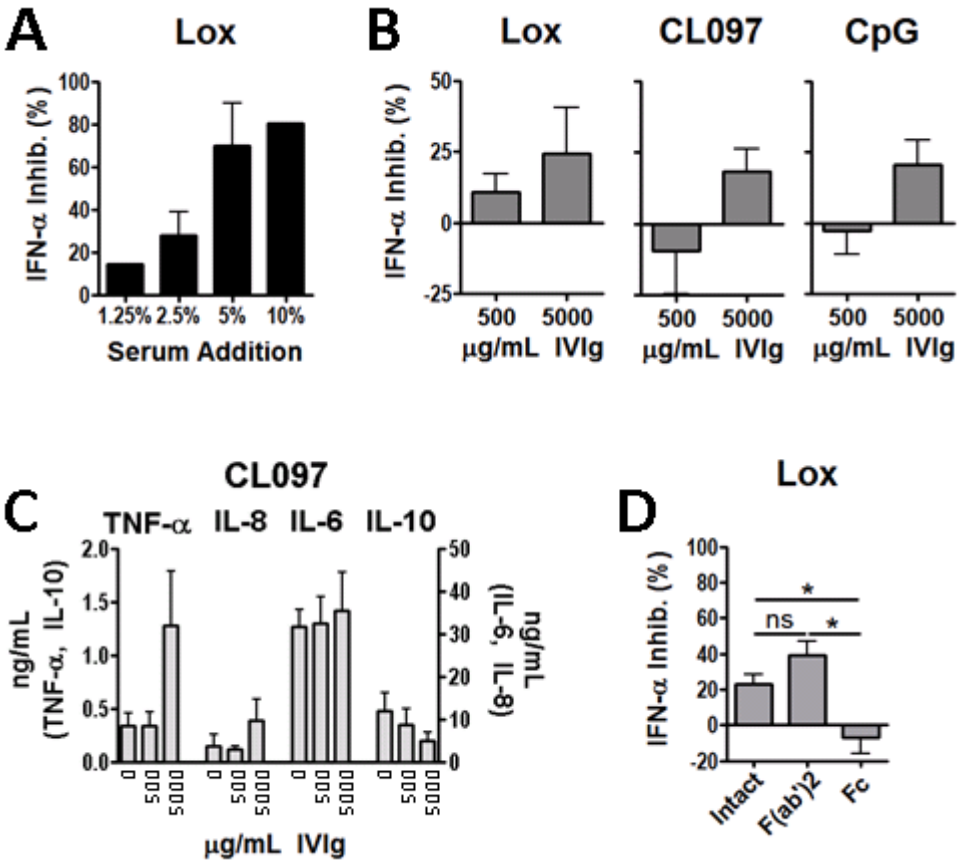

Figure 4.2. IVIg inhibition of TLR agonist-stimulated IFN-α requires F(ab')2. Human PBMCs were stimulated with the TLR agonists Loxoribine (Lox), CL097, or CpG and supernatants collected at 20h and (A-B, D) assayed for IFN-α by ELISA (results expressed as % inhibition compared to no IVIg treatment) after addition of: (A) normal human serum or (B) IVIg at 500 or 5000 µg/mL. (C) CL097-stimlulated PBMC assayed for TNF, IL-8, IL-6, or IL-10 after addition of IVIg at 500 or 5000 µg/mL. (D) Lox-stimulated PBMC assayed for IFN-α after addition of IVIg (5000 µg/mL), F(ab')2 fragments (3333 µg/mL), or Fc fragments (1667 µg/ml). Results represent at least four experiments in each group. Bars represent mean + SEM. *, **, *** represent p-values of <0.05, <0.01, and <0.001, respectively; ns, not significant.

Sialylation-enriched (SNA+) IVIg inhibits IFN-α but does not require the presence of sialic acid

As mentioned above, the sialylated subset of IgG has been implicated as possessing anti-inflammatory properties (99). We therefore addressed whether sialylated IgG was responsible for the IFN-α inhibition. In contrast to the sialylation-independent inhibition of SLE IC-stimulated IFN-α by IgG, we found that the sialylated subfraction (SNA+) of IgG compared to the sialylation-depleted subfraction (SNA-) was a more potent inhibitor of IFN-α induced by the TLR agonists Loxoribine, CL097, and CpG (Figure 4.3A). Also, equimolar F(ab')2 fragments of the SNA+ subset of IgG were more effective at inhibition of IFN-α (Figure 4.3B).

Surprisingly, however, the sialic acid was not critical for inhibition, as neuraminidase treatment of SNA+ intact or F(ab')2 fragments, which cleaved the sialic acid (Figure 3.3B), did not alter IFN-α inhibition (Figure 4.3C). Consistent with these findings, blockade of DC-SIGN by using specific antibody did not alter inhibition (data not shown), nor did addition of the heavily sialylated proteins fibrinogen, fetuin, or transferrin (data not shown). Together, these results suggested that the inhibitory component of SNA+ IgG to TLR agonist stimulation resided in F(ab')2 but that the sialic acid residue itself was not required for inhibition.

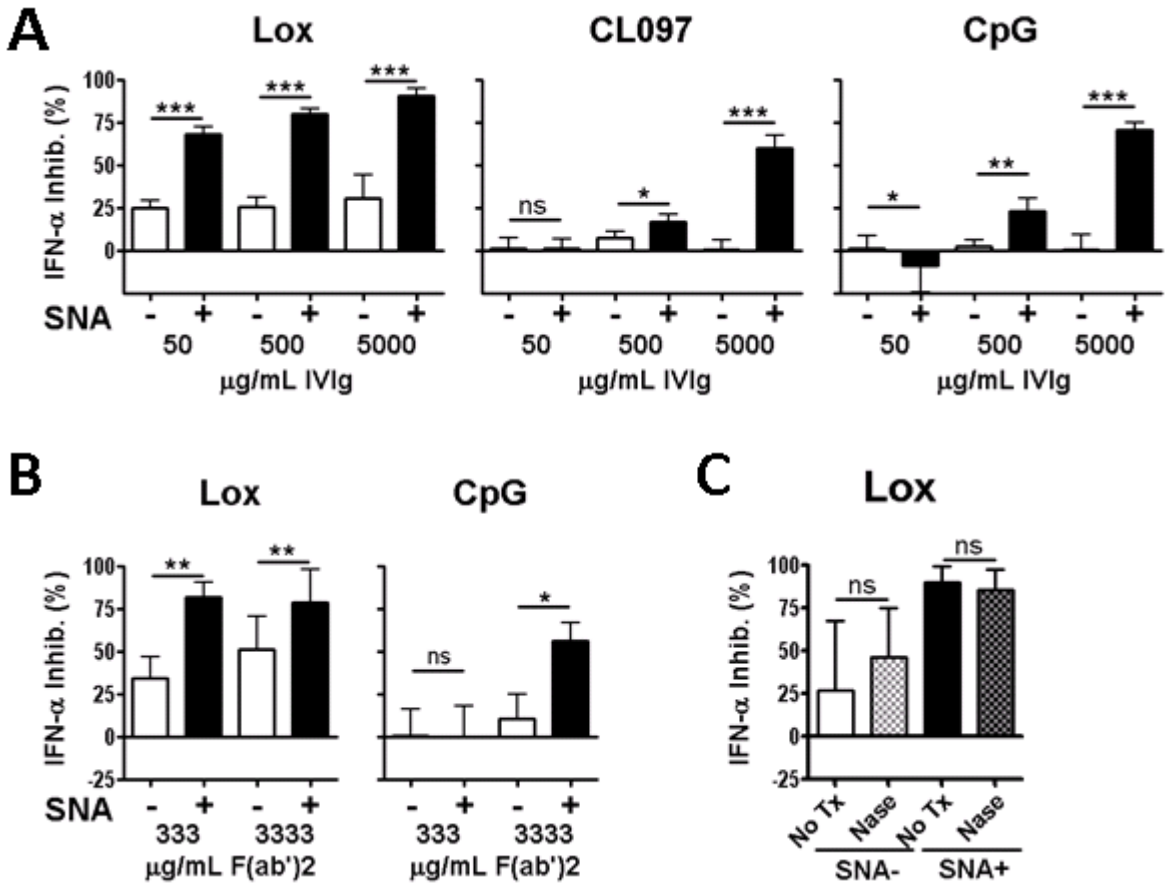

Figure 4.3. Sialylation-enriched (SNA+) IVIg is a potent inhibitor of TLR agonist-stimulated IFN-α. Human PBMCs were stimulated with the TLR agonists Loxoribine (Lox) or CpG and supernatants collected at 20h and assayed for IFN-α by ELISA (results expressed as % inhibition compared to no IVIg treatment) after addition of: (A) 50, 500, or 5000 µg/mL IVIg that was enriched (SNA+) or depleted (SNA-) for the sialylated subfraction. (B) SNA+ or SNA- IVIg (5000 µg/mL) or their F(ab')2 fragments (3333 µg/mL), or (C) SNA+ or SNA- IVIg (5000 µg/mL) with or without treatment with neuraminidase (Nase) to cleave sialic acid. Results represent at least four experiments in each group. Bars represent mean + SEM. *, **, *** represent p-values of <0.05, <0.01, and <0.001, respectively; ns, not significant.

Monocytes are required for the inhibition of TLR agonist-induced IFN-α by sialylation-enriched IgG

To elucidate the mechanism by which the sialylated IgG subset inhibited TLR agonist-induced IFN-α, we first addressed whether SNA+ IVIg could act directly on isolated pDCs. We found that SNA+ IVIg had minimal effects on IFN-α production by isolated pDCs (Figure 4.4A). Therefore, we asked which cell type(s) were required for inhibition by SNA+ IgG. We selectively depleted PBMCs of $CD14^+$ monocytes, $CD56^+$ NK/NKT cells, or $CD19^+$ B cells (<0.5% of the depleted cell type remained). Following TLR stimulation of single cell depleted PBMC cultures, we found that only the depletion of monocytes led to abrogation of the inhibitory activity of SNA+ IgG (Figure 4.4B). Thus, in contrast to the inhibition of SLE IC-induced IFN-α by IVIg which requires the Fc domain and appears to act directly on pDCs, the inhibition of TLR agonist stimulated IFN-α by SNA+ IVIg required the presence of monocytes and did not require Fc.

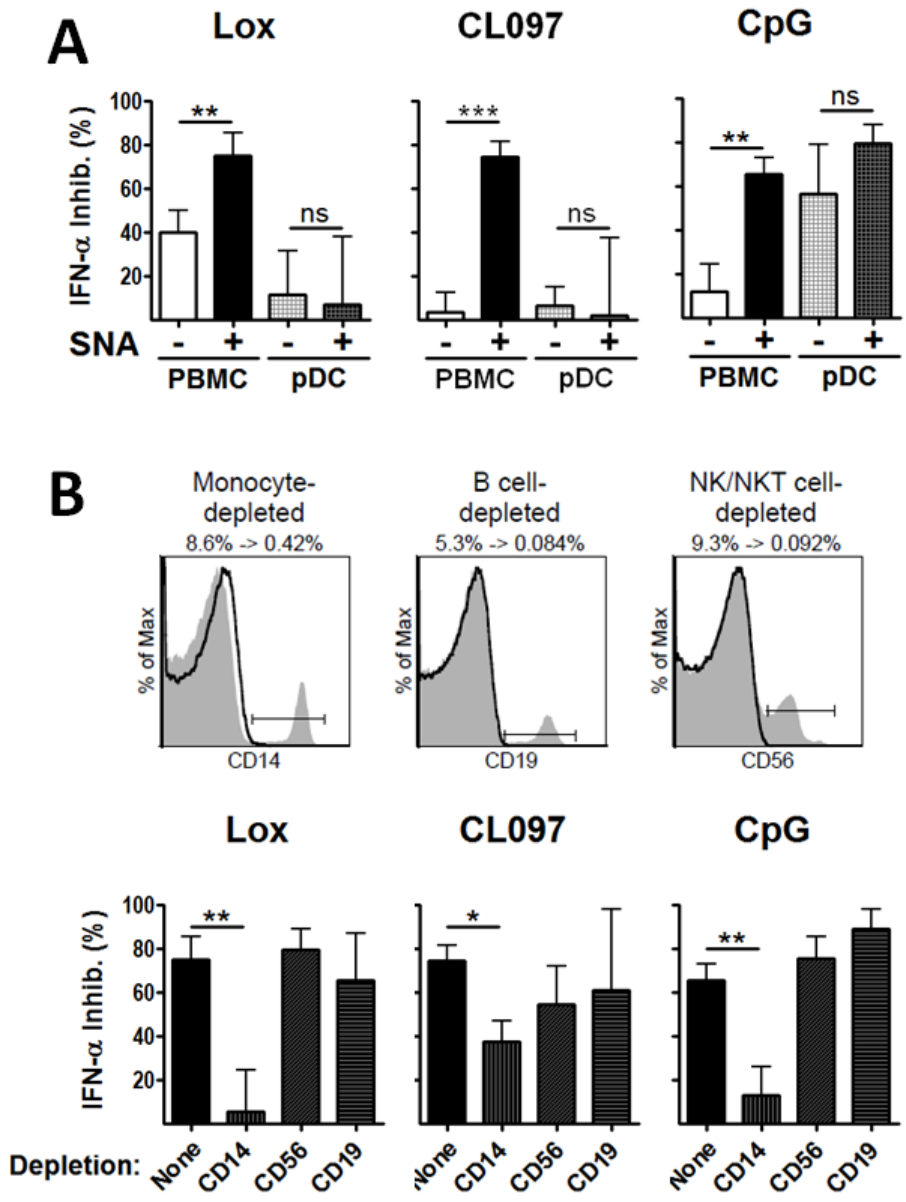

Figure 4.4. SNA+ IVIg inhibition of IFN-α requires monocytes. Human cells were stimulated with the TLR agonists Loxoribine (Lox), CL097, or CpG and supernatants collected at 20h and assayed for IFN-α by ELISA (results expressed as % inhibition compared to no IVIg treatment). (A) Human PBMCs or isolated pDCs were treated with SNA+ or SNA- IVIg (500 μg/mL for Lox, 5000 μg/mL for CpG). (B) PBMC cultures selectively depleted of $CD14^+$, $CD56^+$ or $CD19^+$ cells treated with SNA+ IVIg (500 μg/mL for Lox, 5000 μg/mL for CL097 or CpG). Results represent at least four experiments in each group. Bars represent mean + SEM. *, **, *** represent p-values of <0.05, <0.01, and <0.001, respectively; ns, not significant.

Sialylation-enriched IgG stimulates monocyte production of a soluble factor that can suppress IFN-α production by pDCs

To test whether the IVIg inhibitory activity could be explained by the production of a soluble factor by monocytes, we cultured isolated $CD14^+$ monocytes in the presence of SNA+ or SNA- IgG for 4h, washed the cells extensively to remove unbound IgG (< 1 μg/mL remained by ELISA), added fresh medium and then collected the monocyte supernatants after 20h. These supernatants were then added to cultures of PBMCs or isolated pDCs that had been stimulated with TLR agonists. We found that, indeed, the SNA+ IgG-treated monocytes produced a factor that was inhibitory compared to the SNA- IgG-treated monocytes; this was the case whether PBMCs (Figure 4.5A) or isolated pDCs (Figure 4.5B) were used as responder cells. These trends were also evident using monocyte supernatants generated from F(ab')2 fragments of SNA+ and SNA- IVIg (Figure 4.5C). These results indicate that monocytes produced a soluble inhibitory factor in response to the F(ab')2 fragment of SNA+ IgG, and that the factor could directly inhibit pDCs.

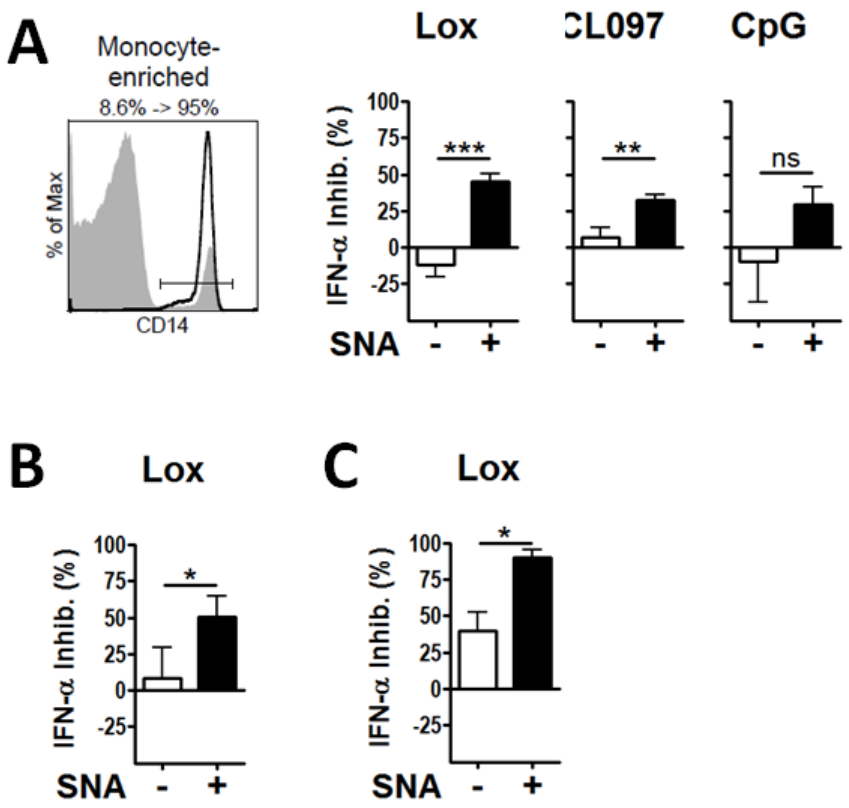

Figure 4.5. SNA+ IVIg induces monocyte production of a soluble factor that inhibits IFN-α. Human cells were stimulated with the TLR agonists Loxoribine (Lox), CL097, or CpG and treated with supernatants (50% v/v) generated from treatment of purified monocytes with IVIg. Culture supernatants were collected at 20h and assayed for IFN-α by ELISA (results expressed as % inhibition compared to monocyte supernatants without IVIg treatment). (A) PBMCs cultured with monocyte supernatants generated using SNA+ or SNA- IVIg (1000 μg/mL). (B) Isolated pDCs cultured with monocyte supernatants as in A. (C) Cultures as in A, but with monocytes treated with F(ab')2 fragments of SNA+ or SNA- IVIg (667 μg/mL). Results represent at least four experiments in each group. Bars represent mean + SEM. *, **, *** represent p-values of <0.05, <0.01, and <0.001, respectively; ns, not significant.

Monocytes produce PGE2 in response to sialylation-enriched IgG

To identify the factor that was produced in response to SNA+ IgG and responsible for the pDC inhibition, we next quantified known inhibitors of IFN-α that can be produced by monocytes including IL-10 (70), TNF-α (71, 72), and PGE2 (72-74). Only low concentrations (pg/mL range) of IL-10 and TNF-α were induced by SNA+ IgG—insufficient to suppress IFN-α production by pDC (72). In contrast, high concentrations (ng/mL range) of PGE2 were induced by the SNA+ IgG but not by SNA- IgG (Figure 4.6A). Such PGE2 concentrations have been shown to inhibit production of IFN-I by acting directly on pDCs (72-74). PGE2 was also produced in response to SNA+ F(ab')2 fragments, but not in the absence of monocytes (data not shown), demonstrating that monocytes were the only cells producing PGE2 in response to SNA+ IgG.

We recognize that endotoxin, if present, may affect our results. However, we took several measures to ensure this was not the case. First, endotoxin levels were rigorously tested and were very low in all IVIg preparations used (<2 EU/mg protein). Additionally, IVIg preparations with extremely low endotoxin (<0.05 EU/mg protein) or incubated with polymyxin B (which neutralizes endotoxin) produced the same inhibitory effect (data not shown). Lastly, TNF-α and IL-10 (which are produced in response to endotoxin) were produced at <0.2 ng/ml in response to any of the IVIg preparations (data not shown). Therefore, the results are highly unlikely to be due to endotoxin contamination.

To test if PGE2 in the concentration range produced by the IVIg-stimulated monocytes was sufficient to inhibit IFN-α production, we added exogenous PGE2 to TLR agonist-stimulated as well as SLE IC-stimulated cultures. The PGE2 induced a dose-dependent inhibition at doses within the range made by the monocytes (Figure 4.6B). In contrast, addition of TNF, IL-

10, IL-8, or IL-6 at doses produced in response to SNA+ IVIg did not inhibit IFN-α production (data not shown). Finally, depletion of PGE2 from monocyte supernatants markedly reduced its inhibitory activity (Figure 4.6C), demonstrating that PGE2 was necessary for SNA+ IgG inhibitory activity; furthermore, blockade of TNF, IL-10, or IL-6 had no effect on the inhibition of IFN-α (data not shown). Thus, PGE2 produced by monocytes in response to SNA+ IVIg is both required and sufficient for the observed inhibition of IFN-α.

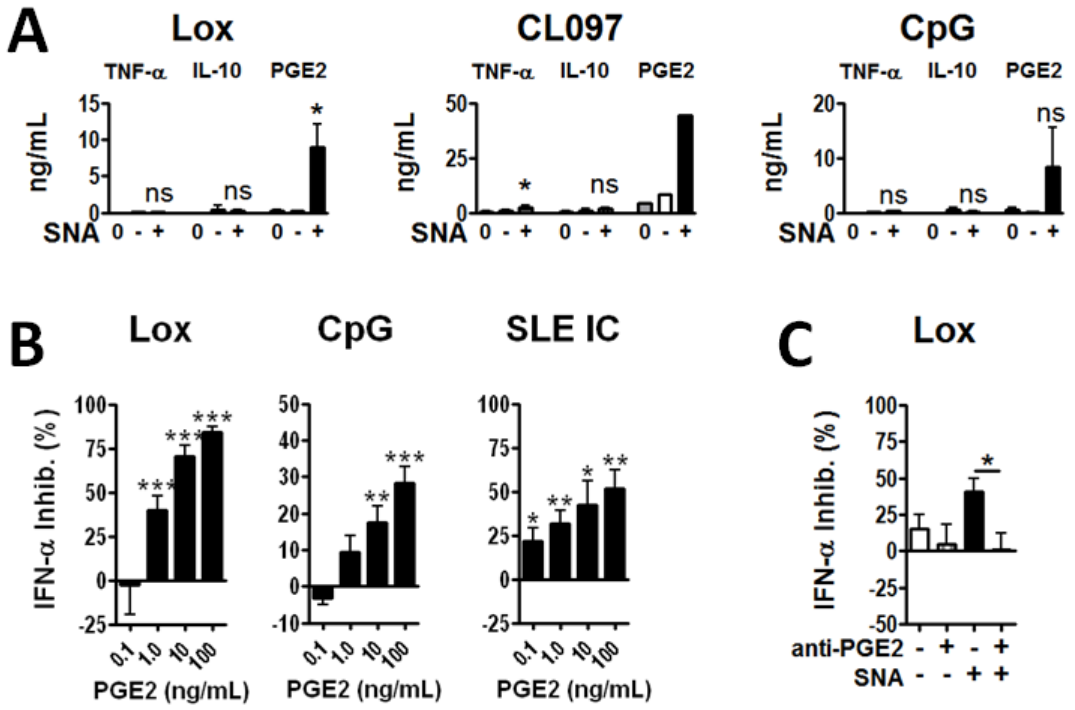

Figure 4.6. PGE2 produced by monocytes in response to SNA+ IVIg is required for IFN-α inhibition. Human PBMCs were stimulated with the TLR agonists Loxoribine (Lox), CL097, CpG, or with lupus immune complexes (SLE IC) and supernatants collected at 20h and assayed for cytokines by ELISA. (A) TNF, IL-10, and PGE2 after addition of SNA+ or SNA- IVIg (5000 μg/mL), (B) IFN-α (results expressed as % inhibition compared to no PGE2 treatment) after addition of doses of PGE2, and (C) IFN-α (results expressed as % inhibition compared to monocyte supernatants without IVIg treatment) after addition of monocyte supernatants as in Figure 4.5B that were depleted of PGE2. Results in B and C represent at least four experiments in each group. Bars represent mean + SEM. *, **, *** represent p-values of <0.05, <0.01, and <0.001, respectively; ns, not significant.

Brief discussion

We found that inhibition of TLR agonist-stimulated IFN-α required much higher doses of IVIg than for SLE IC-induced IFN-α. Surprisingly, we observed that, in contrast to the Fc-dependent inhibition of IC-induced IFN-α, it was the F(ab')2 region that exerted the inhibitory effect. Furthermore, inhibition was enhanced following enrichment of the sialylated subset on SNA lectin columns. While the only conserved N-linked glycosylation site within the IgG molecule is in the CH2 region in the Fc portion of the heavy chain (Asn 297), 10-20% of IgG also contain a glycosylation site in the variable region in the Fab portion (98, 120). The F(ab')2 and Fc of such antibodies are differentially glycosylated, with the F(ab')2 fragment tending to contain fully processed sialylated glycans while the Fc glycan generally lacks sialic acid and predominantly terminates in galactose (121). Because of the high sialylation of F(ab')2 glycans, they are preferentially isolated when sorting by the SNA lectin column (91, 102). Interestingly, neuraminidase cleavage of the sialic acid from SNA+ F(ab')2 had no effect on its inhibitory activity, demonstrating that although sialylated IVIg was more inhibitory, it was not due to the presence of sialic acid itself. The most likely explanation for these findings is that sorting for SNA+ IgG also enriches certain antibody specificities that modulate monocyte function. In support of this hypothesis, it has been demonstrated that sialylation-enriched IVIg has different antibody specificities than unsorted or sialylation-depleted IVIg (91). Furthermore, F(ab')2 N-glycans are known to contribute to antibody binding (122), and we found that cleavage of the N-glycans from IVIg abrogates its inhibitory activity (data not shown). However, the identity of the putative antibody target on monocytes that drives the PGE2 production remains to be determined.

In this study we have demonstrated two distinct mechanisms by which IVIg inhibits IFN-α production by pDCs (summarized in Figure 7.1 of the Discussion). First, IVIg Fc can block IC binding to FcγRIIa on pDCs. Second, IVIg F(ab')2 enriched for the sialylated subset drives monocyte production of PGE2 to inhibit IFN-α produced by pDCs in response to TLR7 and TLR9 agonists. Elucidating these mechanisms points to distinct ways the immune system may be regulated to prevent IFN-α overproduction.

Chapter 5

Monocyte responses to sialylation-enriched (SNA+) IVIg

Brief Introduction

Monocytes (Mo) are versatile cells that defend against pathogens, regulate inflammation, and participate in the induction of adaptive immunity. Blood monocytes are recruited to sites of tissue inflammation where they become activated and differentiate into macrophages (Macs) or dendritic cells. Mo/Macs are an important source of a variety of cytokines, which can mediate both inflammatory and regulatory functions. Mo/Macs also act as antigen presenting cells and, together with their potent ability to synthesize cytokines, they play a central role in initiating immune responses.

The role of monocytes in the development of autoimmune diseases such as lupus is not yet entirely clear. Theories formulated in the 1980s proposed that Mo/Macs from SLE patients have defective phagocytic function, thus enabling accumulation of ICs contributing to autoimmunity; however, recent studies suggest a more active role of Mo/Macs in mediating tissue inflammation (reviewed in (123)). In animal models, activation of monocytes in the context of deficient phagocytosis of cell debris leads to overt autoimmunity (124). In human SLE patients, Mo/Mac infiltration and activation are important indicators of lupus nephritis (125, 126). In addition, monocytes from SLE patients have altered inflammatory responses; they produce increased amounts of nitric oxide (NO) (127) and IL-10 (128, 129), but reduced amounts of IL-12 (130). The increased inflammatory cytokines, and specifically increased IFN-α present in the serum of SLE patients can cause monocytes to differentiate into DCs (131).

Monocytes of SLE patients have increased CD40 expression (132), are able to induce strong response to alloantigen (131), and can promote plasmablast differentiation (133).

Monocytes are a heterogeneous cell population (reviewed in (134)). In humans, three populations of monocytes have been defined: $CD14^{dim}CD16^{+}$, $CD14^{+}CD16^{+}$ and $CD14^{+}CD16^{-}$ (135). It has been proposed that these monocyte populations have different functionalities: the two $CD14^{+}$ monocyte subsets (called "inflammatory monocytes") are most abundant, and respond to TLR2 and TLR4 signals by producing abundant IL-6 and IL-8, whereas the rarer $CD14^{dim}$ "patrolling" monocytes that cling to the vasculature via LFA-1 respond mainly to viruses and nucleic acids to produce TNF and IL-1β (136). These $CD14^{dim}CD16^{+}$ monocytes also respond to SLE immune complexes (136), and accumulate inside glomerular blood vessels in some cases of SLE (137), suggesting that $CD14^{dim}CD16^{+}$ monocytes may play an active role SLE disease.

Therefore, monocytes are poised to be critical regulators of immune responses and have a potential role in the pathogenesis of lupus. We demonstrated in the previous chapter that monocytes play a critical role in the inhibition of IFN-α by SNA+ IVIg after TLR agonist stimulation. Therefore, we wished to further explore the activity of SNA+ IVIg on the different monocyte subsets and on specific monocyte functional properties.

SNA+ IVIg inhibits costimulatory molecule expression on monocytes

Monocytes upregulate expression of both CD86 (Figure 5.1A) and CD40 (data not shown) in response to TLR agonist stimulation in cultures of PBMCs. This activation could be inhibited by treatment with SNA+, but not SNA-, IVIg (Figure 5.1B), suggesting that the sialylated subfraction of IVIg plays an inhibitory role on monocytes. To determine if this inhibition was

due to a direct effect on monocytes, or mediated by other cells present in PBMC cultures, we performed experiments using purified monocytes. Under these conditions, TLR agonists were not sufficient to induce CD86 upregulation, and consequently there was no discernible inhibition by SNA+ IVIg (Figure 5.1C).

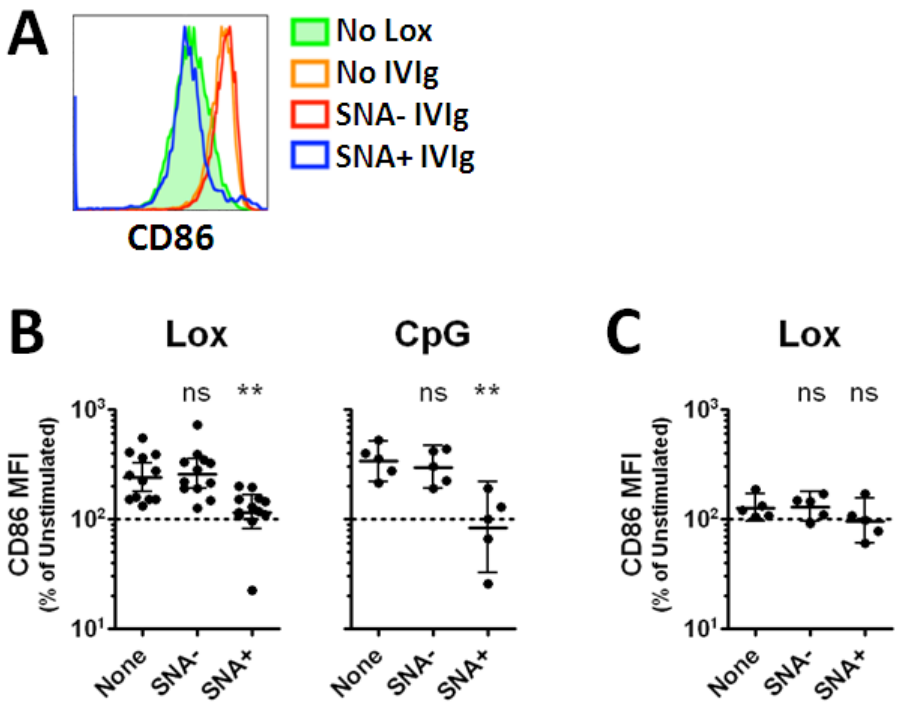

Figure 5.1. Activation of monocytes by TLR agonists is inhibited by SNA+ IVIg. Cells were treated with the TLR agonists Loxoribine (Lox) or CpG in the presence of absence of SNA+ or SNA- IVIg (5000 μg/mL) for 20h, and the expression of the activation marker CD86 on $CD14^+$ monocytes was quantified by flow cytometry. (A) A representative plot of CD86 expression on monocytes from PBMCs after Lox stimulation. (B) Quantification of CD86 MFI on monocytes from PBMCs (represented as a % of MFI on unstimulated monocytes). Statistic represent paired t tests to cultures without IVIg. (C) Isolated monocytes treated and analyzed as in B. *, **, *** represent p-values of <0.05, <0.01, and <0.001, respectively; ns, not significant.

Monocytes have altered cytokine production in response to SNA+ IVIg

Because SNA+ IVIg inhibited monocyte costimulatory molecule expression and induced the production of the inhibitory factor PGE2 by monocytes, we wished to address whether SNA+ IVIg was also capable of suppressing pro-inflammatory cytokines produced by monocytes. We stimulated PBMCs with agonists of TLRs primarily expressed by monocytes: TLR2 (Zymosan), TLR4 (LPS), TLR5 (FliC), or TLR8 (CL097), and then assayed the supernatants for IL-6, IL-8, IL-10, and TNF concentrations after 20 hours. Unexpectedly, addition of SNA+ IVIg did not inhibit the production of these pro-inflammatory cytokines and, if anything, moderately enhanced their production (Figure 5.2A). The same trends of inflammatory cytokine production in response to SNA+ IVIg were found even in the absence of stimulation, or with stimulation by a TLR7 agonist, Loxoribine (Figure 5.2B). To address the monocyte-specific alteration in production of these inflammatory cytokines, we performed the experiments on purified monocytes, but again we found that while SNA+ IVIg modulated cytokine production, it was an increase rather than decrease in production (Figure 5.2C).

Taken together, these data indicate that whereas expression of costimulatory molecules by monocytes is inhibited by SNA+ IVIg, production of several important inflammatory cytokines was not reduced at 20 hours. This is in contrast to the potent inhibitory effect of SNA+ IVIg on IFN-α by induction of PGE2 production by monocytes, suggesting that SNA+ IVIg is not universally anti-inflammatory. However, it remains to be determined if any other pro- or anti-inflammatory cytokines are altered by SNA+ IVIg, or whether there are any inhibitory effects at a later time point.

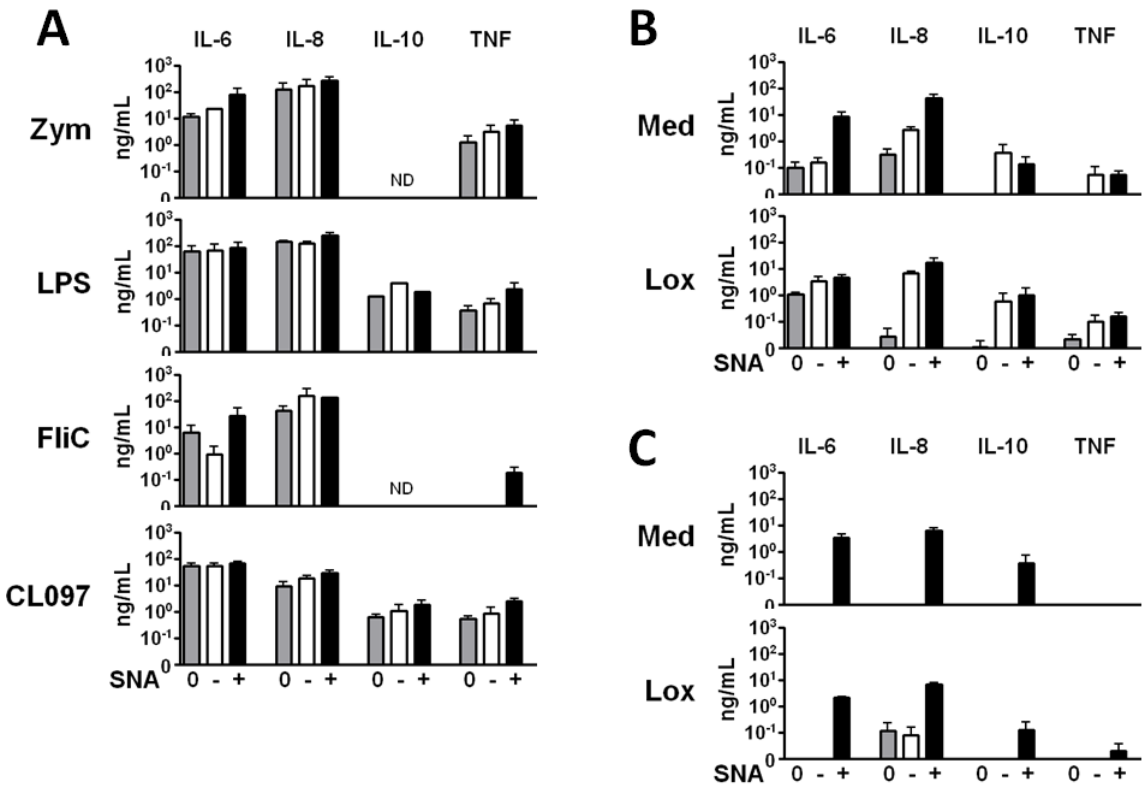

Figure 5.2. Monocytes have altered cytokine production in response to SNA+ IVIg. Cells were stimulated with TLR zymosan (Zym), LPS, flagellin FliC (FliC), CL097, Loxoribine (Lox) or unstimulated (Med) with or without addition of SNA+ or SNA- IVIg (5000 μg/mL), and supernatants were analyzed for cytokines at 20h. Production of the cytokines IL-6, IL-8, IL-10, and TNF by (A and B) PBMCs, or by (C) isolated monocytes. Bars represent mean + SEM. *, **, *** represent p-values of <0.05, <0.01, and <0.001, respectively; ns, not significant.

Monocyte subsets produce PGE2 in response to SNA+ IVIg

In the previous chapter we showed that monocytes produced the inhibitory factor PGE2 in response to SNA+ IVIg. In the current chapter, we also found that monocyte activation was reduced with SNA+ IVIg, but that it increased production of inflammatory cytokines. Since monocytes are in fact a heterogeneous population with subsets that produce different cytokines in response to stimulation, we wished to address which monocyte populations were producing these cytokines. We sorted $CD14^{dim}CD16^{+}$, $CD14^{+}CD16^{+}$ and $CD14^{+}CD16^{-}$ monocytes (Figure 5.3A) and cultured them in the presence of TLR agonists—LPS, a bacterial ligand, and CL097 to represent nucleic acid ligand. Consistent with previous reports (136), we found that the $CD14^{dim}CD16^{+}$ subset was less responsive to LPS but more responsive to nucleic acid stimulation and produced less IL-6 or IL-8 and more TNF and IL-1β compared to $CD14^{+}CD16^{-}$ monocytes (Figure 5.3B).

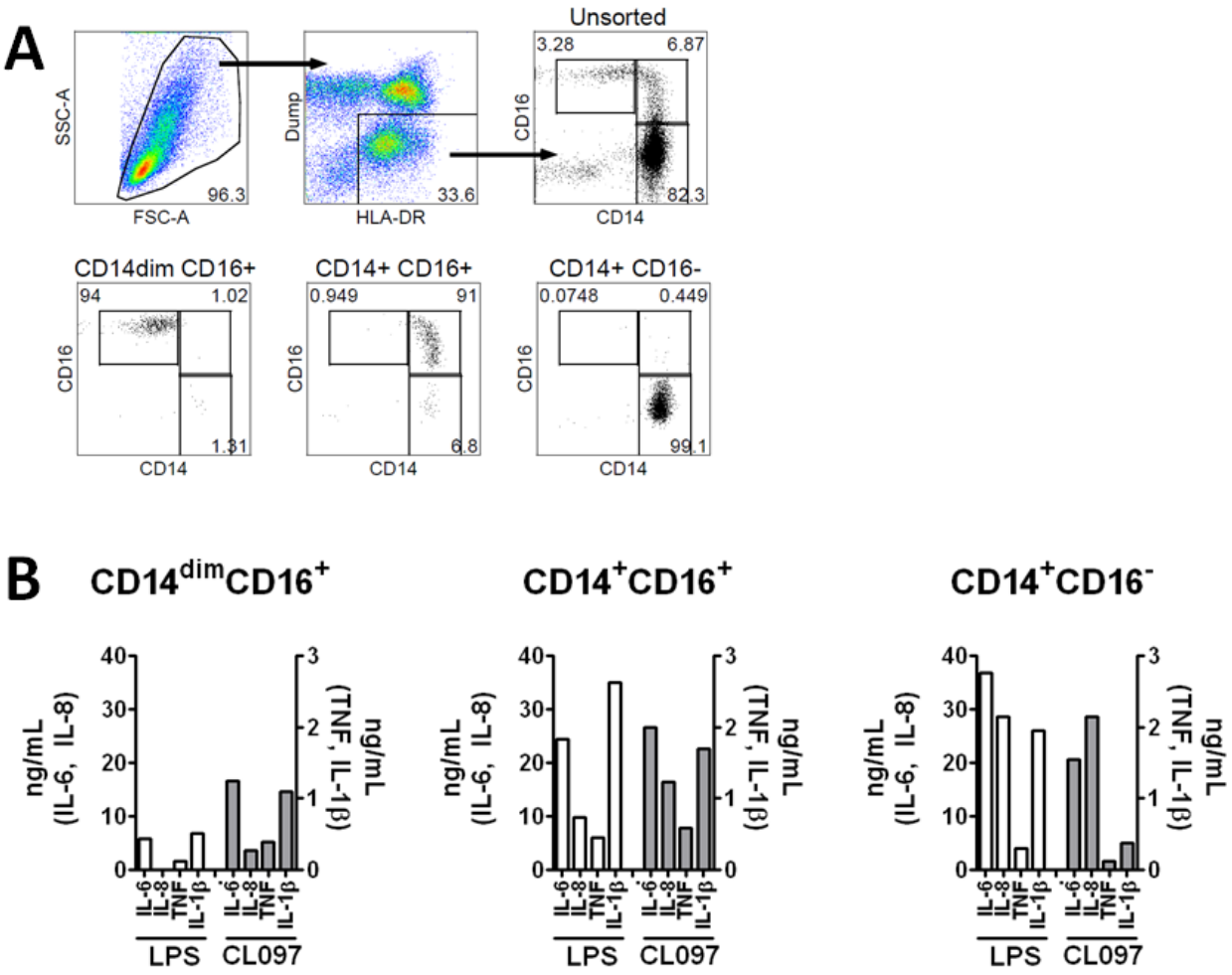

Figure 5.3. Monocyte subsets have differential cytokine production in response to different stimuli. Monocyte subsets ($CD14^{dim}CD16^{+}$, $CD14^{+}CD16^{+}$, and $CD14^{+}CD16^{-}$) were sorted by flow cytometry, simulated by TLR agonists 100 ng/mL LPS (TLR4) or 1 µg/mL CL097 (TLR8) and supernatants analyzed for cytokine production at 20h. (A) Representative flow plot of monocyte subset sort. (B) Production of cytokines IL-6, IL-8, TNF, and IL-1B by monocyte subsets. Results are a representative example of four experiments in A and two experiment in B.

As PGE2 was found as the inhibitory factor induced by SNA+ IVIg we asked if a particular subset was responsible for its production. We found that all three subsets of monocytes produced PGE2 in response to the bacterial ligand LPS, but that the $CD14^{+}$ monocyte subset produced more on a per cell basis than the $CD14^{dim}$ monocyte subset (Figure 5.4A). Interestingly, all three subsets also produced PGE2 specifically in response to SNA+ IgG and not SNA- IVIg (Figure 5.4B). However, it appeared that again the $CD14^{+}CD16^{-}$ population was superior. We therefore asked whether this $CD14^{+}16^{-}$ population, which is more abundant and generally produces more of the inhibitory factor PGE2, was deficient in SLE patients. We found that although the percentage of total monocytes in PBMC was increased SLE (12.8 ± 3.1 vs. 9.5 ± 1.0 for controls), this $CD14^{+}16^{-}$ subset within monocytes was reduced (Figure 5.4C), suggesting that they may have a defective response to SNA+ IVIg. However, despite this deficiency, SLE monocytes can sufficiently respond to SNA+ IVIg, as SNA+ IVIg was a more robust inhibitor of IFN-α on SLE patient PBMC (Figure 5.4D).

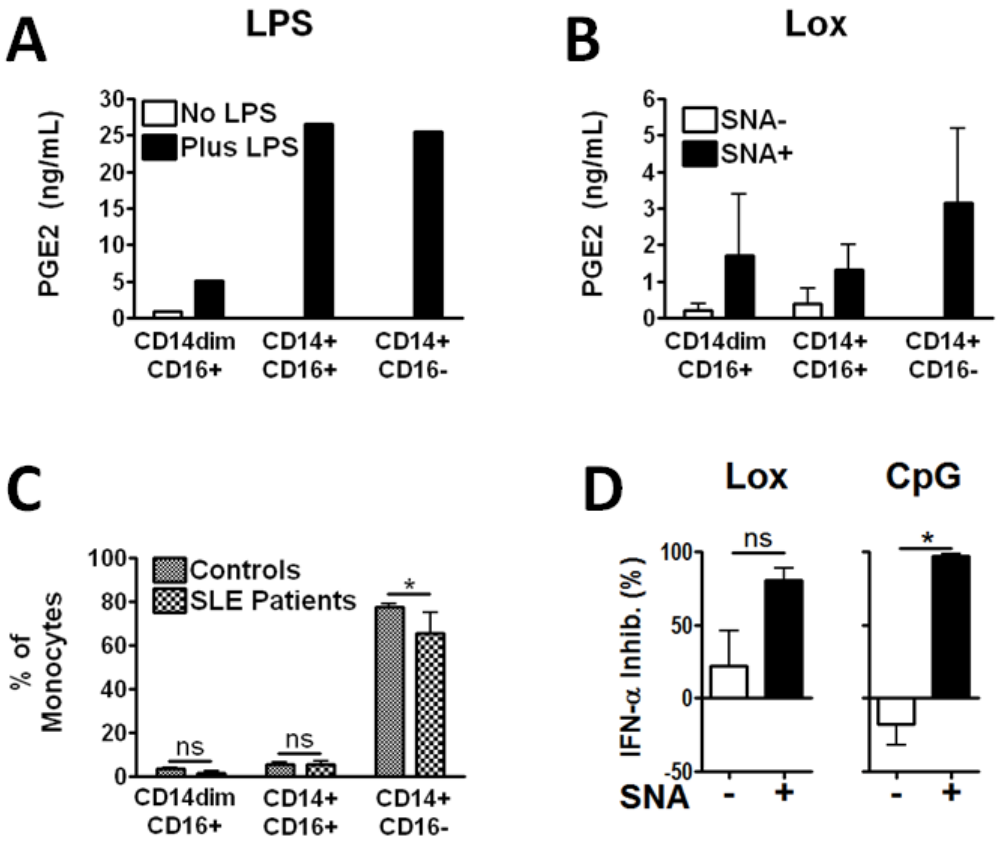

Figure 5.4. Monocyte subsets produce PGE2 in response to SNA+ IVIg. Monocyte subsets were sorted as in Figure 3 and PGE2 in supernatants was analyzed 20h after (A) LPS treatment or (B) SNA+ or SNA- IVIg treatment (500 μg/mL) in the presence of Loxoribine (Lox). (C) Monocyte subset distribution in the PBMCs of healthy controls and SLE patients (represented as a % of HLA-DR^{+} monocytes). (D) IFN-α (represented as % inhibition) was assayed after 20h in the supernatants of SLE patient PBMC cultures that were stimulated with the TLR agonists Loxoribine (Lox) or CpG and treated with SNA+ or SNA- IVIg (5000 μg/mL). Results in A and B are a representative example of two experiments. Results in C and D represent at least four experiments in each group. Bars represent mean + SEM. *, **, *** represent p-values of <0.05, <0.01, and <0.001, respectively; ns, not significant.

Monocytes bind both SNA+ and SNA- IVIg

In the previous chapter we found that SNA+ IVIg F(ab')2 was a more potent inhibitor of IFN-α via a direct interaction with monocytes, the cell responsible for PGE2 production. Based on the studies mentioned in the previous chapter (91), we reasoned that this was likely due to selective enrichment in antibody specificity in the SNA+ selected subset. We therefore wished to address if there was an increased affinity of SNA+ F(ab')2 for total monocytes or a particular monocyte subset. However, we found that fluorescently labeled SNA+ and SNA- F(ab')2 IVIg both bound equally well to monocytes (Figure 5.5A). No significant binding to B cells, T cells, or NK cells was observed (data not shown). In addition, there was no difference in binding by the two subfractions on any of the three subsets of monocytes (Figure 5.5B). Interestingly, however, we found that IVIg binding to the $CD14^{dim}CD16^{-}$ monocyte subset was reduced compared to the $CD14^{+}$ monocyte subsets (Figure 5.5B).

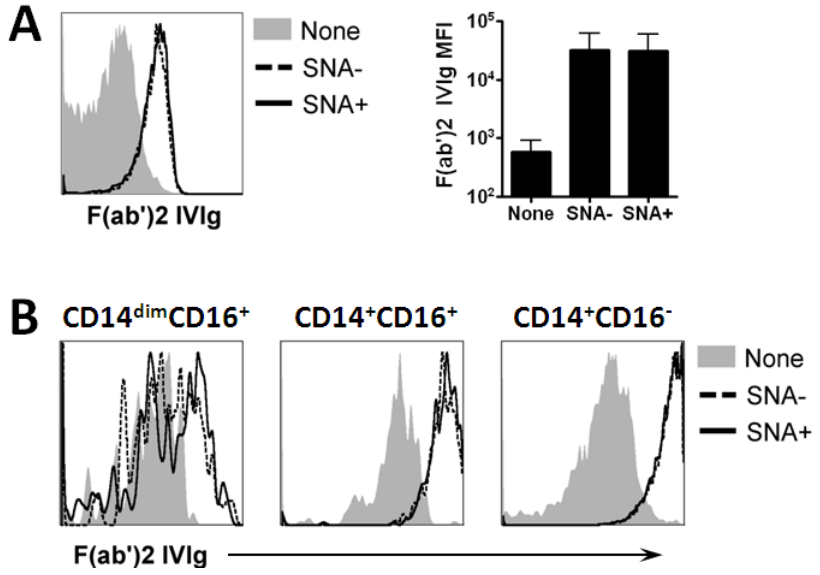

Figure 5.5. Both SNA+ and SNA- F(ab')2 IVIg bind monocytes. Cells were incubated for 30 minutes with fluorescently labeled SNA+ or SNA- F(ab')2 IVIg and then stained for monocyte markers and analyzed by flow cytometry. (A) A representative plot and quantification of the MFI of bound IVIg on total $CD14^+$ monocytes in PBMCs. (B) Representative plots of IVIg bound to monocytes subsets within PBMCs. Results in A represent at least four experiments in each group and results in B are representative of two experiments. Bars represent mean + SEM.

Brief Discussion

Monocytes are critical for SNA+ IVIg-mediated inhibition of IFN-α via production of PGE2. Here we found that SNA+ IVIg could inhibit activation of monocytes, as indicated by reduced expression of CD86 and CD40. Interestingly, however, the production of inflammatory cytokines IL-6, IL-8, IL-10, or TNF after stimulation by monocyte-specific TLR agonists were not decreased with addition of SNA+ IVIg, and in fact resulted in a subtle increase in the expression of these proteins. The induction of these cytokines by SNA+ IVIg did not require stimulation of monocytes by a TLR agonist nor the presence of other cells, as purified monocytes without stimulation were able to produce them. This is consistent with the finding that C1q, another serum factor that was found to inhibit IFN-α, decreased monocyte activation and IFN-α, but not other inflammatory cytokines (97).

Because monocytes are a heterogeneous cell population, we were interested in whether particular monocyte subsets were responsible for the changes in inflammatory cytokine production seen with SNA+ IVIg. Consistent with the report by Cros et al (136), we found that of the three monocyte subsets described, $CD14^{dim}CD16^{+}$ monocytes were more responsive to nucleic acid/viral ligands and produced more TNF and IL-1β, whereas $CD14^{+}CD16^{-}$ monocytes were more responsive to bacterial ligands, producing more IL-6 and IL-8. And while all of these subsets are able to produce PGE2 in response to SNA+ but not SNA- IVIg, we found that it was the $CD14^{+}CD16^{-}$ monocytes that produced the highest concentrations of PGE2. This result is consistent with previous studies showing that the $CD14^{+}CD16^{-}$ monocyte population produces more PGE2 in response to *Candida albicans* (138). Interestingly, SLE patients trend towards a lower percentage of circulating $CD16^{-}$ monocytes (139), and their monocytes produce less PGE2 in response to Concanavalin A (140). In addition, SLE monocytes are less able to inhibit IFN-α

production by pDCs, although the mechanisms were not entirely clear (72). We also found that there was a modest but statistically significant decrease in the percentage of $CD14^{+}CD16^{-}$ monocytes in SLE patients, suggesting that they might have a deficiency in SNA+ IVIg mediated inhibition of IFN-α. However, instead, we observed that after stimulation with TLR ligand to induce IFN-α, SLE patient PBMC exhibited strong IFN-α inhibition following the addition of SNA+ IVIg, confirming that these cellular processes are intact in the blood immune cells of patients with SLE.

In the previous chapter, we showed that it was the F(ab')2 fragment of SNA+ IVIg that was responsible for mediating PGE2 production by monocytes and inhibiting IFN-α production. Because Käsermann et al. (91) demonstrated different antibody binding specificities within SNA+ versus SNA- IVIg preparations and we observed that cleavage of glycans disrupted antibody binding, we postulated that the likely explanation for the inhibitory activity of SNA+ IVIg was that the SNA selected F(ab')2 fragments bound to structures on monocytes that induced PGE2 production. Here we tested whether there was a difference in binding of SNA+ or SNA- F(ab')2 IVIg to monocytes or to monocyte subsets. While we found that SNA+ and SNA- IVIg both bound monocytes equally well, it was clear that the strongest binding of F(ab')2 IVIg was to $CD14^{+}CD16^{-}$ monocytes, while the "patrolling" $CD14^{dim}CD16^{+}$ monocytes that have been implicated in the pathogenesis of SLE bound the least.

Taken together, these results confirm that SNA+ and SNA- IVIg have differential effects on monocytes in culture. Both subfractions of IVIg bind monocytes equally well, but SNA+ IVIg dampens expression of the costimulatory molecule CD86 while SNA- IVIg does not. However, SNA+ IVIg did not inhibit the inflammatory cytokines IL-6, IL-8, IL-10 or TNF that had been induced by various TLR ligands. While all monocyte subsets were able to produce

PGE2 in response to SNA+ IVIg, the $CD14^{+}CD16^{-}$ population was superior, and it was this population that was reduced in SLE patients. However, SNA+ IVIg was still able to robustly inhibit IFN-α production in SLE PBMCs stimulated by the TLR agonist, indicating that this pathway is intact in SLE patients. Interestingly, while all monocyte subsets bound IVIg, IVIg bound least to the $CD14^{dim}CD16^{+}$ "patrolling" monocytes that are implicated in lupus disease activity and pathogenesis, and also become activated upon stimulation with immune complexes. Perhaps the lack if IVIg binding to this subset of monocytes explains its reduced production of PGE2. However, more work is required to identify the receptor for SNA+ IVIg on monocytes and whether the expression of the receptor is reduced on the $CD14^{dim}CD16^{+}$ monocyte subset.

Chapter 6

Mechanism of pDC IFN-α inhibition by PGE2

Brief Introduction

We demonstrated that PGE2 was the crucial factor induced by SNA+ IVIg that was responsible for inhibition of pDC IFN-α production. Though it has been demonstrated that PGE2 can inhibit pDC functions (72-74, 141), the molecular mechanism for the inhibition specific to this cell type is poorly understood, in part because of the difficulty obtaining the number of cells required for mechanistic studies. PGE2 is believed to interact with pDCs via two receptors, EP2 and EP4 (73, 74). Both of these receptors evoke intracellular responses via increased cAMP and subsequent PKA activation (reviewed in (142)). Consistent with this is the finding that pDCs are inhibited by forskolin, an inducer of cAMP and thus activator of PKA (74). However, the mechanism by which PKA prevents pDC activation is not clear.

Recently it was found that the mTOR pathway can play a significant role in the production of IFN-α through the regulation of the transcription factor, IRF7 (outlined in Figure 6.1). It was shown in mice that mTOR has two functions supporting IRF7 activity. First, mTOR, as part of a mutli-protein complex, can promote the translation of IRF7 mRNAs into protein via the phosphorylation of 4E-BP (143), which releases eIF4E, activating a complex that promotes translation. Second, the mTOR complex drives S6K activity (144), which stabilizes the endosomal TLR-MyD88 complex. Thus, activation of mTOR can increase both the protein expression of IRF7 as well as promote the activation/phosphorylation of IRF7 and subsequent translocation to the nucleus, ultimately promoting an increase in IFN-α production.

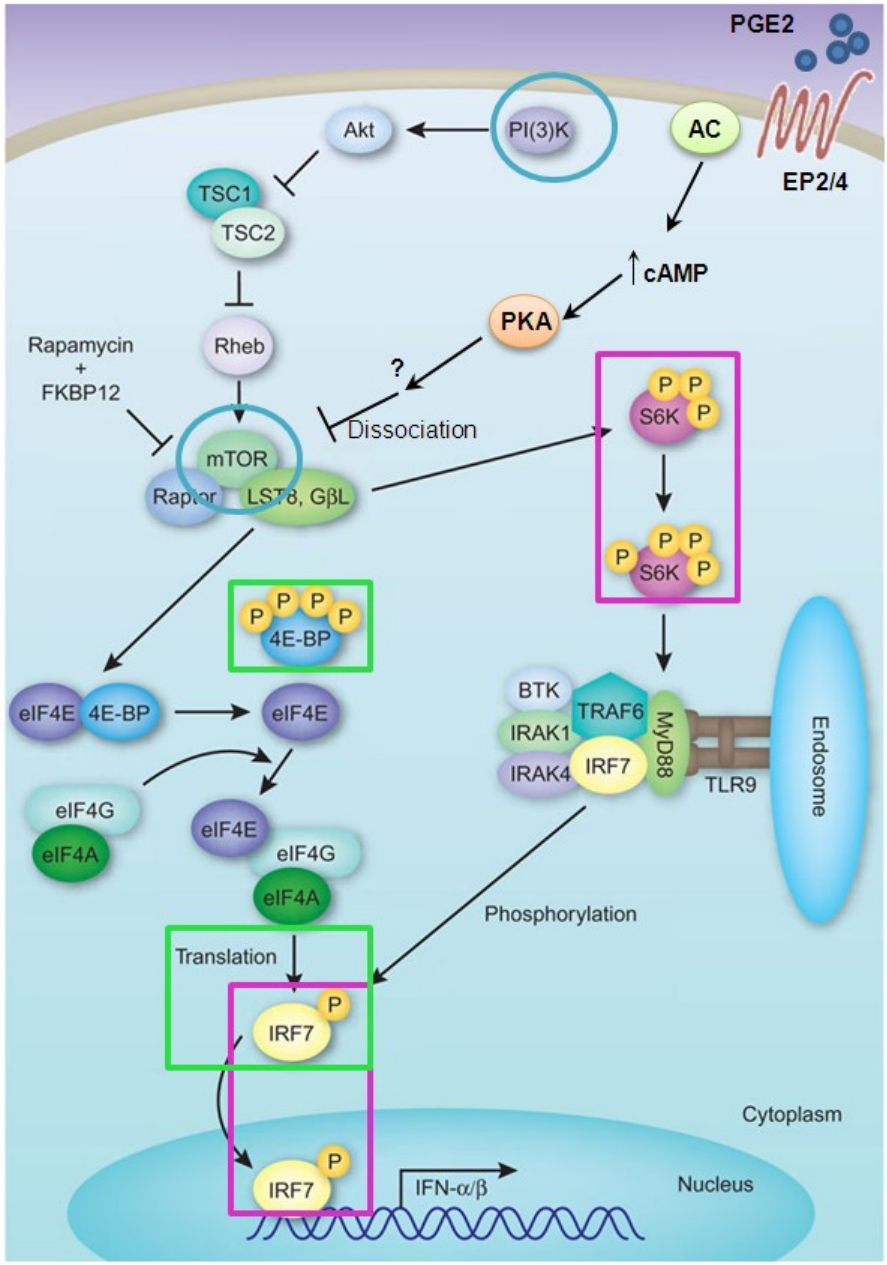

Figure 6.1. Putative signaling model for PGE2 inhibition of IFN-α production by pDCs. PI3K promotes activation of mTOR (blue circles), part of the multiprotein mTOR complex, which can contribute to IFN-α production in pDCs by two mechanisms: (1) mTOR phosphorylation of 4E-BP frees eIF4E to promote IRF7 translation (green boxes) and (2) mTOR phosphorylation of S6K promotes TLR9/MyD88 complex stability resulting in increased IRF7 activation and nuclear translocation (pink boxes). PGE2 can engage EP2 and EP4 expressed on pDCs, which promotes cAMP and PKA activation. PKA activation may contribute to disassociation of the mTOR complex and thus the downstream signaling leading to IFN-α production. Adapted from (145).

One group has shown that in mouse embryonic fibroblasts and human embryonic kidney cells, activation of PKA inhibits mTOR activity by disrupting the mTOR complex, independent of many of the known regulators of mTOR (146). However, this link between PKA activation and mTOR inhibition has not been established in other cell types. We hypothesized that PGE2 inhibits pDCs via this mechanism, with the consequence ultimately being a reduction in IRF7 activation and IFN production.

IFN-α is inhibited by PGE2 or by a suppressor of mTOR

To further explore the mechanism by which PGE2 inhibits signaling in pDCs resulting in a reduction in IFN-α production, we first confirmed that PGE2 could act directly on pDCs. Using either purified primary pDCs (Figure 6.2A) or the pDC line Gen2.2 (Figure 6.2B), we found that IFN-α induced by TLR agonist stimulation was inhibited by the addition of PGE2. Next we sought to confirm that PKA activation and mTOR were required for IFN-α production. We stimulated PBMCs with a TLR agonist and added either PGE2, a cell-permeable cAMP analog that activates PKA (dibutyryl-cAMP), or a mTOR suppressor (Rapamycin). With any of the three treatments, IFN-α production was inhibited (Figure 6.2C), demonstrating that PKA and mTOR pathways contribute to IFN-α regulation. PGE2 was used at 10 ng/mL to represent the quantity of PGE2 induced by SNA+ IVIg. The chosen concentration of 100 μM dibutyryl-cAMP is commonly used, but was also the highest concentration at which the DMSO carrier control wells did not elicit IFN-α inhibition; a lower dose of 10 μM dibutyryl-cAMP did not significantly inhibit IFN-α (data not shown). Rapamycin at 10 ng/mL is the approximate targeted serum concentration of this drug in kidney transplant patients; Rapamycin inhibited IFN-α production over a broad range of doses, from 100 pg/mL to 1 μg/mL (data not shown).

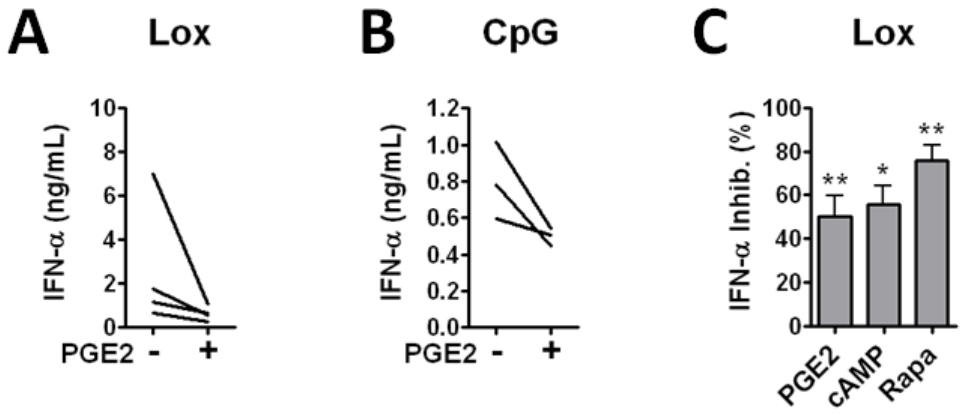

Figure 6.2. PGE2, PKA activator, or mTOR inhibitor suppress IFN-α. Cells were stimulated with TLR agonists Loxorobine (Lox) or CpG and supernatants analyzed for IFN-α after 20h. IFN-α production with or without addition of PGE2 (10 ng/mL) to (A) isolated primary pDCs or (B) a pDC line (Gen2.2). (C) PBMCs with addition of PGE2 (10 ng/mL), dibutyryl-cAMP (100 μM), or Rapamycin (10 ng/mL). Results represent at least three experiments in each group. Bars represent mean + SEM. Symbols above bars represent a comparison of IFN-α concentration in cultures without and with the indicated treatment. *, **, *** represent p-values of <0.05, <0.01, and <0.001, respectively; ns, not significant.

PGE2 reduces IRF7 translocation to the nucleus of pDCs

Phosphorylated IRF7 that translocates to the nucleus is responsible for the induction of IFN-α gene expression in pDCs. Impaired IRF7 translocation is hypothesized to result from inhibition of the mTOR complex. We asked whether PGE2 would also impair IRF7 translocation in pDCs. Using isolated primary human pDCs, we showed that IFN-α, but not IL-6 production was inhibited by PGE2 (Figure 6.3A and 6.3B). Using confocal microscopy, we determined that IRF7 translocation to the nucleus was increased with CpG stimulation, but that with the addition of PGE2, translocation was reduced (Figure 6.3C). CpG stimulation did not result in NFκB p65 translocation to the nucleus (data not shown). A reduction in IRF7 intensity after PGE2 treatment was also seen by fluorescence microscopy of both Loxoribine-stimulated primary pDC (data not shown) as well as CpG-stimulated Gen2.2 pDC line, whereas no change in NFκB p65 intensity was observed (Figure 6.3D).

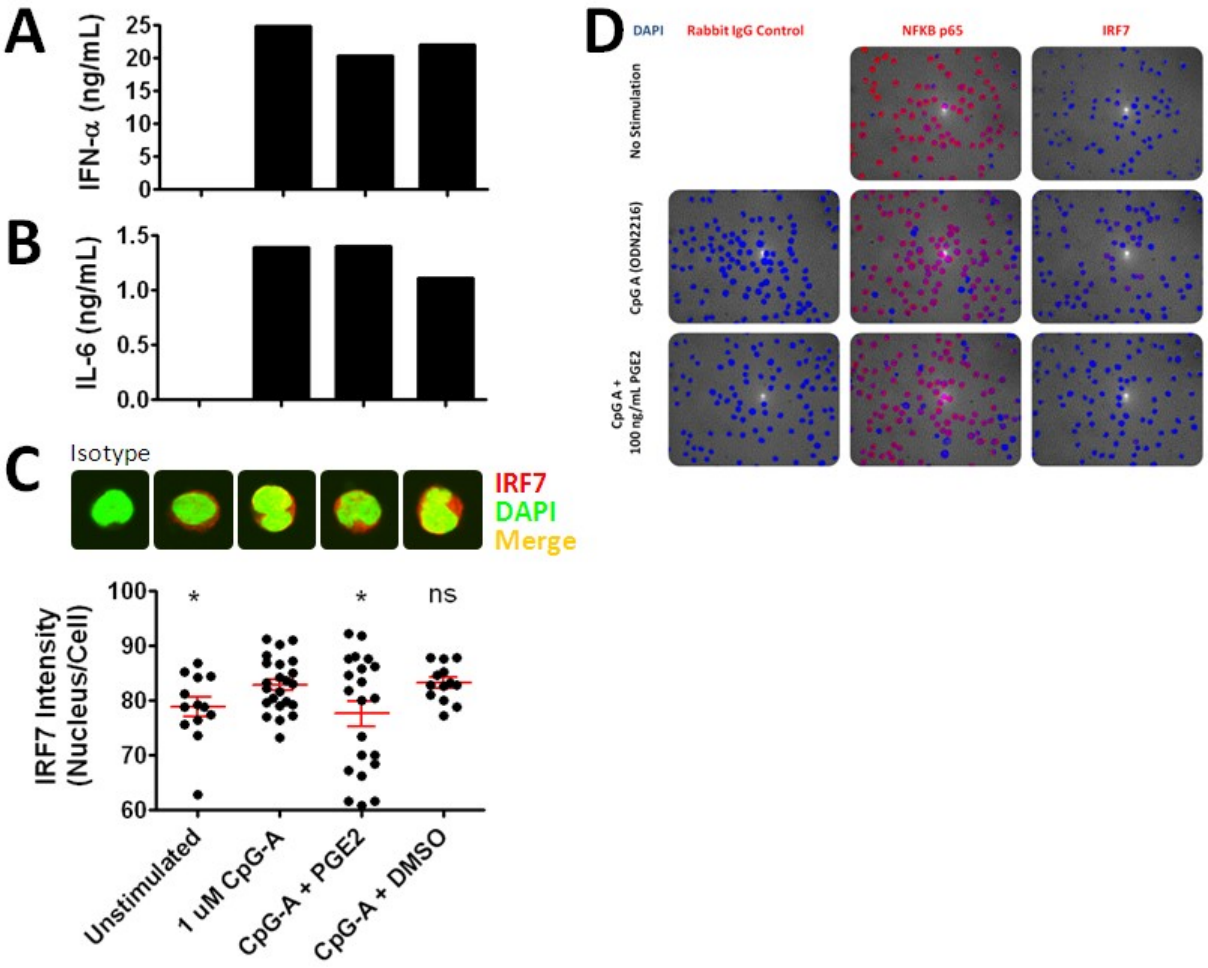

Figure 6.3. PGE2 inhibits CpG-induced IRF7 nuclear translocation and IFN-α production by pDCs. Isolated primary pDCs were stimulated with CpG with or without PGE2 (100 ng/mL) or DMSO carrier control and (A) supernatants analyzed at 20h for IFN-α. and (B) IL-6, or (C) pDCs were harvested at 1h, affixed to a slide, stained with IRF7 (red) and DAPI (green), and analyzed by confocal microscopy for intensity of IRF7 in the nucleus (% of intensity of IRF7 in the entire cell). Results represent one experiment with at least ten cells in each group. (D) Cells from the Gen2.2 pDC line were with CpG with or without PGE2 (100 ng/mL) and then harvested at 1h, affixed to a slide, stained with antibodies to NFκB, IRF7, or isotype control (red) and DAPI (blue), and analyzed by fluorescence microscopy. In C, statistics represent t test comparison versus CpG treatment alone. *, **, *** represent p-values of <0.05, <0.01, and <0.001, respectively; ns, not significant.

Brief Discussion

Though it is known that PGE2 can inhibit IFN-α production by pDCs, the effects of PGE2 on the signaling required for this response have not been explored. We propose a mechanism whereby PGE2 via interaction with EP2 or EP4 increases intracellular cAMP and activates PKA which can disrupt the activity of the mTOR complex, ultimately leading to reduced IRF7 expression and reduced translocation of IRF7 to the nucleus. Consistent with our hypothesis, we found that PGE2 directly inhibited pDC production of IFN-α, and that a surrogate activator of PKA, dibutyryl-cAMP, or an mTOR inhibitor, Rapamycin, both inhibited IFN-α production. Additionally, we show that PGE2 reduces IRF7 expression and nuclear translocation that is induced by TLR agonist stimulation of pDCs.

These results demonstrate an involvement of both the PKA and mTOR pathways in the regulation of IFN-α by pDCs, and may represent novel targets for immune modulation in diseases exacerbated by IFN-I. The next chapter includes a deeper analysis of the consequences of these findings, and the relevance for disease.

Chapter 7

Discussion and Future Directions

The goals of this dissertation were to investigate the role of IVIg in the regulation of pDC IFN-α production, and to elucidate the importance of IgG sialylation in this process. These are important questions as IVIg has been used in autoimmune diseases in which pDCs and IFN-I are thought to play a role, including in SLE (84). While the beneficial effects of IVIg in lupus have been demonstrated in several case studies, in small clinical trials (reviewed in (147, 148)) and in one small randomized trial in lupus nephritis (149), the role for IVIg in the regulation of IFN-α has not been addressed. Elucidating the inhibitory component(s) and the mechanism by which they act could reduce the use of this limited resource in favor of small amounts of the more potent components and may offer novel targets for therapy in SLE and other IFN-I-driven autoimmune diseases. We and others had previously found that IgG could inhibit lupus immune complex (IC)-induced IFN-α (67, 97), though the specific mechanism had not been elucidated, nor had the importance of sialylation been addressed.

The data presented in this dissertation demonstrated that IVIg Fc fragments significantly inhibited IC-induced IFN-α by blocking their binding to pDCs. The affinity of IgG for FcγRs varies, and has divided the FcγRs into high-affinity and low-affinity groups. The affinity for FcγRI is highest, approximately $1x10^{8}$ M^{-1} (150), and it is known that this receptor binds monomeric IgG and can be saturated at serum concentrations. The other FcγRs—FcγRIIa, FcγRIIB/C, FcγRIIIA, and FcγRIIIB—all exhibit at least 10-fold lower affinity, and thus have been purported to only bind multimeric IgG. FcγRIIa is the only FcγR expressed by pDCs (67, 104), and therefore it was surprising to find that monomeric IVIg Fc preparations could inhibit

the binding of ICs to this receptor. However, other evidence suggests monomeric IgG can compete for binding to low-affinity FcγRs. Following isolation by gel filtration, monomeric IgG without dimers was able to bind, but not activate, human FcγRIIa (105), and monomeric IgG can inhibit binding of dimeric or oligomeric IgG to FcγRIII on neutrophils (107). In addition, IC binding to low-affinity FcγR in mice can be blocked by IVIg at high concentrations (thousands of times greater than the concentration of ICs) (106). In addition to pDCs, monocytes and NK cells also express low-affinity FcγRs; we found that IVIg Fc could also block IC binding to these cell subsets (data not shown). Because pDCs express only FcγRIIa, and IVIg Fc inhibited SLE IC-induced IFN-α by acting directly on pDCs, other FcγR cannot be implicated in this inhibitory process.

In a series of elegant studies using the K/BxN mouse model of arthritis, Ravetch and colleagues showed that the sialylated subset of IVIg was the anti-inflammatory component of IVIg (151). Thus, we wished to address whether the small sialylated subset of human IgG was responsible for the inhibition of IC-induced IFN-α production by human pDCs. Glycosylation of IgG at the conserved site in the Fc (Asn 297) affects its affinity for FcγR. A complete lack of the glycan abrogates its effector function (98), and consistent with this, we found that after being cleaved of its N-linked glycan by PNGase, IgG no longer could block IC binding to FcγRIIa on pDCs. Subtler changes in Fc glycosylation can also have effects on FcγR interaction. The absence of a fucose residue or the presence of a bisecting GlcNAc which both increase affinity for activating FcγR (111-114), whereas the presence of a sialic acid residue reduces the affinity (115). This may be important for immune complex inhibition, as glycosylation of IgG Fc can be modified following B cell stimulation (152), and patients with chronic inflammatory diseases are reported to have reduced galactosylation (116, 117) and sialylation (118) on serum IgG Fc.

These changes would result in an increased affinity for activating FcγR. We found, however, that inhibition by IVIg Fc fragments treated with neuraminidase to cleave sialic acid was no less effective than inhibition by untreated IVIg. In addition, recombinant Fc fragments that were engineered to be hypersialylated were equipotent in IFN-α inhibition. Together, these data demonstrate that sialylation is dispensable for the inhibition of IFN-α that is induced by ICs. Similarly, galactose was also not required, as cleavage of this residue with galactosidase had no effect on the inhibitory activity of IVIg Fc.

The mechanism we identified of IVIg inhibition of SLE IC-induced IFN-α differs significantly from the mechanism of IVIg anti-inflammatory activity elucidated by Ravetch and colleagues in the K/BxN mouse model of arthritis. In the K/BxN model, immune complex-mediated joint-specific inflammation is induced by transfer of serum from mice that develop antibodies recognizing glucose-6-phosphate isomerase. In this model, they showed that pre-treatment with high-dose human IVIg can prevent joint swelling (153). Additionally, Fc fragments of IVIg also conferred the same protection (154), and the sialylated subset of Fc was a much more potent inhibitor while desialylated IVIg lost its anti-inflammatory activity (155), demonstrating that sialic acid was critical. Subsequent research in the same model identified a subset of "regulatory" macrophages (156) or DCs that express the receptor for sialylated IVIg—SIGN-R1 (86) or DC-SIGN (the human homologue)—that is required for the anti-inflammatory effects of sialylated IVIg. These regulatory cells produced IL-33 in response to sialylated IVIg, which drove basophils to produce IL-4, and finally the upregulation of the expression of the inhibitory FcR, FcgRIIb, on macrophages, ultimately reducing their threshold of activation by immune complexes (87). We found that SLE IC-induced IFN-α inhibition by IVIg, like the model described by Ravetch et al., required the Fc domain of Ig, but in contrast, did not require

sialylation nor the presence of a separate regulatory cell expressing DC-SIGN. These differences in the mechanisms of inhibition of ICs may simply indicate that there are fundamental differences in the way in which inflammation is mediated in the mouse model of joint inflammation, which requires macrophages, and in vitro cultures of SLE IC-induced IFN-α, which requires pDCs. Interestingly, in mouse models of immune thrombocytopenic purpura (ITP), sialylation-enriched IVIg, neuraminidase-treated (desialylated) IVIg and normal IVIg did not differ in their efficacy to alleviate ITP (88, 89), bringing into question the importance for sialylation of IVIg in diseases other than the K/BxN mouse model for arthritis. Alternatively, the mechanistic differences may reflect the timing of IVIg administration—while Ravetch's group treated mice with IVIg prior to induction of disease, we added IVIg simultaneously with IC stimulation. Another possible cause for differences is the use of a human blood product in mice. In our system, inhibition of IC-induced IFN-α by IVIg was through blockade of FcγRIIa, and thus depends crucially on the ability of IVIg Fc to bind FcγR. Mouse FcγRs have reduced ability to bind human IgG, and thus in a mouse system, the effects of FcγR blockade by monomeric IVIg may be lost.

Variation in the IgG subclasses (IgG1, 2, 3, and 4) is also known to affect affinity for FcγRs. All low affinity receptors tend to have reduced affinity for IgG2 and IgG4 (150). FcγRIIa, in particular, has the highest affinity for IgG1, whereas affinity for IgG2, IgG3, and IgG4 is at least 5-fold lower (150). Furthermore polymorphic variants of FcγRIIa affect affinity—IgG and especially IgG2 have increased affinity for the H131 variant compared to the R131 variant (150); and the frequency of the R131 variant is increased in SLE (157, 158). While the distribution of IgG subclasses in total IgG from SLE patients is no different than what is found in healthy human donors (159, 160), the subclass distribution among autoantibodies can

vary significantly. For instance, the IgG3 subclass is enriched in anti-DNA, anti-Smith, and anti-histone antibodies (161), whereas anti-RNP antibodies are predominantly of the IgG2 subclass (160). Because of the high affinity for FcγRIIa, IgG1 and IgG3 subclasses of Fc may be the most potent inhibitors of binding by ICs to pDCs. No differences in the subclass distribution was observed between the SNA+ and SNA- subsets of IVIg (91) that were used in our experiments. However, further studies will be required to explore the role of IgG subclass of normal and autoantibody IgG in competition for FcγRs in SLE.

SLE patients have increased serum IgG (159) yet this is not sufficient to inhibit IC stimulation of IFN-α. There are several reasons that may explain this seemingly paradoxical situation. First, while there is an increase in IgG concentrations in sera from SLE patients, much of this increase is accounted for by autoantibodies (162). Therefore, this shift will result in more IC formation such that the 'normal' IgG cannot protect from IFN-α induction. Treatment with IVIg may shift this balance back to a healthy ratio. IVIg is administered to hypogammaglobulinemic patients at 100-500 mg/kg every 3-4 weeks to maintain a serum concentration of 5 mg/mL (163), and the high-dose treatment, which is used in certain inflammatory diseases, is up to 10 times higher (1-2 g/kg), such that after two weeks there remains an increase of serum IgG of about 8 mg/mL (164). An influx of normal IVIg of this magnitude may well counteract the increased autoantibody concentration found in SLE and prevent stimulation of IFN-α by ICs. Alternatively, the IFN induced by ICs in SLE may be a consequence of the quality of the IgG. As described previously, IgG glycosylation and isotype is often altered by B cell stimulation (152) and/or inflammatory conditions (165). Consequently, SLE IgG autoantibodies have altered glycosylation and are enriched for certain isotypes which possibly contribute to increased affinity for FcγRIIa expressed on pDCs. Restoring the

concentration of normal IgG could offer sufficient competition for binding to FcγR such that it blocks IC binding and subsequent IFN-α induction.

The discovery that binding to FcγRIIa is required for pDC activation by ICs has led to the creative idea of incubation of IgG with EndoS to enzymatically remove the glycan from the heavy chain of all four human IgG subclasses resulting in reduced binding to FcγRIIa (166). EndoS treatment of ICs inhibited their uptake by pDCs and also the resultant IFN-α production (167). How this could be exploited for treatment of human SLE is difficult to envisage as therapeutic administration of this enzyme would result in cleavage of glycans from all IgG (and other molecules) and humans would develop an immune response to the bacterial enzyme. Our data suggest that an alternate method may be to provide an abundance of Fc fragments such that the balance of normal IVIg Fc and that of IgG in immune complexes is restored.

IgG Fc is attractive as a therapeutic because it can be produced using recombinant methods, and the glycosylation and subclass can be controlled. Whereas IVIg as a human product has a limited availability, recombinant Fc could be produced in abundance. Additionally, its smaller size may offer increased therapeutic efficacy. Multiple studies have shown that in diseases associated with IFN-I, there are decreased numbers of pDCs in peripheral blood (168-172), most likely because pDCs have migrated to sites of inflammation. This is confirmed by detection of infiltrating pDCs in biopsy specimens from skin (173), kidneys (174), and lymph nodes (175) of patients with SLE, minor salivary gland biopsy specimens from patients with primary Sjögren's syndrome (176), muscle biopsy specimens from patients with myositis (177), and synovial fluid from patients with rheumatoid arthritis (172). Thus, a therapeutic that can reach these tissues is critical. The rate of diffusion of a molecule is approximately inversely proportional to the cube root of molecular weight (178). Therefore, Fc

being only one third of the weight of intact IgG would likely have a diffusion rate that is nearly 30 times greater. Future studies are required to determine optimal glycosylation and IgG subclass, and whether this Fc can modulate IFN-α-mediated disease in vivo.

We also wished to determine whether IVIg had any inhibitory effects independent of Fc blockade. To this effect, we induced IFN-α by TLR agonists, which circumvents the need for FcR interaction, and found that IVIg also inhibited IFN-α production, although the inhibitory effects were more modest. The concentration of IVIg required for inhibition was more than 10-fold that seen for ICs, suggesting that it may be a particular subset of IVIg that was responsible for inhibition. Indeed, in contrast to the Fc-dependent, sialylation-independent inhibition of IC-induced IFN-α, we discovered that TLR agonist-induced IFN-α was more robustly inhibited by sialylation-enriched (SNA+) IVIg F(ab')2 fragments. Interestingly, this inhibition appeared specific for IFN-α, as little or no inhibition was observed for IL-6, IL-8, IL-10, or TNF. This could be an important consideration for a potential therapeutic as the goal is to prevent autoimmunity with as little perturbation of protective immunity as possible. Enrichment of the sialylated subset of IVIg might therefore offer selective advantages.

The finding that the F(ab')2 fragment was inhibitory was curious, as this precluded a requirement for interaction with Fc receptors. We wondered, therefore, whether the sialic acid might be the crucial component, as sialic acid is known to be required for binding to several receptors that typically elicit inhibitory effects, including DC-SIGN (151) and the family of sialic acid-binding immunoglobulin-like lectins (Siglecs) (179). However, neuraminidase cleavage of sialic acid from the SNA+ subset had no effect on its ability to inhibit IFN-α. Also, addition of other sialylated proteins such as fibrinogen, fetuin, or transferrin did not inhibit IFN-α. Another possibility was that the specificity of the sialylated antibodies was critical. SNA+ antibodies are

enriched with certain specificities such as those directed towards cytomegalovirus or Epstein-Barr virus, while decreased among antibodies specific for rubella virus, varicella zoster virus, and red blood cells antigens (91). Consistent with the importance for antibody specificity, cleavage of the N-glycans using PNGase, which can abrogate binding when present in the variable region (122), diminished the inhibitory activity of F(ab')2 IVIg. Therefore, it appeared that the inhibitory activity of SNA+ IVIg F(ab')2 was due to an enrichment of an unidentified antibody reactivity.

The specificity for IFN-α regulation, and not other cytokines, led us to ask whether SNA+ IgG was inhibiting pDCs directly. However, TLR stimulated IFN production by isolated pDCs was not efficiently inhibited by SNA+ IVIg. Selective depletion of cell subsets from PBMC that are known to regulate pDCs including monocytes, NK cells and B cells (72, 180-182), revealed that the inhibitory activity of SNA+ IVIg was consistently lost only in the absence of monocytes. Further exploration into how monocytes were inhibiting IFN-α revealed that PGE2 production was critical. PGE2 was significantly and robustly produced in response to SNA+ but not SNA- IVIg, in contrast to other cytokines known to inhibit IFN-α, including IL-10 and TNF (70-72). Only PGE2 at the concentrations induced by SNA+ IVIg was able to inhibit both TLR agonist- and IC-induced IFN-α, and could act directly on isolated pDCs. Interestingly, another group recently also found that regulatory T cells were expanded by IVIg, and such inhibition was also mediated by PGE2 induction by F(ab')2 fragments (90), calling into question Ravetch's model which implicates the Fc region as possessing the anti-inflammatory activity. The ability of SNA+ IVIg F(ab')2 to induce production of a factor that could inhibit pDC IFN-α regardless of the kind stimulation and expands regulatory T cells implies that enrichment of the

sialylated subset of IVIg or F(ab')2 fragments may provide a therapeutic that is more robust than whole IVIg for use in SLE.

The observation that sialylated IgG could attenuate IFN-α production raises important questions: (1) Is this pathway deficient in SLE? and (2), what is the target on monocytes that is responsible for PGE2 induction?

A role for monocytes in the regulation of pDCs has been demonstrated here and elsewhere (72). Interestingly, the monocytes of SLE patients have been found to be defective in their inhibition of pDCs (72), though the mechanisms are unclear. Interestingly, SLE monocytes produce less PGE2 in response to Concanavalin A (140). Three human monocyte populations have been described: $CD14^{dim}CD16^{+}$, $CD14^{+}CD16^{+}$ and $CD14^{+}CD16^{-}$ (136), with the $CD16^{+}$ monocytes being more responsive to nucleic acids, and implicated in SLE. While we showed that all three subsets of monocytes were able to produce PGE2 in response to SNA+ but not SNA- IVIg, we found that it was the $CD14^{+}CD16^{-}$ monocytes that produced the highest concentrations of PGE2. This result is consistent with previous studies showing that the $CD14^{+}CD16^{-}$ monocyte population produces more PGE2 in response to *Candida albicans* (138). Interestingly, SLE patients trend towards a lower percentage of circulating $CD16^{-}$ monocytes (139), and we too found that there was a modest but statistically significant decrease in the percentage of $CD14^{+}CD16^{-}$ monocytes in SLE patients, suggesting that they might have a deficiency in SNA+ IVIg mediated inhibition of IFN-α. Experimental testing, however, revealed that after stimulation with TLR ligand to induce IFN-α, SLE patient PBMC exhibited strong IFN-α inhibition following the addition of SNA+ IVIg. Therefore, these cellular processes appear to be intact in blood cells of lupus patients. Further study is required to determine if

SNA+ IVIg and PGE2 are inhibitory at common sites of inflammation in lupus patients such as the kidney or skin.

To attempt to identify the target of SNA+ F(ab')2 IVIg on monocytes, we first compared the binding to monocytes by SNA+ versus SNA- IVIg, with the prediction that SNA+ would bind more highly, but we found no difference. Because it appeared that the $CD14^{+}CD16^{-}$ monocytes were the most robust producers of PGE2, we analyzed the binding of SNA+ versus SNA- IVIg to the various monocyte populations, but again found no difference. While SNA+ and SNA- IVIg bound equivalently within each subset, binding by IVIg was reduced on the $CD16^{+}$ monocytes, and particularly the $CD14^{dim}CD16^{+}$ subset, which, interestingly, has been implicated in SLE (136, 137). Further study will be required to determine if this difference is functionally significant. Though levels of binding are equivalent between the SNA+ and SNA- subsets, this does not necessarily imply that they have the same binding targets. To determine if there is a unique specificity among the SNA+ subset, the two IVIg preparations could be applied to monocyte surface antigens, immunoprecipitated, and analyzed by mass spectrometry to identify the target proteins. Candidates for targets would be those that are found in greater abundance by pulldown with SNA+ IVIg. Interestingly CD83-Ig induces PGE2 (183), suggesting that the ligand of CD83 may be a good candidate, but the ligand is not currently known.

Lastly, we wished to further elucidate how PGE2 inhibits pDCs, as very little is known about this process. Prostaglandin E2 (PGE2) is a major end product of cyclooxygenase conversion of arachidonic acid in myeloid and stromal cells. Though it can be produced by most cell types, the heterogeneous effects of PGE2 are reflected by differential expression of its four different receptors, designated EP1, EP2, EP3, and EP4. The signaling through the two Gs

coupled receptors, EP2 and EP4, is mediated by the adenylate cyclase-triggered cAMP/PKA/CREB pathway, whereas EP3 is a G_i-coupled receptor that inhibits adenylate cyclase, and EP1 signaling involves $Ca2^+$ release. Though originally thought to be pro-inflammatory because of its upregulation in inflammatory conditions, PGE2 has since been appreciated as playing a mostly anti-inflammatory role, particularly in macrophages and macrophage-like cells of various organs (184). We and others have demonstrated the ability of PGE2 to inhibit pDC production of IFN-α (72-74, 141), but the mechanism of this inhibition remains poorly understood.

PGE2 acts primarily through the receptors EP2 and EP4 on pDCs (73, 74)that would be predicted to stimulate cAMP and PKA activation as determined by studies in other cell types bearing these receptors (142). Consistent with this prediction, PKA activators such as forskolin or the cAMP analog dibutyryl-cAMP can also inhibit IFN-α ((74, 185) and our own work). There are conflicting results on whether PGE2 simply kills pDCs (73, 74, 141), particularly because IFN-α is a pro-survival factor for pDCs (72, 83). The only publication offering a mechanism to link PGE2 with inhibition of IFN-α production revealed that IRF7 mRNA levels were significantly reduced in CpG-A-stimulated pDCs treated with PGE2 compared to untreated pDCs (73); however, this is complicated by the fact that IRF7 is also an interferon-response gene (186), and accordingly, IRF7 expression was lowest in unstimulated pDC cultures.

We wished, therefore, to understand how PKA activation might modulate signaling pathways in pDC that lead to IFN-α production. IFN-α expression in pDCs is induced by TLR7 or 9 ligation which, via MyD88 and signaling mediators, leads to the phosphorylation and translocation of IRF7 to the nucleus where it promotes the transcription of IFN-α genes (187). More recently it has also been shown that the PI3K/mTOR/p70S6K pathway is constitutively

active and required for TLR-mediated IFN-I induction in pDCs by promoting TLR9/MyD88 interactions (144, 188). In addition, mTOR also regulates the eIF4E binding proteins (4E-BPs) which normally bind to and blocks the eIF4E protein that promotes translation of a subset of mRNAs including that encoding IRF7 (143). Indeed, it was found that IFN-I expression is higher in pDCs from mice deficient in 4E-BP1 and 4EBP2 (143). Therefore, mTOR activity plays a significant role in the IFN-α induction in pDCs. Though no research has been done to link PKA activity with interference in the mTOR pathway in pDCs, one group has shown in mouse embryonic fibroblasts and in human embryonic kidney cells that cAMP inhibits mTOR by dissociation of its complex in a PKA-dependent mechanism which is independent of other known regulators of mTOR such as TSC2, Rheb, and Rag GTPases (146). Thus, I hypothesized that mTOR deactivation was the mechanism by which PGE2, via EP2/EP4 upregulation of cAMP and activation of PKA, inhibited IFN-α production. Consistent with this hypothesis, I found that IFN-α production was inhibited by PGE2, dibutyryl-cAMP (a cAMP analog which activates PKA), or Rapamycin (an mTOR suppressor). Furthermore, I demonstrated by confocal microscopy an increase in nuclear translocation of IRF7 in pDCs with CpG-A stimulation that is significantly reduced after treatment with PGE2. By fluorescence microscopy it appears that IRF7 expression is also diminished. Future research will focus on the effects of PGE2 on the mTOR pathway by analyzing phosphorylation of S6Ks and 4E-BPs. Additionally, we will determine the importance of PKA activation in the process by addition of the PKA inhibitor, H89. Lastly, we will determine whether PGE2 and Rapamycin have similar effects on IRF7 expression, phosphorylation, and nuclear translocation.

The discovery that PGE2 was the factor responsible for pDC inhibition upon treatment with SNA+ IVIg reveals a novel target for IFN-α modulation in SLE and other IFN-I-associated

diseases. While PGE2 had a potent inhibitory effect on IFN-α, it had relatively little effect on other cytokines. This could be beneficial to inhibit the pathogenic cytokine while allowing others to remain intact and therefore less likely to jeopardize protective immunity. Whereas quite stable in vitro, PGE2 has a very rapid turnover rate in vivo and is rapidly eliminated from tissues and circulation (189). Additionally, because PGE2 effects are dependent on the receptor expression of various cell targets, there is a strong possibility for off-target effects. Clinical use of PGE2 (dinoprostone) is limited to vaginal suppository for preparation of the cervix for labor. However, EP2 or EP4 antagonists such as butaprost or 11-deoxy-PGE1 may represent more stable and more specific treatments for targeting pDCs. Consistent with a role in preventing autoimmunity, drugs that induce the release of PGE2, such as cetirizine, were reported to have effects that may be of benefit in autoimmune diseases (190). Additionally, the importance of PGE2 in controlling IFN-α production may explain occasional reports of disease flares and aseptic meningitis in SLE patients (191) and induction of SLE-like symptoms by cyclooxygenase inhibitors (192), but also the improvement of such symptoms by treatment with PGE1 (193) and PGE analogs (194). Also, while SLE monocytes are reported to have reduced production of PGE2 (140), SLE pDC inhibition by PGE2 is as good as or better than pDCs from healthy controls (73 104). Together, our research indicates that SNA+ IVIg, PGE2, or receptor agonists specific to EP2 or EP4 may be attractive novel treatments for IFN-I-mediated autoimmune diseases such as SLE.

The mTOR pathway may also be an interesting target for the treatment of SLE. Treatment with Rapamycin ameliorated signs of disease in mouse models of lupus as they demonstrated prolonged survival, maintained normal renal function, and reduced anti-dsDNA titers (195, 196). Rapamycin is also being used to generate T_{regs} from patients with autoimmune

disease (197). Importantly, Rapamycin is approved by the FDA and has been used safely and effectively to treat renal transplant rejection since 1999. A small study of SLE patients found that they responded well to Rapamycin, leading to a decrease in disease activity and prednisone requirement (198), but this study was not well-controlled, and while they demonstrated that T cells were more resistant to activation, there was no analysis for effects on IFN-I. Rapamycin may prove to be a promising therapy for SLE. Future studies focusing on a broader range of outcome measures, including levels of IFN-I, will demonstrate whether it can be used as an ancillary therapy in this disease in view of its known side effects.

The objective of this dissertation was to investigate the role of IVIg in the regulation of pDCs and IFN-α production, and to elucidate the importance of IgG sialylation in this process. We found that two mechanisms by which IVIg could inhibit IFN-α production by pDCs (Figure 7.1). SLE IC-induced IFN-α was inhibited by IVIg Fc fragments—independent of sialylation—by blocking binding of the ICs to FcγRIIa on pDCs. In contrast, we made the novel discovery that IFN-α induced by TLR agonists, which circumvents interaction with FcγR, was inhibited by higher concentrations of IVIg F(ab')2 fragments specifically of the sialylation-enriched subset. The activity was not direct on pDCs, but instead required monocytes which produced PGE2, a potent inhibitor of IFN-α. Questions that still remain are the identity the monocyte subset and particular antigen target(s) responsible for inducing the PGE2 production, and the mechanism by which PGE2 inhibits pDC production of IFN-α. We have found that the inhibitory pathways we described are intact in lupus patient PBMCs, suggesting that their monocytes are competent to produce PGE2 in response to SNA+ IVIg and that their pDCs are responsive to this inhibitory factor. Also, we provide evidence to support our hypothesis that PGE2 disrupts mTOR activation, thus inhibiting downstream IFN-α induction. Further elucidation of these pathways

and exploitation of the therapies mentioned in this dissertation may lead to more efficient immune modulation in SLE and other diseases where pDC-derived IFN-I is thought to play an important role.

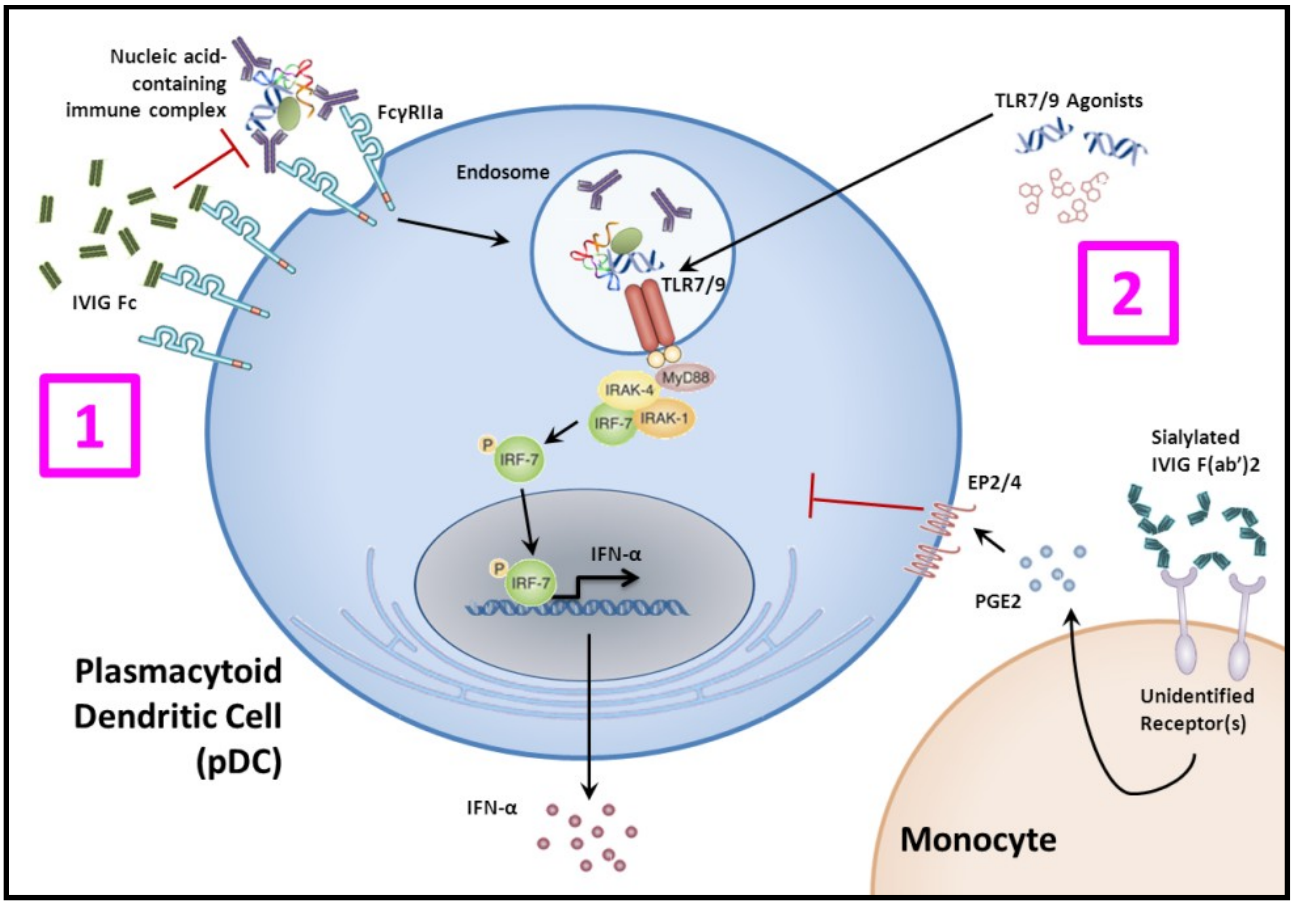

Figure 7.1. Model of the two mechanisms by which IVIg inhibits pDC IFN-α production. IFN-α is produced by pDCs in response to SLE ICs endocytosed via FcγRIIa which can trigger TLRs 7 and 9 and downstream signaling leading to expression of IFN-α genes. [1] The Fc fragment of IgG inhibits SLE IC-induced IFN-α by preventing the binding of ICs to FcγRIIa on pDC. [2] The F(ab')2 fragment of sialylation-enriched (SNA+) IgG inhibits TLR agonist-induced IFN-α independent of sialic acid by inducing monocyte production of PGE2.

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