Integrin αvβ3 limits Toll-like receptor type I interferon production by plasmacytoid dendritic cells and restricts autoimmunity

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Abstract

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Plasmacytoid dendritic cells (pDCs) are strongly implicated as a major source of type I interferon (IFN-I) in systemic lupus erythematosus (SLE), triggered through Toll-like receptor (TLR)-mediated recognition of nucleic acids released from dying cells. However, relatively little is known about how TLR signaling and IFN-I production are regulated in pDCs. Here I describe a role for integrin αvβ3 in regulating TLR responses and IFN-I production by pDCs in mouse models. I show that αv and $\beta3^{-/-}$ pDCs produce more IFN-I and inflammatory cytokines than controls when stimulated though TLR7 and TLR9 *in vitro* and *in vivo.* This dysregulated TLR signaling results in activation of B cells and promotes germinal center B cell and plasma cell expansion. Furthermore, in a mouse model of TLR7-driven lupus-like disease, deletion of αvβ3 from pDCs causes accelerated autoantibody production and pathology. I therefore identify a pDC-intrinsic role for αvβ3 in regulating TLR signaling and preventing activation of autoreactive B cells. As αvβ3 serves as a receptor for apoptotic cells and cell debris, I hypothesize that this regulatory mechanism provides important contextual cues to pDCs and functions to limit responses to self-derived nucleic acids.

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Dedication

To Luke, who fills my life with joy
and makes the hard days bearable.

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List of Abbreviations

SLE:	Systemic lupus erythematosus
pDC:	Plasmacytoid dendritic cell
TLR:	Toll-like receptor
DNA:	Deoxyribonucleic acid
RNA:	Ribonucleic acid
ds:	double-stranded
ss:	single-stranded
SNP:	Single nucleotide polymorphism
ODN:	Oligodeoxynucleotide
TNF:	Tumor necrosis factor
IFN:	Interferon
IFN-I:	Type I Interferon
IL-6:	Interleukin-6
Itgav:	Integrin αv
Itgb3:	Integrin β3
Ptpn22:	Protein Tyrosine Phosphatase Non-Receptor Type 22

Bank1: B cell scaffold protein with ankyrin repeats 1

Dnase1: Deoxyribonuclease 1

Atg5: Autophagy Related 5

IRF5: Interferon regulatory factor 5

IRF7: Interferon regulatory factor 7

Stat4: Signal transducer and activator of transcription 4

EBV: Epstein-Barr Virus

C1Q: Complement component 1q

NET: Neutrophil extracellular traps

PS: Phosphatidylserine

MFG-E8: Milk fat globule-EGF factor 8 protein

MZ: Marginal Zone

CDC: Conventional dendritic cell

OX40L: OX40 ligand

TFH: T follicular helper cell

GC: Germinal center

TREG: T regulatory cell

IgG: Immunoglobulin G

ISG: Interferon-stimulated gene

IC: Immune complex

MHC-II: Major histocompatibility complex- class II

IPC: Inteferon-producing cell

DTR: Diphtheria toxin receptor

TCF4: Transcription factor 4

CCL19: Chemokine (C-C motif) ligand 19

Ab: Antibody

BCR: B cell receptor

ASC: Antibody secreting cells

AFC: Antibody forming cell

PC: Plasma cell

MyD88: Myeloid differentiation primary response 88

Nf- κb: Nuclear factor κ-light-chain-enhancer of activated B cells

IRAK4: Interleukin-1 receptor-associated kinase 4

TRAF: TNF receptor-associated factor

IKKα: Inhibitor of nuclear factor κ-B kinase subunit α

AP-3: Adaptor Protein-3

LC3: Microtubule-associated protein 1A/1B-light chain 3

LIR: Leukocyte immunoglobulin-like receptor subfamily B

SRC: Src family kinase

SYK: Spleen tyrosine kinase

ROS: Reactive oxygen species

CD11c: *Itgax* gene

C57BL/6: C57 Black 6 mice

BDCA2: C-type lectin domain family 4 member C

RPMI: Roswell Park Memorial Institute

HEPES: N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

Flt3L: FMS-like tyrosine kinase 3 ligand

FACS: Fluorescence-activated cell sorting

A647: Alexa fluor 647

FITC: Fluorescein isothiocyanate

PBS: Phosphate-buffered saline

RBC: Red blood cell

CXCR5: CXC motif chemokine receptor 5

PD-1: Programmed cell death protein 1

PNA: Peanut agglutinin

IMQ: Imiquimod

Ifit3: Interferon induced protein with tetratricopeptide repeats 3

Mx1: MX dynamin like GTPase 1

BM: Bone marrow

BSA: Bovine serum albumin

ICAM-1: Intercellular adhesion molecule 1

LFA-1: Lymphocyte function associated antigen 1

LAP: LC3-associated phagocytosis

Rubicon: Run domain Beclin-1-interacting and cysteine-rich domain-containing protein

NOX2: NADPH oxidase 2

R848: Resiquimod

CRE: Causes recombination

EF: Extrafollicular

T1: Transitional type 1

T2: Transitional type 2

Fo: Follicular

SmRNP: Smith ribonucleoprotein

C3: Complement component 3

RUNX2: Runt-domain transcription factor 2

SARS-CoV2: Severe acute respiratory syndrome coronavirus 2

WT: Wild-type

KO: Knockout

PAMP: Pathogen-associated molecular pattern

NCA: Non-canonical autophagy

Dock 2: Dedicator of cytokinesis 2

AC: Apoptotic cell

IFNAR: Interferon α/β receptor

HSV: Herpes simplex virus

CARD9: Caspase recruitment domain-containing protein 9

TRIF:	TIR domain containing adaptor molecule 1MAPK
SCARF1:	scavenger receptor class F member 1
RGD:	Arginylglycylaspartic acid
TIRAP:	TIR domain containing adaptor protein
TRIF:	TIR-domain-containing adapter-inducing interferon-β
TRAM:	TRIF-related adaptor molecule
TCF4:	Transcription factor 4

Chapter 1: Introduction

1.1 Lupus is a multifactorial disease with diverse clinical manifestations.

Systemic Lupus Erythematosus (SLE), commonly referred to as Lupus, is a complex autoimmune disease affecting millions of people worldwide. It represents a significant health burden due to devastating clinical manifestations of diverse symptoms, affecting multiple organ systems including the skin, heart, kidneys, and joints. SLE is chronic and patients often require treatment for the course of their lifetime which may mean immunosuppressive medications leading to further adverse effects. In the last 50 years the 10-year survival rate has increased dramatically to more than 70%, but the clinical heterogeneity of the disease remains a hurdle for treatment and management of the disease (Tsokos 2020). It has now become clear that the diverse manifestations of this disease can be attributed to multiple pathogenic processes and is caused by a complicated interplay between genetic and environmental conditions.

Due to the high heritability of SLE to first-degree relatives of patients, it is clear that there are genetic factors at play. Some genes, such as complement component *C1q*, *TREX1*, or *DNASE1L3*, are capable of causing disease with their deficiency alone (Moulton, Suarez-Fueyo et al. 2017). However, it is more often the case that it is the interaction of multiple polymorphisms at several gene loci that leads to the diverse clinical manifestations of SLE. Some of the pathways implicated in SLE are involved in degradation of self-derived nucleic acids and clearance of dead and dying cells, such as *DNASE1* and *ATG5* (Moulton, Suarez-Fueyo et al. 2017). Additionally, there are genes

like *IRF5, IRF7,* and those encoding endosomal TLRs (*TLR7, TLR8,* and *TLR9),* that are involved in sensing of DNA and downstream signaling for the production of Type I Interferons that have also been associated with increased risk of SLE. Finally, there have been single-nucleotide polymorphisms (SNPs) in certain genes regulating T and B cell function, like *PTPN22* and *BANK1* (Moulton, Suarez-Fueyo et al. 2017) identified, which likely increase activation of lymphocytes when disrupted, leading to an inflammatory environment in response to autoantigens. Overall, the genes altered in SLE are complicated and disease is rarely the result of a single polymorphism, however those that have been identified point to the uptake of and signaling pathways for nucleic acid self-ligands by immune cells as a major focus of research.

It is widely accepted that SLE has a strong genetic component to pathogenicity, but it is clear that there are environmental triggers that contribute as well. For example, UV exposure has been implicated in development of disease and induction of flares (Menke, Hsu et al. 2008) as well as various hormonal factors. Microbial colonization and infection have also been shown to be involved due to the contribution of both pathogens and commensals. Epstein-Barr virus (EBV) infection can lead to cross-reactivity with self-ligands, leading to cytokine production and autoantibody formation (Pisetsky 2023). Furthermore, certain commensal bacteria present normally in the microbiome, such as *Propionibacterium propionicum*, have been shown to shape the T cell response in SLE patients through cross-reactivity with Ro60 autoantigens, suggesting these microbes as drivers of lupus pathogenesis (Greiling, Dehner et al. 2018).

In addition to genetic and environmental factors that contribute to SLE development, there is a strong sex bias skewed towards women. The vast majority of SLE patients are women of child-bearing age, and it is known that people with Klinefelter's disease, who have a third X chromosome, are highly susceptible to SLE. Several of the genetic loci identified as SLE risk variants, such as *TLR7,* are located on the X chromosome (Tsokos 2020). Furthermore, estrogen alters B and T cell activation to promote autoinflammation, indicating hormonal involvement in disease development. Ancestry has also been identified as a determinant of disease outcome, with patients of African ancestry having far worse kidney damage than those of European descent (Tsokos 2020). Further work is necessary to understand the complex genetic, microbial, and environmental risk components. There are many factors at play during the development of lupus and it is most likely that a combination of innate immune intolerance to self-ligands, alongside microbial and environmental insults, drives disease.

1.2 Dysregulation of innate immune receptors leads to autoimmunity.

SLE is characterized by the loss of tolerance to self-nucleic acids (Kirou, Lee et al. 2005) and improper clearance of self-derived autoantigens has been shown to be a strong contributor to SLE disease. These anti-nuclear antibodies, formed in response to DNA, RNA, ribonucleoproteins, histone, or other nuclear antigens (Båve, Magnusson et al. 2003), can come from a variety of sources, and are indicative of a failure to tolerate otherwise harmless self-derived nuclear material. One such source of autoantigens comes from neutrophils, which in SLE patients produce more neutrophil extracellular traps (NETs) and are less able to clear those NETs (Frangou, Chrysanthopoulou et al. 2019,

Tsokos 2020). Additionally, the loss of receptors involved in phagocytosis of dead and dying cells, like those binding complement protein C1q (Elkon and Santer 2012), leads to a build-up of dead and dying cells and causes SLE in mice. Furthermore, the loss of certain scavenger receptors, like scavenger receptor type F family member 1 (SCARF1), that recognize and engulf apoptotic cells through C1q, lead to lupus-like disease (Ramirez-Ortiz, Pendergraft et al. 2013). The onset of SLE following the loss of phagocytic receptors underscores the importance of dead and dying cells, like those undergoing apoptosis, as a source of self-reactive nuclear material.

At homeostasis, it is estimated that, in the human body, roughly 50 billion cells die by apoptosis and are cleared every day (Raj, Brash et al. 2006), but when that clearance is disrupted autoinflammation occurs. An inability to properly clear apoptotic cells, which should be immunologically silent, seems to be a key driver of autoimmune disease (Fig. 1). Some autoantigens highly implicated in SLE, such as the ribonucleoproteins, Ro and La, preferentially localize to the surface of apoptotic cells (Casciola-Rosen, Anhalt et al. 1994), implicating apoptotic cells as a source of self-reactive nuclear material. Additionally, the systemic exposure of mice to apoptotic cells led to antinuclear autoantibodies and IgG deposition in the kidney glomeruli (Mevorach, Zhou et al. 1998), indicative of lupus-like disease in mice. The relevance of these clearance defects to lupus disease position apoptotic cell receptors and uptake pathways as potential candidates for the regulation of autoimmunity.

One method of internalization of apoptotic cells is through the binding of externalized phosphatidylserine (PS) motifs with the C2 domain of Milk fat globule epidermal growth

factor 8 (MFG-E8). MFG-E8 forms a bridge between the apoptotic cell and the phagocyte (Hanayama, Tanaka et al. 2004), allowing for efficient phagocytosis of dying cells. This phagocytosis requires the integrin αvβ3, which was the first receptor identified for apoptotic cells (Savill, Dransfield et al. 1990). Integrin αvβ3 connects MFG-E8 to the phagocyte through binding to the RGD motifs on its E2 domain, allowing for internalization of the apoptotic cell (Savill, Dransfield et al. 1990). SLE patients have been shown to have higher levels of MFG-E8 in the blood and MFG-E8$^{-/-}$ mice exhibit splenomegaly and lupus-like disease (Kruse, Janko et al. 2010), positioning apoptotic receptors as key regulators of autoimmune disease. Additionally, SLE patients exhibit higher numbers of circulating apoptotic cells (Ramirez-Ortiz, Pendergraft et al. 2013), further suggesting the contribution of impaired clearance to the development of lupus.

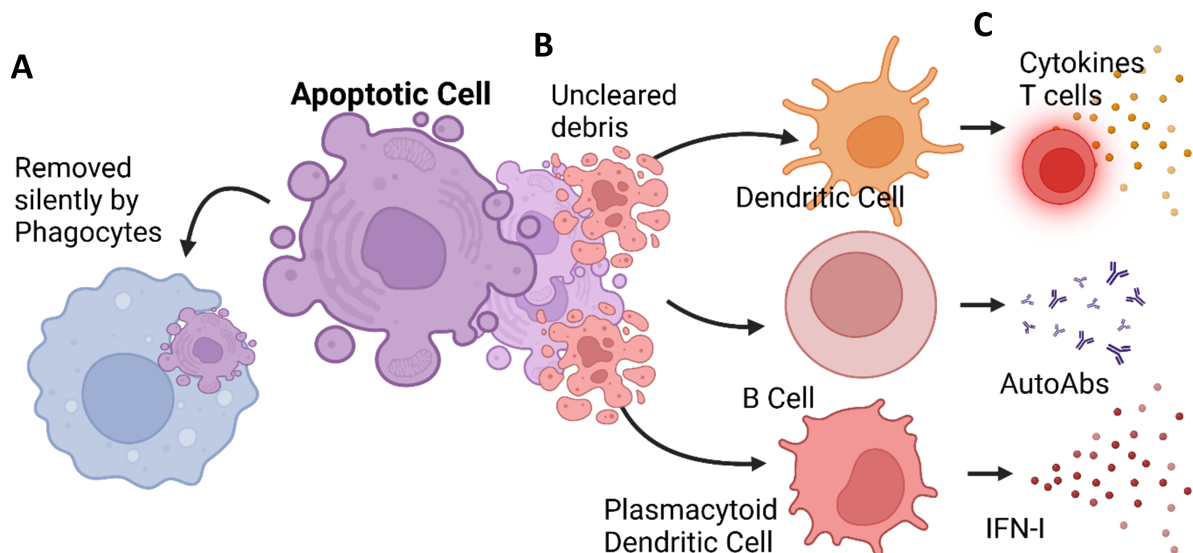

Figure 1: Response of immune cells to build-up of self-ligand: **(A)** At homeostasis, apoptotic cells are removed silently by phagocytosis**. (B)** When apoptotic cells build up due to a loss of clearance receptors or an influx of ligand, the uncleared debris promotes immune responses from **(C)** conventional dendritic cells (cytokine production, T cell activation), B cells (autoantibody production), and plasmacytoid dendritic cells (IFN-I production).

This accumulation of self-ligand in SLE, due to improper clearance of dead cells, influences the immune system in many ways.

Self-ligands released from apoptotic cells, but not phagocytosed, can affect both the innate and adaptive immune response to lead to autoimmune disease. The failure to clear dying cells can lead to a build-up of secondary necrotic cells, causing inflammation to induce conventional dendritic cells to become activated and begin aberrant and inflammatory cytokine production. These mature dendritic cells can then present antigens to self-reactive T cells, leading to a loss of T cell tolerance and induction of an autoimmune response (Mahajan, Herrmann et al. 2016), rather than "silently" removing the self-antigens as should happen at homeostasis (Fig. 1). Furthermore, defects in clearance of dying cells lead to the accumulation of apoptotic cells in the germinal center, causing increased B cell activation and the survival of autoreactive B cells (Baumann, Kolowos et al. 2002). Therefore, both a build-up of self-material and a loss of tolerogenic signals from phagocytic receptors contribute to the unabated inflammation seen with SLE (Fig. 1).

Unlike macrophages and conventional dendritic cells, very little is known about how plasmacytoid dendritic cells (pDCs) interact with apoptotic cells. One group had previously shown that pDCs respond directly to apoptotic cells in conjunction with IgG from SLE patients to produce more IFNα (Båve, Magnusson et al. 2003). In addition, a more recent study found that in response to apoptotic cells alone, pDCs produce tolerogenic cytokines like IL-6 and IL-10, and further activate T cells to make IL-10, in order to suppress inflammation. When apoptotic cells are combined with the TLR9

ligand, CpG-A, however, pDCs produce significantly increased IFNα (Simpson, Miles et al. 2016), suggesting that the pDC response to apoptotic cells depends on the inflammatory context. These studies suggest that apoptotic cells represent one source of self-antigens that can stimulate pDCs to produce IFN-I which drives SLE (Fig. 1).

In response to these autoantigens, often in the form of immune complexes (ICs), pDCs produce massive amounts of type I interferon (IFN-I), leading to further immune dysregulation. It is well-known that active SLE is associated with elevated levels of type I Interferons (IFN-I) in the serum and expression of IFN-stimulated genes (ISGs) (Baechler, Batliwalla et al. 2003, Bennett, Palucka et al. 2003), which correlate with titers of anti-DNA antibodies and disease severity (Bennett, Palucka et al. 2003, Kirou, Lee et al. 2005, Weckerle, Franek et al. 2011). An increase in the serum concentration of one type I IFN, IFNα, was one of the first cytokine abnormalities identified in SLE patients (Båve, Magnusson et al. 2003). Although all cells have the capacity to produce IFN-I, plasmacytoid DCs (pDCs) are highly specialized to rapidly produce large amounts of IFN-I in response to viral infection, and despite making up only 0.05% of peripheral blood mononuclear cells, they produce more than 95% of the IFN-I circulating in the serum (Lombardi, Khaiboullina et al. 2015), estimated to be 1000 times more than any other cell type. There is mounting evidence that the aberrant IFN-I produced by pDCs in response to autoantigens is highly relevant to the development of SLE and the downstream clinical manifestations; however, many questions about pDC development and origin remain, the answers to which could help determine how to prevent and treat SLE.

1.3 Plasmacytoid dendritic cells bridge innate and adaptive immunity.

Traditionally, immunity is thought to be divided into two classifications: innate immunity, which is made up of myeloid cells such as monocytes or macrophages capable of responding directly to pathogens and the damage that they cause, and adaptive immunity comprised of lymphocytes capable of memory responses to pathogens experienced in the past. Largely distinguished by their massive IFN-I production, but with a morphology more closely resembling a lymphocyte, pDCs occupy a unique position as a bridge between innate and adaptive immunity. In fact, despite more than a half-century of study, it is still debated whether these cells are developmentally lymphoid or myeloid in origin. Because of their plasticity, the ability to secrete cytokines as well as present antigen, they have at various times been called T cells, B cells or monocytes, and are now, likely a misnomer, referred to as dendritic cells.

When pDCs were first discovered in the 1950's, as clusters of cells localized to T cell-rich areas of lymph nodes, they were called "lymphoblasts" due to their lymphoid-like phenotype, as well as their huge secretory behavior, similar to that of a B cell (Jegalian, Facchetti et al. 2009). However, by the 1990's it was discovered that in culture when stimulated, they grew long processes, much like those found on dendritic cells. It was discovered that in this "dendritic" state they lost their cytokine-producing capacity and upregulated MHC-II, allowing for activation of T cells. There was then a distinction between so-called "Interferon-producing cells (IPCs)" and "plasmacytoid dendritic cells" (Jegalian, Facchetti et al. 2009). Now, both states are recognized as the same cell type, plasmacytoid dendritic cells (pDCs); however, there is still debate whether pDCs are

lymphoid or myeloid cells, with one group reporting that pDCs are descended from lymphoid progenitors (Dress, Dutertre et al. 2019), and another asserting that they have a shared origin with DC1s (Feng, Pucella et al. 2022). More recently there has even been a push to reclassify pDCs as innate lymphocytes and revert to the term "interferon-producing cells" once more (Ziegler-Heitbrock, Ohteki et al. 2023). These disputes are indicative of the multi-faceted nature of pDCs and underscore the need for further study into their development and origin.

1.4 pDCs are key drivers in the onset and development of lupus.

Although largely appreciated for their anti-viral capacity, pDCs are increasingly implicated as critical contributors to the pathology of SLE. In mouse models, depletion of pDCs in SLE-prone BXSB.DTR mice led to reduced activation and autoantibody production by B cells, as well as less glomerulonephritis when pDCs are depleted early and transiently in disease (Rowland, Riggs et al. 2014), suggesting that pDCs are critical during the onset of SLE. A similar finding was reported by the Reizis group when they reduced gene dosage of *Tcf4*, specifically impairing pDCs, and observed a reduction in many symptoms of SLE-like disease in the mice, as well as a reduction in germinal center formation (Sisirak, Ganguly et al. 2014). Loss of pDC IFN-I production in lupus-prone mice (Baccala, Gonzalez-Quintial et al. 2013) reduced autoantibody production and splenomegaly, further supporting the role of IFN-I in the development of SLE in a pDC-specific manner. Finally, pDCs activated by self-derived nucleic acids and auto-antigen immune complexes promote the expansion of autoreactive B cells (Soni, Perez et al. 2020), suggesting a direct interaction between pDCs and autoantibody-producing B cells.

The relationship between pDCs and SLE is less clearly established in humans. Although there are reports that numbers of pDCs are increased in the blood in SLE patients (Jin, Kavikondala et al. 2008), the majority of studies find that pDCs are reduced in blood but increased in target organs such as kidneys and skin (Blomberg, Eloranta et al. 2001, Farkas, Beiske et al. 2001, Migita, Miyashita et al. 2005, Fiore, Castellano et al. 2008, Tucci, Quatraro et al. 2008), suggesting that pDCs are recruited to sites of inflammation. pDCs also show phenotypic and functional changes in SLE patients which have been shown to contribute to disease. SLE patients exhibit changes in IFNα production in response to TLR9 ligands (Kwok, Lee et al. 2008), suggesting ongoing IFN-I signaling during disease. SLE pDCs have also been reported to have increased expression of certain activation and migration markers, such as CCL19 (Gerl, Lischka et al. 2010). Additionally, pDCs from SLE patients increase T cell activation and proliferation and fail to induce immunosuppression by regulatory T cells (Tregs) as is seen with healthy pDCs (Jin, Kavikondala et al. 2010). Furthermore, a recent clinical trial determined that a humanized monoclonal antibody against pDC-specific BDCA2 reduced joint swelling and tenderness in SLE patients compared to placebo (Furie, van Vollenhoven et al. 2022).

Clinical trials have been conducted targeting many points of the IFN-I signaling pathway, with some success. One drug, Sifalimumab, a fully human monoclonal antibody against IFNα had moderate clinical activity and good tolerability (Thanou and Merrill 2014), while another antibody against IFNα, Rontalizumab, lead to an improvement in symptoms and flare rates of SLE patients (Thanou and Merrill 2014). Studies have also been conducted attempting to lower the IFNα signature of SLE patients with a humanized monoclonal IgG4 antibody against IFNα with promising results (Thanou and Merrill

2014). Recently, a monoclonal antibody blocking all IFN-I was shown to improve many aspects of clinical disease in SLE patients, including the severity of skin disease (Morand and Furie 2020). However, the diverse manifestations of this disease, with some patients having a very high IFNα signature and some low, have conflated clinical trials and further study is required to determine a treatment appropriate for both groups. Further complicating clinical trials are the many medications required to manage symptoms of SLE. Treating SLE patients with IFN-I intervention seems promising, despite the complications from conventional agents. However, this intervention requires an understanding of how IFN-I affects other arms of the immune response.

1.5 Type I interferon production by pDCs impacts B cell development.

IFN-I is a potent activator of innate and adaptive immune responses and is required for autoimmune disease in mouse models of SLE (Santiago-Raber, Baccala et al. 2003, Agrawal, Jacob et al. 2009). In SLE, B cells are major targets of IFN-I, and high levels of IFNα have been correlated to increased SLE-associated autoantibodies (Weckerle, Franek et al. 2011). IFN-I promotes B cell activation and proliferation by inducing higher sensitivity to BCR stimulation (Braun, Caramalho et al. 2002), as well as by increasing Ig secretion. IFN-I stimulation also promotes autoreactive germinal center (GC) formation and the appearance of IgG antibody forming cells (AFCs) in the spleen (Liu, Bethunaickan et al. 2011, Mathian, Gallegos et al. 2011). Additionally, IFN-I signaling promotes autoreactive AFC and GC B cell responses and the production of pathogenic IgG2a/c antibodies (Domeier, Chodisetti et al. 2018) implicating IFN-I in the initial loss of tolerance to self-antigens.

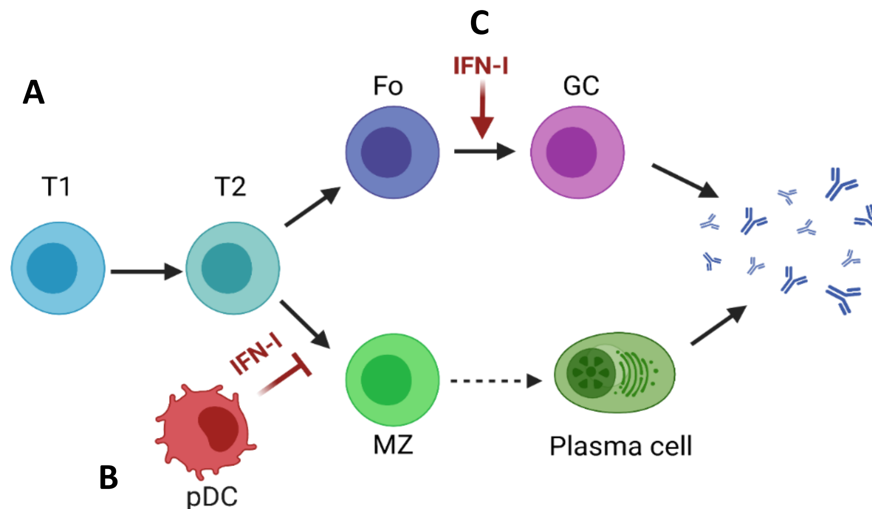

Figure 2: IFN-I from pDCs impacts B cell development and activation: **(A)** B cells move through transitional states (T1, T2) of development until they differentiate into either follicular B cells, to enter the Germinal Center for long-lived antibody production, or are shuttled to the Marginal Zone (MZ) where they may become short-lived plasma cells. **(B)** pDC-derived IFN-I limits MZ B cell development and **(C)** promotes germinal center formation and autoantibody production.

As the primary producers of IFN-I, particularly IFNα, pDCs have been highly implicated in SLE pathology and development, and recently their contribution to B cell activation and autoantibody production has been explored. In response to cytokines IL-6 and IFNα produced by pDCs, B cells have been shown to differentiate and class switch into antibody-producing plasma cells (Jego, Palucka et al. 2003). Additionally, IFN-I from pDCs limits MZ B cell formation (Giltiay, Chappell et al. 2013), suggesting that IFN-I does promote GC formation. Finally, pDC-derived IFN-I promotes anti-DNA reactivity by driving continuous extrafollicular differentiation of ASCs (Soni, Perez et al. 2020), further implicating pDCs in the development of autoimmune disease (Fig. 2). Therefore, IFN-I production and signaling are attractive potential targets for treatment in SLE, but developing effective therapies requires a better understanding of the mechanisms leading to dysregulated IFN-I production and autoimmune responses. The interaction between

pDC cytokine production and the development of autoreactivity requires further study to understand precisely what role IFN-I plays in autoantibody production.

1.6 Toll-like receptor signaling contributes to the development of lupus.

pDCs and B cells sense nucleic acids *via* the endosomal toll-like receptors (TLRs) TLR7 and TLR9, that bind single-stranded RNA and double-stranded DNA, respectively. TLR7, in particular, is heavily implicated in mouse lupus models and in SLE. Deleting TLR7 or the critical TLR-signaling component Myd88 protects mice from lupus-like autoimmunity in multiple models (Christensen, Shupe et al. 2006, Teichmann, Schenten et al. 2013), and overexpression of TLR7 promotes SLE in mice (Pisitkun, Deane et al. 2006, Subramanian, Tus et al. 2006) and humans (García-Ortiz, Velázquez-Cruz et al. 2010). Deletion of TLR9 also reduces development of anti-nucleosome autoantibodies, but causes increased lupus-like disease in some models, suggesting a potential tolerogenic role for TLR9 (Christensen, Shupe et al. 2006, Sharma, Fitzgerald et al. 2015). This has been attributed variously to roles for TLR9 in B cell tolerance, or in competition between endosomal TLRs, leading to hyperactive TLR7 in TLR9-knockout settings. Recent work from the Schlomchik lab determined that in B cells, TLR9 exhibits a complex multi-level "scaffold" protection against lupus through both MyD88-dependent and -independent proinflammatory roles (Leibler, John et al. 2022). However, in a mouse model of SLE driven by DNA autoantigens, both TLR7 and TLR9 contribute to disease, and TLR9 is required for autoantibody production and pathology when TLR7 is deleted (Soni, Perez et al. 2020). Hence, it is likely that both TLR7 and TLR9 contribute

to IFN-I production, and play overlapping and partially redundant roles in promoting SLE-like disease.

1.7 Endosomal trafficking determines the rate and route of TLR signaling.

Downstream of TLRs, signaling pathways can be altered depending on the localization of binding to ligands and by the specific signaling adaptors or co-receptors involved. The cell-surface Toll-like receptor, TLR4, can signal through either a MyD88-dependent pathway involving TIRAP for the activation NF-κB and production of inflammatory cytokines or through a TRIF-dependent pathway involving TRAM for activation of IRF3 and the production of IFN-I (Kagan, Su et al. 2008). Interestingly, what the Medzhitov group discovered was that TLR4 activated these pathways sequentially, in a manner dependent on endosomal location, with the TIRAP-MyD88 pathway occurring at the plasma membrane and TRAM-TRIF signaling coming only once the ligand has been internalized and localized to early endosomes (Kagan, Su et al. 2008). Thus, they conclude that the intracellular location of signaling molecules and ligand determine the signaling outcomes, and that these processes can happen sequentially, not simultaneously from the plasma membrane as it was previously thought.

While conventional dendritic cells express many TLRs (TLR1,2,4,5) which respond to microbial particles or pathogen-associated damage (Takeda, Kaisho et al. 2003), pDCs express only endosomal TLRs, TLR7 and TLR9, which bind to nucleic acids from viruses under normal circumstances, or self-NA in the case of autoimmunity. Endosomal TLRs, such as TLR7/9 on B cells and pDCs, activate IFN-I transcription through a

signaling pathway distinct from that used for production of inflammatory cytokines such as IL-6 and TNFα. Transcription of inflammatory cytokines downstream of endosomal TLRs (Fig. 3) occurs first through recruitment of an adaptor protein, MyD88, which associates with interleukin-1 receptor-associated kinase (IRAK) family members for binding of TNF receptor-associated factor 6 (TRAF6) leading to NF-κB and mitogen-activated protein kinases (MAPK) activation (Uematsu, Sato et al. 2005). Expression of IFN-I genes requires different signaling molecules downstream of MyD88, including IRAK1 (Uematsu, Sato et al. 2005), TRAF3 (Häcker, Redecke et al. 2006, Oganesyan, Saha et al. 2006), IKKα (Hayashi, Taura et al. 2018), dedicator of cytokinesis 2 (DOCK2) (Gotoh, Tanaka et al. 2010), and osteopontin (Shinohara, Lu et al. 2006), leading to the activation of IRF7 (Fig. 3). The rapid production of large amounts of IFN-I by pDCs is thought to be due to their ability to recruit IRF7-activating signaling components to TLR-containing endosomes, which can occur through a number of mechanisms dependent on the size of the TLR ligand and uptake pathway (White, Caswell et al. 2007).

Spatio-temporal regulation of IFN-I production was demonstrated through the use of TLR9 ligands of differing size, yielding distinct signaling outcomes. The TLR9 ligand, CpG DNA, can exist in multiple forms with CpG-A existing as large aggregates, and CpG-B in a monomeric form. Honda et al, showed that while CpG-A elicited a robust IFN-I response from pDCs, CpG-B only led to the production of pro-inflammatory

cytokines (Honda, Ohba et al. 2005). They attributed this to a prolonged association of CpG-A ligand with the MyD88 signaling pathway, leading to sustained activation, whereas CpG-B was transferred rapidly to lysosomal vesicles, where cargo is degraded and signaling is terminated. These studies very elegantly showed that trafficking of cargo in pDCs can determine the signaling outcome.

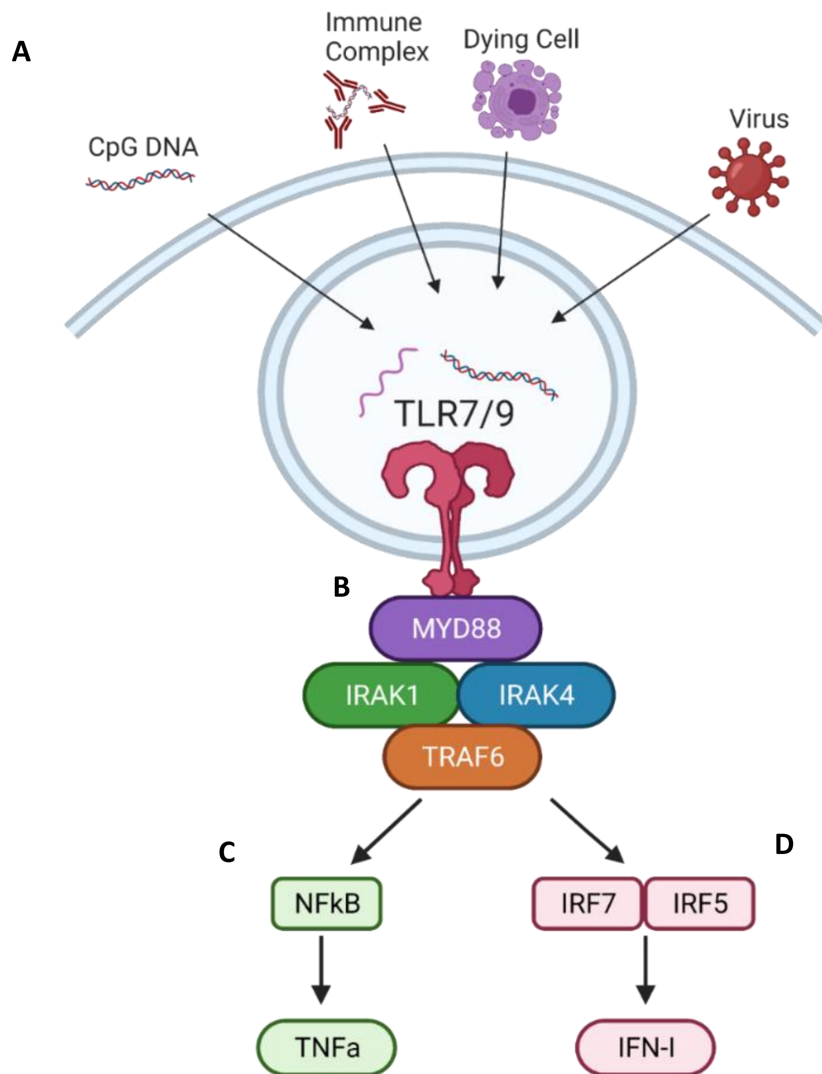

Figure 3: Endosomal TLR signaling pathway in response to various ligands: **(A)** Various ligands are internalized through a variety of receptors into the TLR-containing endosome where they are degraded and sensed by TLR7/9 leading to **(B)** signaling through MyD88 which phosphorylates kinases IRAK1 and IRAK4 leading to TRAF6 recruitment and **(C)** translocation of transcription factors NFkB or IRF7 leading to **(D)** pro-inflammatory cytokines, like TNFα, or to IFN-I production, respectively.

Furthermore, certain members of the non-canonical autophagy pathway can affect localization of TLR ligand and the outcome of signaling. Non-canonical autophagy, or LC3-associated phagocytosis (LAP), is distinct from autophagy by the reliance on Rubicon (RUN domain protein as Beclin-1 interacting and cysteine-rich containing). Rubicon stabilizes the Class III PI3K complex, termed the LAPosome, and induces the assembly of NADPH oxidase 2 (NOX2). This complex is required for generation of ROS for the killing of pathogens and downstream signal transduction (Martinez 2018). Early work in macrophages found that in response to TLR stimulation, the ubiquitin-like protein Microtubule-associated protein 1A/1B-light chain 3 (LC3) was rapidly recruited to the phagosome, but rather than the conventional double-membrane structure of the compartment, a single-membrane phagosome fused with lysosomes (Sanjuan, Dillon et al. 2007), leading to degradation of cargo and termination of signaling.

Early studies implicated the clathrin adaptor AP-3 in responses of pDCs to the TLR9 ligand CpG-A (Sasai, Linehan et al. 2010), but for other ligands the endosomal trafficking requirements differed. IFN-I production in response to nucleic-acid containing immune complexes requires recruitment of LC3 to phagosomes through a non-canonical autophagy pathway (Henault, Martinez et al. 2012). Further studies identified LC3 as an anchor for the recruitment of IKKα through the LIR-2 and LIR-3 domains, bringing this kinase in proximity to and allowing activation of IRF7 (Hayashi, Taura et al. 2018). IFN-α production by pDCs is increased in SLE patients and associated with localization of TLR7 to late endosomal compartments (Murayama, Furusawa et al. 2017) suggesting that dysregulation of TLR trafficking may contribute to autoimmune disease.

1.8 Integrin αvβ3 regulates Toll-like receptor signaling to prevent lupus.

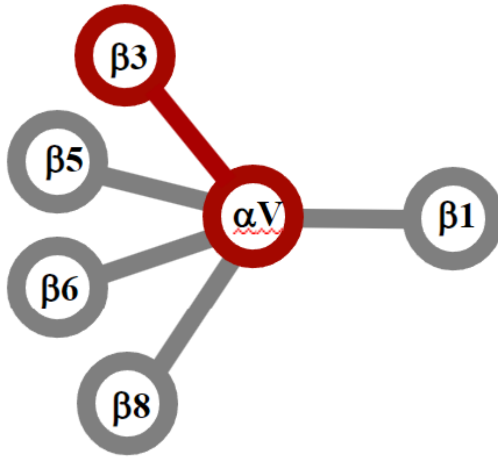

Figure 4: αv integrin binding to five β-subunit binding partners: β1, β3, β5, β6 and β8. αvβ6 and αvβ8 play important roles in maintaining gut homeostasis and αvβ3 on B cells protects against autoimmune disease.

.Cell-surface receptors involved in internalization of TLR ligands can play distinct roles in determining trafficking and signaling outcomes by acting as "co-receptors" that can either bind directly with TLRs, such as CD14 (Lee, Dunzendorfer et al. 2006) or bind elsewhere on a more complicated ligand. Additionally, TLRs, such as TLR2 and TLR6, can cooperate to discriminate between various bacterial components (Takeuchi, Kawai et al. 2001). Recently implicated as tolerance co-receptors, αv integrins are heterodimeric membrane proteins (Fig. 4) that signal both by binding ligand, "inside-out," as well as "outside-in" allowing for cellular migration and spreading, as well as survival and proliferation of all blood cells (Shen, Delaney et al. 2012). Our group has shown that the apoptotic cell receptor integrin αvβ3 regulates TLR7 and TLR9 signaling in B cells by promoting formation and trafficking of IRF7-competent endosomes (Acharya, Sokolovska et al. 2016). αvβ3 activates LC3 conjugation to TLR-containing endosomes, via a non-canonical autophagy pathway involving the Src-family kinase Syk, reactive oxygen species (ROS) and ATG5. LC3 recruitment, in turn, allows activation of IRF7

downstream of TLR7 and TLR9, but also promotes the fusion of endosomes with lysosomes, leading to termination of TLR signaling. Deletion of αv or β3 integrin from B cells delayed LC3 recruitment to TLR-containing endosomes, leading to increased and prolonged TLR signaling (Fig. 5). *In vivo*, B cell-specific deletion of αv integrins caused increased autoantibody production and autoimmunity in a TLR7-driven model of SLE, suggesting that this mechanism restricts responses to self-derived nucleic acid TLR ligands (Acharya, Raso et al. 2020). Because of the contribution of pDCs to the dysregulated cytokine production seen in SLE, we speculated that this increased

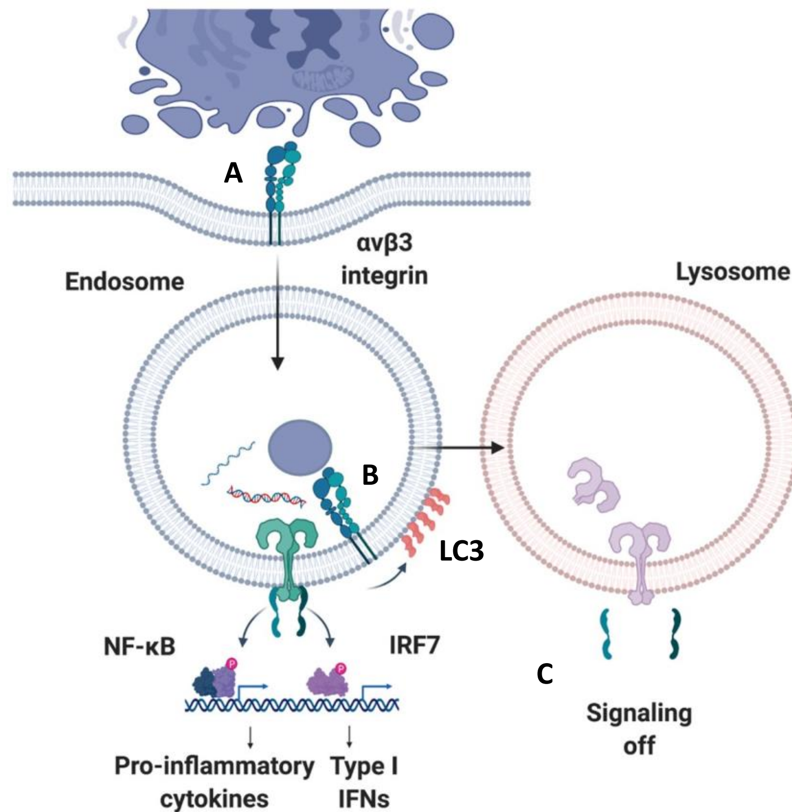

Figure 5: Model of αvβ3 regulation of B cell signaling: **(A)** Apoptotic ligands are internalized, along with αvβ3 integrin, at the cell surface into the TLR containing endosome where ligand is presented to TLR7/9. **(B)** The presence of αvβ3 allows for recruitment of LC3 to the endosome, leading to **(C)** maturation of the endosome, degradation of cargo, and termination of signaling.

inflammation may be sufficient to cause disease in the absence of integrin αvβ3 (Fig. 6), which we posit acts as a tolerance co-receptor in response to self-ligand.

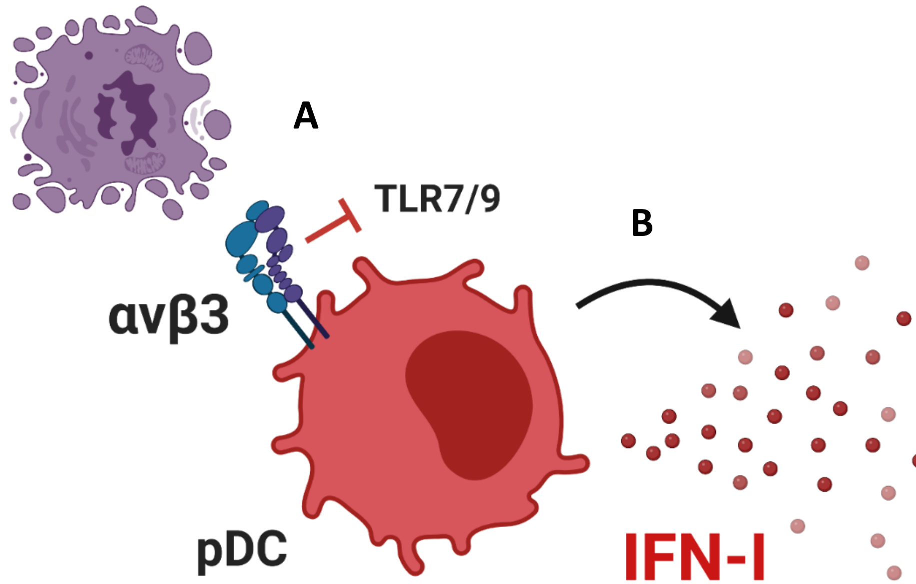

Figure 6: Hypothesis of Integrin αvβ3 regulation of IFN-I production in pDCs: **(A)** In response to self-ligand, αvβ3 integrin limits TLR7/9 signaling to **(B)** reduce IFN-I production by pDCs, preventing the development of autoimmune disease.

1.9 Rationale

Here, I used αv and β3-condtional knockout mouse models to examine the role of αvβ3 in TLR responses in pDCs. I found that in response to *in vitro* TLR stimulation, αvβ3-knockout pDCs produce more IFN-I and proinflammatory cytokines. *In vivo*, mice lacking αvβ3 on pDCs have increased serum IFN-I after stimulation with TLR7 and TLR9 ligands. I further show that increased production of IFN-I by pDCs directly impacts B cell responses, leading to increased marginal zone (MZ) B cell activation and germinal center (GC) formation. Finally, I show that deletion of αvβ3 from pDCs results in increased autoantibody production and autoimmune pathology in a mouse model of TLR7-driven autoimmunity. Therefore, in this study, I show that αvβ3 limits TLR responses in pDCs, and this restrains the development of autoimmunity. Furthermore, these data provide clear evidence that dysregulated TLR signaling in pDCs promotes autoimmune B cell expansion and autoimmune disease.

1.10 Significance

There is increasing evidence that increased IFN-I production by plasmacytoid DCs contributes to the development of systemic autoimmunity, but the mechanisms by which this is regulated remain largely unknown. Here I identify Integrin αvβ3 as a cell-intrinsic regulator of TLR signaling in pDCs, and show that loss of αvβ3 in pDCs results in increased IFN-I production and exacerbates B cell activation and development of lupus-like autoimmunity in a mouse model. These findings identify a mechanism of pDC regulation and demonstrate that increased pDC IFN-I production is sufficient to promote autoantibody production and autoimmune disease.

Chapter 2: Methodology

2.1 Mice

αv-flox, αv-tie2, αv-CD11c and β3-ko mice were all used on C57Bl/6 background and have been previously described (Hodivala-Dilke, McHugh et al. 1999, Lacy-Hulbert, Smith et al. 2007, Acharya, Mukhopadhyay et al.). β5-ko mice were on a mixed C57Bl6/129 background (Huang, Griffiths et al. 2000). Mice with β3 integrin floxed mice (Morgan, Schneider et al. 2010) were obtained from Jackson Labs (Strain 028232) and crossed to CD11c-Cre BAC-transgenic mice (Caton, Smith-Raska et al. 2007). BDCA2-DTR mice and B6.SJL mice were acquired from Jackson Labs (Strains 014176, 002014 respectively). Littermate mice were used as controls. All animal experiments were performed under appropriate licenses and institutional review within local and national guidelines for animal care.

2.2 pDC BMDC Culture

Bone marrow (BM) cells were cultured in pDC media (RPMI 1640,10% FCS, penicillin/streptomycin, L-glutamate, nonessential amino acids, HEPES, sodium pyruvate, and 2-ME) containing 200 ng/ml Flt3L (Peprotech) for 6 days and purified by sorting as $B220^{+}PDCA1^{+}$ cells by FACS or sorted with EasySep™ Mouse Plasmacytoid DC Isolation Kit (Stemcell Technologies- cat. 19764). 20-50 x 10^3 purified pDCs per well were plated in 96-well plates in pDC media with 200 ng/ml Flt3L. Cells were rested for 2 hours and then stimulated for 6-24 hours with CpG-A ODN1585 (2 μM) (Invivogen).

Cytokines were measured using LEGENDplex Mouse Anti-Virus Response Panel (Biolegend, cat. 740621).

2.3 CpG-ODN Uptake and Acidification

Spleens were harvested in RPMI, manually disrupted, and digested for 25 minutes at 37° C with Collagenase, Type IV (Worthington) in HEPES (Thermo Fisher) and 10% FBS, followed by 5 minutes with Cell Dissociation Buffer (Invitrogen) in a shaker-incubator. Dendritic cells were sorted with CD11c Positive Selection kit (Stemcell technologies cat. 18780), plated at $2x10^5$ cells per well in media containing Flt3L, then stimulated with fluorescently labeled ODN1585 with A647 or FITC (6FAM) attached to the 5' end for various durations of stimulation. Cells were then washed 2x with ice cold PBS and stained for FACS.

2.4 Flow Cytometry

Spleens were digested as above, splenocytes depleted of red blood cells (RBC lysis buffer, Sigma-Aldrich), and single cell suspensions were blocked with Fc Block (BD Biosciences) and stained with fluorochrome tagged antibodies for surface markers (1: 200 dilution) at 4 °C for 30 minutes. Samples were acquired using LSRII or FACSymphony (BD Biosciences) and analyzed by FlowJo software (Tree Star Inc.).

2.5 Antibodies and Reagents

Biotin hamster anti-mouse CD61 (2C9.G2) and Biotin rat anti-mouse CD51 (RMV-7) were acquired from BD Biosciences. Anti-mouse B220-Af594 and B220-PerCP/Cy5.5 (RA3-6B2), anti-mouse SiglecH-PE (551), anti-mouse CD8α-BV711 (53-6.7), anti-mouse CD86-PE-Cy7 (GL-1), anti-mouse CD45.1-APC (A20), anti-mouse CD45.2-A700 (104), anti-mouse CD23-PE (EBVCS-5), anti-mouse CD24-FITC (M1/69), and anti-mouse PD-1-PE-Cy7 (RMP1-30) were obtained from Biolegend. Anti-mouse I-A/I-E-BV650 (M5/114.15.2), anti-mouse CD11c-BV605 (HL3), anti-mouse CD19-BV650 (1D3), anti-mouse CD21/CD35-BV510 (7G6), anti-mouse CD95-PE (JO2), and anti-mouse CXCR5-Biotin (2G8) were obtained from BD Biosciences. Anti-mouse PDCA1-PerCp-eFluor 710 (eBio927) was obtained from Thermo Fisher, anti-mouse PNA-Fluorescein (FL-1071) came from Vector Laboratories, and anti-mouse IgG2c-FITC (1078-02) came from Southern Biotech.

2.6 *In vivo* TLR stimulation

For short term TLR responses, mice were injected with 100mg R848 or 10mg CpG-A ODN1585 complexed with the lipid DOTAP (20mg, Avanti) intravenously and bled submandibularly at given timepoints. Cytokines were measured in serum using Legendplex assay. For chronic TLR7 stimulation, β3-CD11c mice were treated epicutaneously with 5% Imiquimod cream on the ear (3mg/ear) three times a week for up to 12 weeks.

2.7 Quantitative RT-PCR

Blood RNA was extracted using RNeasy Protect Animal Tubes (Qiagen) and converted to cDNA with High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher). Taqman primers *Ifit3* (Mm01704846_s1) and *Mx1* (Mm00487796_m1) were used to measure gene expression with TaqMan Fast Advanced Master Mix (cat. 4444556)(all ThermoFisher). Samples were run on QuantStudio5 and analyzed with QuantStudio software.

2.8 Bone Marrow Chimeras

Irradiated (600rad x2) B6.SJL recipient mice were injected intravenously with a 1:1 mixture of BM cells from αv-tie2, αv-CD11c or β3-ko mice (all CD45.2/2) with BM from BDCA2-DTR mice (CD45.1/2) (5 x 10^6 BM cells in total). For control 'reverse' chimeras, irradiated mice were reconstituted with a 1:1 mixture of BM from WT mice and either αv-tie2.BDCA2-DTR or β3-ko.BDCA2-DTR mice. After 6 weeks for reconstitution, mice were then depleted of pDCs by intraperitoneal injection of Diphtheria toxin (DT- List Biological Laboratories) every 48 hours for up to 2 weeks.

2.9 Autoantibody ELISA

Immulon 2HB microtitre plates (DYNEX) were coated and dried overnight at 37 °C with ssDNA (Sigma D8273), calf thymus DNA (Sigma D3664) or RNP-Sm (Arotec). After blocking for 2 hours at 37 °C (2% BSA, 2% fetal calf serum, 0.1% Tween-20 and 0.02% sodium azide in PBS) sera diluted 1:25 in diluent buffer (1:2 dilution of block) were diluted 1:4 four times and incubated overnight at 4ºC. Wells were then washed and

stained with alkaline phosphatase-conjugated goat anti-mouse IgG (Southern Biotech) in blocking buffer for 60 minutes at 37 °C. Secondary antibodies were detected by using disodium p-nitrophenyl phosphate substrate (Sigma-Aldrich) and absorbance (OD) read at 405 nm. Serum autoantibodies were calculated as relative titers using serum from autoimmune mice (TLR7.1 transgenic mice, as described (Acharya, Raso et al. 2020)).

2.10 Immunofluorescence staining of kidney sections

Cryosections of OCT embedded kidneys were incubated in cold acetone for 5 minutes and air dried. Slides were then blocked in PBS with 5% BSA, 0.05% Tween, 0.02% azide for 10 minutes, and stained with antibodies against IgG2c-FITC (Southern Biotech cat. 1079-02) at 1:100 dilution or C3-FITC (MP Biomedicals, cat. 855500) at 1:200 for 30 minutes. Slides were washed in PBS, stained with DAPI to visualize nuclei, mounted and visualized on Echo Revolve microscope. Glomeruli were identified based on nuclear stains, and green fluorescence measured (ImageJ Software).

2.11 Clustering Experiments

pDCs were grown in culture as previously stated and stimulated with ODN1585 for two hours. Cells were then counted by light microscope, with a cluster defined as three or more cells in direct contact with each other. The cells were treated with LFA-1 inhibitor (RWJ50271, catalog number: 4227) for 30 min prior to stimulation. For the ICAM-1 experiments, plates were coated overnight with soluble ICAM-1 (product number: 796-IC) prior to stimulation with ODN1585.

Chapter 3: Integrin αvβ3 limits pDC activation and cytokine production

3.1 Introduction:

We have previously shown that αvβ3 integrin plays a crucial role in limiting TLR signaling in B cells by regulating the trafficking of TLR-containing endosomes. Deletion of αvβ3 leads to increased antibody responses to TLR ligands, as well as increased responsiveness to TLR stimulation. In response to TLR ligands, both NF-κB and IRF7 signaling are increased in the absence of αvβ3 on B cells, which translates to increased B cell proliferation and activation. It was determined from this work that signaling occurs sequentially from an early NF-κB-signaling endosome, then transitioning to IRF7 signaling in a late endosomal compartment, eventually transitioning to lysosomes where signaling is terminated.

This regulation occurs through the activation of the LC3-associated phagocytosis pathway for the efficient trafficking and processing of TLR ligands, leading to termination of signaling. In the absence of αvβ3 on B cells, increased TLR signaling, autoantibody production, and SLE disease pathology have been observed, leading to the conclusion that αvβ3 limits autoimmunity by altering the trafficking of signaling components. Due to previous work indicating that endosomal trafficking in pDCs impacts TLR signaling and the expression of the same endosomal receptors in pDCs and B cells, I sought to explore the role of αvβ3 on pDCs in regulating TLR cytokine production and signaling.

Here I examine the role of αvβ3 on pDCs in the regulation of cytokine production in response to TLR stimulation. My findings indicate that in the absence of αvβ3, pDCs produce more inflammatory and IFN-I cytokines *in vitro* and *in vivo*. Additionally, I observed a significant delay in both cytokine production and uptake and acidification of ligand. I conclude that αvβ3 on pDCs is critical for the regulation of TLR cytokines by hastening the degradation of cargo, thereby limiting the duration of signaling.

3.2 pDCs express αvβ3 and produce more TLR cytokines in its absence.

Integrin αv partners with 5 β subunits (β1,3,5,6 & 8) to form heterodimers with overlapping ligand specificities and cellular function. Primary pDCs purified from spleen or grown from bone marrow precursors express transcripts for αv and β3 integrins (*ITGAV* and *ITGB3*), and both could be detected at uniformly high levels by FACs (Fig 7A,B; Supp Fig 1A). pDCs also expressed *ITGB5*, the gene for β5, but the lack of suitable antibodies prevented direct measurement of αvβ5 protein. To assess the relative amounts of surface αvβ3 and αvβ5 on pDCs, I measured αv and β3 in pDCs from integrin knockout mice. As expected, pDCs from mice lacking αv in immune cells (αv tie2) (Lacy-Hulbert, Smith et al. 2007) did not stain for αv or for β3, confirming that β3 is only present as αvβ3 heterodimers. $\beta3^{-/-}$ pDCs also lost almost all surface expression of αv, while $\beta5^{-/-}$ pDCs had no reduction in αv (Fig 7C). I therefore concluded that αvβ3 was the major αv integrin expressed on the surface of pDCs.

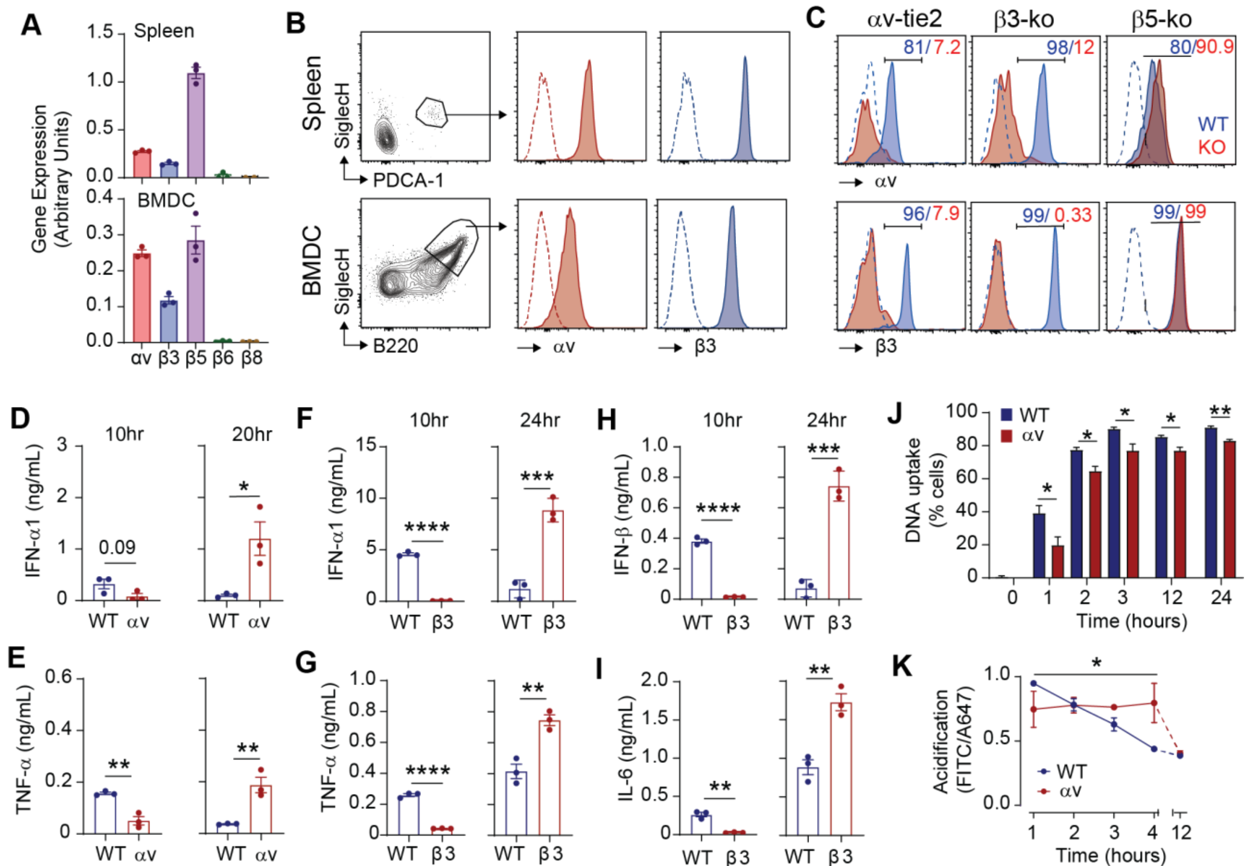

Figure 7: Integrin αvβ3 pDCs limits TLR responses *in vitro:* (A) qPCR of *Itgav, Itgb3, Itgb5, Itgb6* and *Itgb8* (encoding integrins αv, β3, β5, β6, and β8 respectively) from pDCs sorted from the spleen (B220+PDCA1+SiglecH+ cells; see Supp. Fig 1A for full gating strategy) or grown in culture from bone marrow precursors (SiglecH+ B220+ cells; BMDC). **(B)** Surface expression of integrin αv and β3 on spleen pDCs or BMDCs. Histograms show staining for αv and β3 (solid line) and Streptavidin alone control (dashed line). **(C)** Surface expression of αv and β3 integrin on spleen pDCs from αv-tie2 (left), β3-ko (middle), and β5-ko (right) mice. Expression in wild type (WT) cells is in blue and respective knockouts (KO) in red. Dashed line is streptavidin-only control. Numbers show the percentage of cells in indicated positive gates. **(D-I)** Concentration of cytokines measured in cell culture supernatant after CpG stimulation of pDCs from αv-tie2 (D,E) and β3-ko (F-I) mice. Data are mean +/-SD of replicate cultures from one experiment. For all data shown, similar results were seen in at least three independent experiments. p values are as shown or *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001 (Student's T test). **(J)** Uptake of fluorescently-labelled CpG-A by wild type (WT) and αv-tie2 primary spleen pDCs, expressed as percentage of B220+ PDCA1+ pDCs that are positive for CpG-A (Gating shown in Supp Fig 1). Data are mean +/-SD of replicate pDC preparations from 3 experiments. *p<0.05 and **p<0.01 from data analyzed with Student's T test. **(K)** Acidification of CpG-A-containing compartments in pDCs, measured as ratio of fluorescence from FITC and A647 dyes (Supp Fig. 2). Data are mean +/-SD of replicate pDC preparations from 3 experiments. *, *p<0.05* (Two-Way ANOVA).

To determine whether integrin αvβ3 affects cytokine production by pDCs, bone marrow-derived pDCs were stimulated with a TLR9 agonist, CpG-A. αv-ko pDCs initially produced less IFNα than controls at early timepoints (10 hours) after CpG-A treatment, but at 20 hours IFNα production was significantly increased compared with controls (Fig. 7D). TNFα secretion showed a similar pattern (Fig. 7E). These effects were due to loss of αvβ3, as β3-ko BM-derived pDCs also showed delayed but increased IFN-α and TNF-α compared with control pDCs, and this extended to IFN-β and IL-6 (Fig. 7F-I). These data indicate αvβ3 has a cell-intrinsic role in regulating cytokine production in pDCs, and that loss of this integrin resulted in delayed but increased cytokine production.

The delay in TLR signaling in αv and β3-knockout cells led us to speculate that αvβ3 may mediate uptake or intracellular traffic of CpG-DNA. To test this, αvβ3-knockout pDCs were incubated with fluorescently labelled CpG-A, and uptake measured by flow cytometry. Both αv and β3-knockout pDCs showed reduced uptake of CpG-DNA compared with wild-type pDCs, particularly at early time points (1-3 hours) (Fig 7J; Supp Fig 1B-C), indicating that αvβ3 promotes CpG-DNA uptake. I have previously shown that αvβ3 also promotes the intracellular traffic of TLR ligands to acidic lysosomes, limiting TLR signaling. To follow trafficking of internalized CpG-DNA, pDCs were incubated with oligos labeled with both pH-resistant Alexa 647 (A647) and pH-sensitive fluoresceine isothyocynate (FITC), which loses fluorescence at low pH. In WT pDCs, the ratio of FITC to A647 fluorescence progressively dropped with time, indicating trafficking of CpG-DNA to acidic lysosomes. In contrast, FITC/A647 ratio remained stable in αv and β3-knockout pDCs over 4 hours (Fig 7K; Supp Fig 1D-F). These data therefore support a role for αvβ3 in both uptake and intracellular trafficking of nucleic

acid TLR ligands, consistent with the pattern of delayed but increased cytokine production.

3.3 pDC activation is increased in mice lacking αvβ3.

To determine the role of αvβ3 on pDCs *in vivo*, I initially made use of αv-tie2 (Lacy-Hulbert, Smith et al. 2007) and $β3^{-/-}$ (Hodivala-Dilke, McHugh et al. 1999) mice to target αv and β3 respectively. αv-tie2 mice and littermate controls had similar numbers of pDCs in the spleen at 10 weeks of age (Fig. 8A), but αv-tie2 mice had increased proportions of CD8α- pDCs (Fig. 8B). CD8α- pDCs are considered to be more activated than CD8α+ pDCs and are the predominant cytokine producing subset of pDCs (Bar-On, Birnberg et al. 2010, Lombardi, Speak et al. 2012, Zhan, Kong et al. 2020), so I also measured surface expression of MHC-II and CD86, which are additional indicators of pDC activation (Asselin-Paturel, Boonstra et al. 2001). pDCs from αv-tie2 mice had similar expression of MHC-II to control pDCs, but I observed a trend toward increased CD86 for both CD8α+ or CD8α- pDCs from αv-tie2 mice (Fig. 8C). β3-ko mice showed broadly similar differences in pDCs compared with controls to those seen in av-tie2 mice (Fig. 8D-F), with increased expression of MHC-II and CD86, but unlike av-tie2 mice, showed no change in proportion of CD8α+ pDCs (Fig. 8E). I therefore concluded that αvβ3 does not affect pDC generation but may negatively regulate their activation.

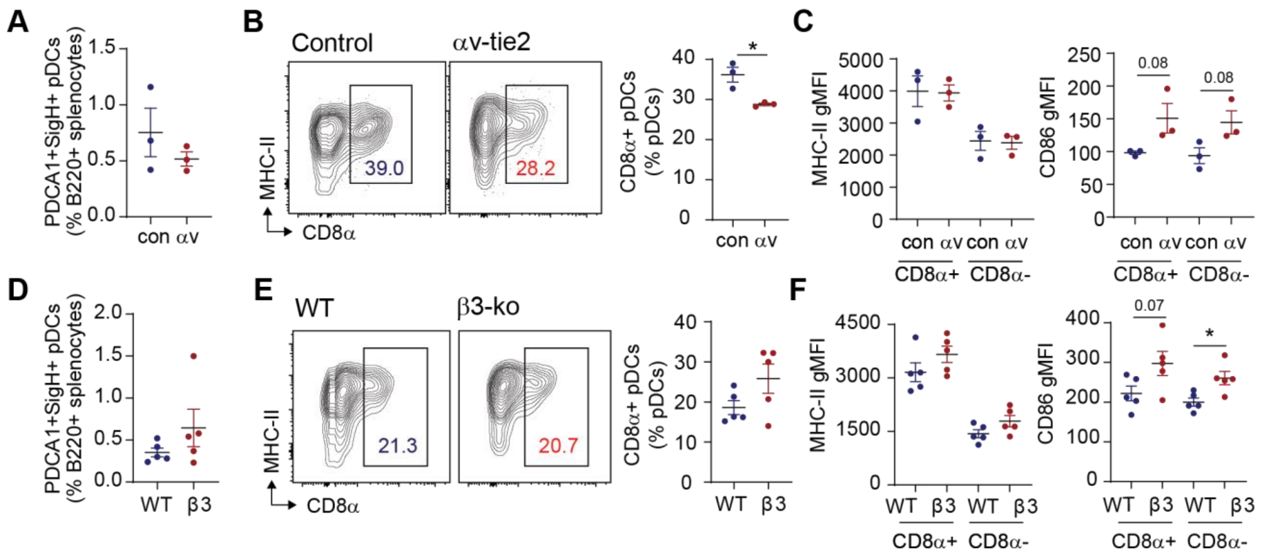

Figure 8: αvβ3 regulates pDC activation *in vivo*: **(A-F)** Analysis of pDCs from spleen of αv-tie2 **(A-C)** and β3-ko **(D-F)** mice and matched controls (con) or wild-type (WT) mice at 10-12 weeks of age. **(A,D)** pDC (PDCA1+SiglecH+) frequency shown relative to all B220+ cells in spleen for αv-tie2 **(A)** and β3-ko **(D)** mice. **(B-E)** Representative FACS plots and combined analysis of CD8α+ pDCs (% of total pDCs) for αv-tie2 **(B)** and β3-ko **(E)** mice. **(C,F)** Levels of surface MHCII and CD86 (gMFI) on CD8α+ and CD8α- spleen pDCs from αv-tie2 **(C)** and β3-ko **(F)** mice. shown as mean +/- SEM**.** Similar results were seen in 3 independent experiments. Each data point represents an individual mouse in a single experiment (n=3 for **A-C**, n=5 for **D-F**). *p*-values are as indicated or *p<0.05. All analyses were done with Student's T test, with values shown as mean +/- SEM**.** Similar results were seen in 3 independent experiments.

3.4 αvβ3 limits TLR cytokine production *in vivo*.

I next asked whether αvβ3 regulates IFN-I production by pDCs *in vivo*. Although I could not detect circulating IFNα or IFNβ in unchallenged $\beta3^{-/-}$ mice, blood immune cells from these mice had increased expression of the Interferon stimulated gene (ISG) *Ifit3* (Fig. 9A), suggestive of increased production of IFN-I. Despite their relatively small numbers, pDCs have been shown to be the predominant initial source of systemic type I IFN in response to TLR9 stimulation (Swiecki, Wang et al. 2011). Using the BDCA2-DTR transgenic mouse strain, which allows selective depletion of pDCs by injection of diphtheria toxin (DT) (Swiecki, Wang et al. 2011), I confirmed that early production of systemic IFNα and IFNβ in response to the TLR7 ligand R848 was likewise dependent

on pDCs, whereas pDCs only contributed partially to TNFα production (Fig. 9B). I next injected αv-tie2 and $\beta3^{-/-}$ mice with R848, and measured production of cytokines in the serum. Both strains showed increased serum IFN-a compared with littermate controls, at 2 and 5 hours after injection (Fig 9C,D). I also measured IFN-b in αv-tie2 mice, which was similarly increased, along with production of TNF-a in both αv-tie2 and $\beta3^{-/-}$ mice (Fig. 9C,D). To test whether these effects extended to TLR9, αv-tie2 mice were injected with CpG-A complexed to the lipid DOTAP. After 3 hours of stimulation, αv-tie2 mice had significantly higher serum levels of IFNβ and TNFα (Fig. 9E) than control mice, although I did not observe a significant change in IFNα levels at this timepoint. To confirm that the increased IFN-I in αvβ3-knockout mice was derived from pDCs, and no other αvβ3-deficient immune cells, I generated αv-tie2: BDCA2-DTR mice. Ablation of pDCs by treatment with DT completely blocked type I IFN production in αv-tie2: BDCA2-DTR mice after treatment with R848 (Fig 9F). Hence, deletion of αv or β3 resulted in increased production of IFN-I by pDCs after *in vivo* stimulation with TLR7 and TLR9 ligands.

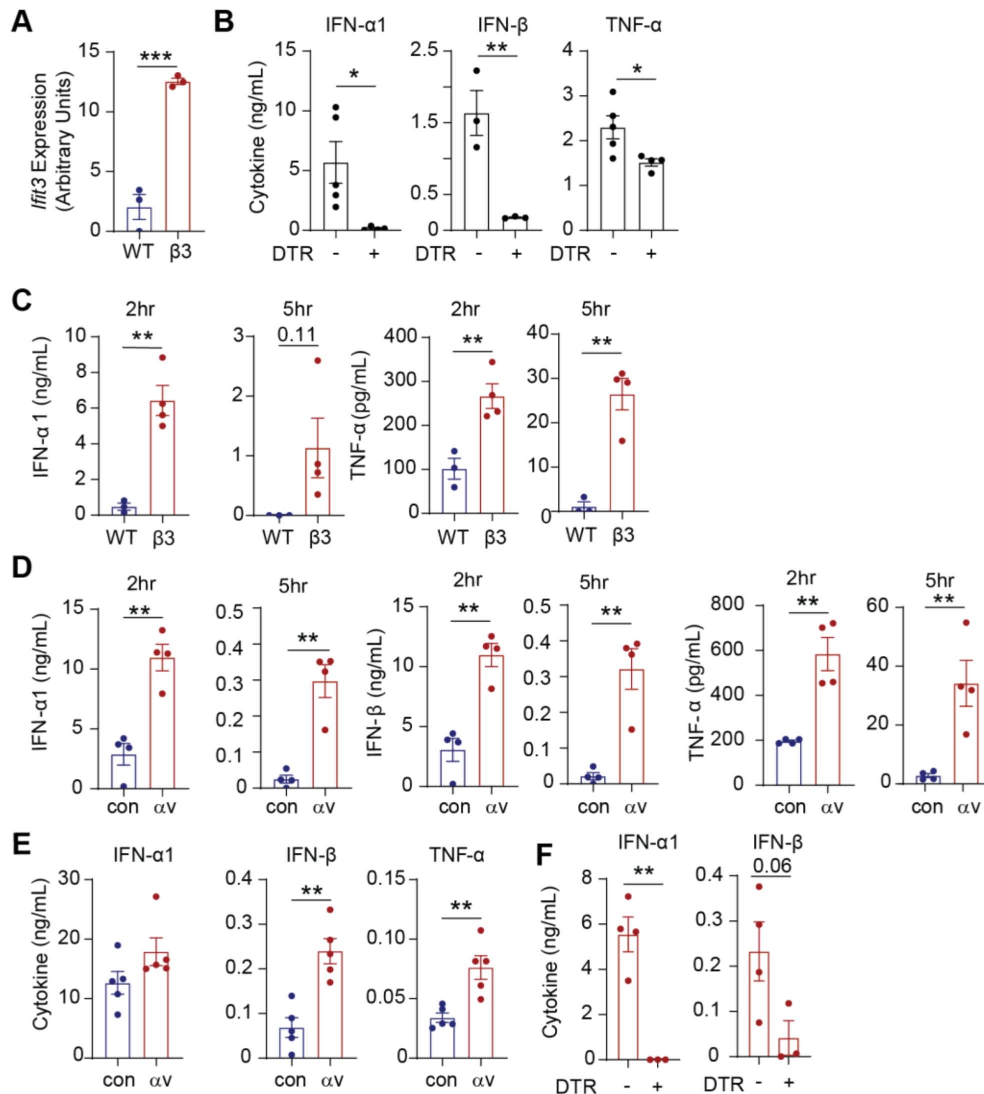

Figure 9: Integrin αvβ3 limits TLR cytokines in vivo: **(A)** *Ifit3* expression measured by qPCR of whole blood RNA in WT and β3-ko mice. **(B)** Serum cytokine concentrations in BDCA2-DTR mice (DTR+) and controls (DTR-) injected with DT to deplete pDCs (24 hours) and then R848 (1 hour). **(C,D)** Serum cytokine concentration 2 or 5 hours post-injection with R848 in β3-ko **(C)** or αv-tie2 **(D)** mice and matching wild type (WT) or control (con) mice. **(E)** Serum cytokine concentration 3 hours post-injection with CpG-A complexed to the lipid DOTAP in αv-tie2 mice. **(F)** Serum cytokine concentrations in αv-tie2 mice with or without BDCA2-DTR allele (DTR+ and DTR- respectively), treated with DT (24 hours) followed by R848 (2 hours). Each data point is an individual mouse (n=3-5), with mean +/- SEM. *p* values are as shown or *$p<0.05$, **$p<0.01$, and ***$p<0.001$ (Student's T test), with values shown as +/- SD. Data are representative of at least three independent experiments.

3.5 TLR effects from loss of αvβ3 are pDC-intrinsic.

Although our data support a role for αvβ3 in regulating pDC responses to TLR stimulation, the αv-tie2 and $\beta3^{-/-}$ mouse strains used thus far delete avb3 in all immune cells, and have additional phenotypes due to loss of other integrin heterodimers which complicate analysis of effects of avb3-knockout pDCs on the immune system. Notably, deletion of αv from DCs results in activation of conventional DCs (cDCs) and loss of peripheral regulatory T cells (Lacy-Hulbert, Smith et al. 2007, Acharya, Mukhopadhyay et al. 2010, Païdassi, Acharya et al. 2011, Boucard-Jourdin, Kugler et al. 2016), due to loss of the αvβ8 heterodimer. $\beta3^{-/-}$ mice, in contrast, have bleeding defects and anemia due to the loss of aIIbβ3 integrin from platelets (Hodivala-Dilke, McHugh et al. 1999). To avoid these phenotypes and investigate the specific role of αvβ3 on pDCs in immune homeostasis, I sought to delete αvβ3 integrins selectively in pDCs. In the absence of pDC-specific CRE lines, I used a bone marrow (BM) chimera approach (Rowland, Riggs et al. 2014), in which 50% of cells in chimeras came from BDCA2-DTR mice and 50% from αv-tie2 or $\beta3^{-/-}$ donors (Fig. 10A). For simplicity, in our initial experiments, I focused on chimeras generated with $\beta3^{-/-}$ bone marrow. Administration of DT ablated all pDCs derived from BDCA2-DTR donor bone marrow, leaving the $\beta3^{-/-}$ pDCs intact. Other immune cells were unaffected, and remained present as a 1:1 mixture of BDCA2-DTR-derived and $\beta3^{-/-}$ cells (Fig. 10B). After DT treatment, β3-DTR chimeras had similar total numbers of pDCs to control DTR-chimeras (Fig. 10C), but a reduced proportion of $\beta3^{-/-}$ pDCs were $CD8a^{+}$ compared with controls (Fig 10D), similar to results in $\beta3^{-/-}$ mice (Fig 8E,F). After treatment with R848, β3-DTR chimeras produced more serum IFN-α

than control chimeras (Fig. 10E). 'Reverse' BM chimeras in which $\beta 3^{-/-}$ pDCs were specifically depleted, did not show significantly different levels of IFN-α production from control chimeras (Fig. 10F,G), confirming that this increased IFN-α was derived from $\beta 3^{-}$

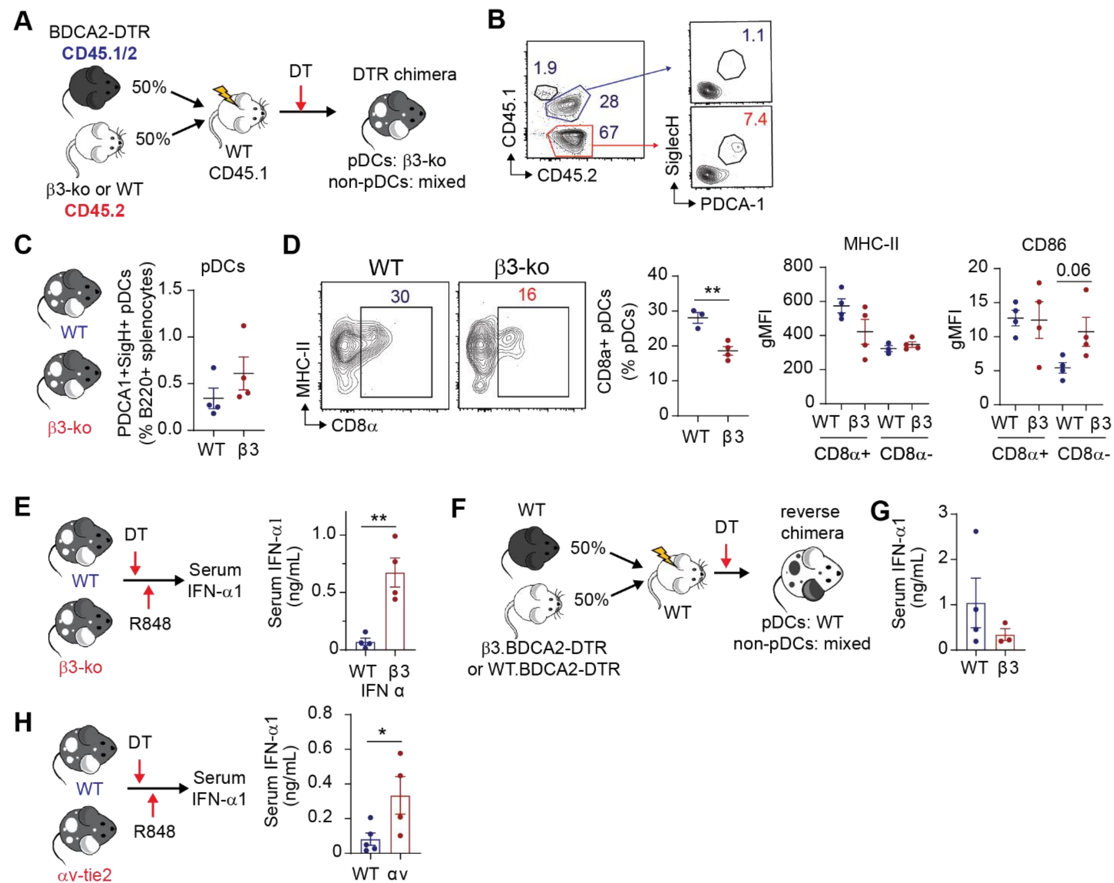

Figure 10: αvβ3 TLR effects are pDC-intrinsic: **(A)** Schematic of bone marrow (BM) chimera strategy for pDC-specific gene deletion. Mixtures of BM from BDCA2-DTR mice and β3-ko mice were injected into irradiated WT mice, followed by DT administration, resulting in mice in which pDCs are selectively deleted for β3. **(B)** Representative FACS indicating that pDCs from 45.1/2 mouse (BDCA2-DTR donor) are ablated, while pDCs from 45.2 mouse (β3 ko or matched WT controls) remain. **(C)** Spleen pDC frequency (SiglecH+ PDCA1+ cells as % of B220+ splenocytes) in WT and b3-ko chimeras**.** **(D)** Representative FACS plots of CD8a and MHC-II expression on spleen pDCs from WT and b3-ko chimeras, and combined analysis of CD8α+ pDCs (% of total pDCs) and expression of surface MHC-II and CD86 for both CD8α+ and CD8α- pDCs**.** **(E)** Serum IFN-a cytokine concentration in β3-ko and WT chimeras 1 hour post-injection with R848**.** **(F)** Schematic of strategy to generate 'reverse' chimeras in which mice have mixed β3-ko and WT immune cells but all pDCs are derived from WT mice. **(G)** Serum IFN-a cytokine concentration in β3-ko and WT reverse chimeras 1 hour post-injection with R848**.** **(H)** Serum IFN-a cytokine concentration in av-tie2 and control chimeras 1 hour post-injection with R848. Each data point represents an individual mouse in a single experiment (n=3-4) with mean +/- SEM. *p*-values are as indicated or * *p*<0.05, ** *p*<0.01 (Student's T test). Similar results were seen in 3 independent experiments.

$^{/-}$ pDCs. I confirmed these results in αv-DTR chimeras, which showed similar increases in serum IFN-α after R848 treatment (Fig 10H). I therefore concluded that both the relative increase in CD8a$^-$ DCs in unstimulated mice, and increased IFN-a production in response to TLR stimulation that I observed in αv-tie2 and β3-ko mice are due to pDC-intrinsic effects of αvβ3 deletion.

3.6 Discussion:

Here I have identified a role for αvβ3 integrin in the regulation of TLR cytokine production by pDCs. In the absence of αvβ3 on pDCs, I observed increased TLR cytokines both *in vitro* and *in vivo*. This delayed but increased production of cytokines corresponds with a delay in uptake of TLR ligand. Additionally, these data indicate decreased acidification of the endosomal compartment, demonstrating a delay in lysosomal fusion and termination of signaling. This reinforces previous data from my group showing similar regulation of TLR signaling by αvβ3 on B cells. In B cells, αvβ3 promotes recruitment of LC3 for the activation of the non-canonical autophagy machinery and hastened degradation of cargo and termination of TLR signaling.

Our data indicate that αvβ3 may regulate TLR signaling through two mechanisms. First, the reduced internalization of CpG oligonucleotides and delayed initial production of cytokines are consistent with a role for αvβ3 in the uptake of nucleic acids to TLR-containing endosomes. The mechanisms of nucleic acid uptake by pDCs are not well established, but I propose that αvβ3 promotes CpG-oligonucleotide uptake either *via* serum opsonins, or in concert with other cell surface receptors, as has been reported for numerous other ligands, including apoptotic cell debris, viral particles and bacterial components. For example, pDCs express several scavenger receptors, such as CD36 (Parcina, Schiller et al. 2009) which can work in concert with αv integrins to bind and internalize apoptotic bodies as well as apoptotic microparticles (Schiller, Parcina et al. 2012, Simpson, Miles et al. 2016, Ainola, Porola et al. 2018).

Second, $\alpha v\beta 3^{-/-}$ pDCs had greatly delayed acidification of TLR ligand-containing vacuoles. This likely reflects retention of CpG oligonucleotides in TLR-containing endosomes instead of fusion with highly acidic lysosomes and corresponds with delayed cytokine production. Delayed lysosomal fusion has been shown to result in increased IFN-I production by pDCs (Honda, Ohba et al. 2005), similar to the increased cytokine production I observe in $\alpha v\beta 3^{-/-}$ pDCs.

I propose that αvβ3 normally regulates TLR signaling in pDCs by promoting LC3 recruitment to TLR endosomes through a non-canonical autophagy pathway (Acharya, Sokolovska et al. 2016, Raso, Sagadiev et al. 2018, Muir, Sagadiev et al. 2022), which has two opposing roles in TLR signaling. First, LC3-conjugation to TLR-containing endosomes promotes IFN-I production (Henault, Martinez et al. 2012) by mediating recruitment of the signaling components IKKα and TRAF3, leading to activation of the transcription factor IRF7 and transcription of IFN-I genes (Hayashi, Taura et al. 2018). However, LC3 also promotes fusion of endosomes with lysosomal membranes, leading to destruction of TLR ligands and termination of signaling. Our group has shown that these processes occur sequentially, with IRF7 activation preceding lysosomal fusion (Acharya, Sokolovska et al. 2016). In B cells, deletion of αvβ3 impaired both processes, resulting in prolonged but delayed activation of IRF7 (Acharya, Sokolovska et al. 2016, Muir, Sagadiev et al. 2022). I see similar increased but delayed IFN-I production in $\alpha v\beta 3^{-/-}$ pDCs, leading us to conclude that αvβ3 and LC3 conjugation likely play similar roles in pDCs.

Chapter 4: αvβ3 on pDCs limits TLR responses to prevent autoreactivity and autoimmunity.

4.1 Introduction:

Both B cells and pDCs play critical roles in the development and progression of autoimmune disease, such as SLE. IFN-I derived from pDCs affects B cell activation, proliferation, autoantibody production, and class switching, and has been shown to affect disease development, especially in its early stages. Even transient ablation of pDCs, early in disease, reduced splenomegaly, decreased activation of lymphocytes, limited anti-nuclear antibodies and improved pathology of the kidneys (Rowland, Riggs et al. 2014), a key organ targeted during SLE disease. Similarly, decreasing the gene dosage of pDC-specific *Tcf4* nearly abolished several clinical manifestations of SLE including autoantibody production and glomerulonephritis (Sisirak, Ganguly et al. 2014), as well as reducing spontaneous germinal center reactions.

Further reports of pDC IFN-I contribution to autoimmunity comes through their interactions with B cells. Previous work had identified IFN-I signaling as a driver of B cell autoreactivity, specifically in the germinal center (Domeier, Chodisetti et al. 2018), and it was further proven to drive autoimmunity through extrafollicular B cell autoreactivity. pDC IFN-I was shown to be required for expansion of activated extrafollicular B cells and increased generation of antibody-forming cells (Soni, Perez et al. 2020) and was necessary for downstream clinical manifestations of SLE in a *Dnase1l3*$^{-\backslash-}$ model of autoimmune disease. IFN-I production from pDCs has been shown

to be a contributor to disease with effects on B cell autoantibody production, but it is less clear if dysregulated pDC activation is sufficient to activate autoreactive B cells.

Due to the effects of αvβ3 on pDC IFN-I production seen both *in vitro* and *in vivo*, I propose that B cell activation and SLE disease development may be increased and worsened, respectively. In the absence of αvβ3 on pDCs, I observe increased B cell activation and germinal center formation, as well as an increase in disease pathology associated with SLE, such as increased autoantibody production and kidney damage. These data indicate that αvβ3 on pDCs promotes tolerance to self-ligands and restricts aberrant autoreactive B cell activation and development of autoimmune disease.

4.2 Deletion of αvβ3 on pDCs promotes B cell activation.

IFN-I produced by pDCs has been shown to activate differentiation and migration of B cells in the marginal zone (MZ), leading to a reduction in MZ B cells and promoting expansion of germinal center (GC) and extrafollicular (EF) plasma cells (Asselin-Paturel, Brizard et al. 2005, Swiecki, Gilfillan et al. 2010, Sisirak, Ganguly et al. 2014, Li, Fu et al. 2015, Soni, Perez et al. 2020). The ability to effectively induce deletion of αvβ3 in pDCs with BDCA2-DTR chimeras allowed us to investigate how dysregulated IFN-I production by pDCs affected B cell activation. For initial experiments, I used both αv-DTR and β3-DTR chimeras interchangeably, and similar effects were seen in both. I induced pDC activation and IFN-I production by injecting mice with R848 twice over a period of two weeks (Fig 11A). R848 stimulation caused an increase in spleen size and in total numbers of spleen B cells in both control and αv-DTR chimeras (Fig 11B), and a

robust IFN-I response as assessed by expression of ISG *Mx1* in circulating immune cells, which was significantly increased in αv-DTR chimeras compared with controls (Fig 11C).

To determine effects of pDC αvβ3 deletion on B cell phenotype, and exclude B cell-intrinsic effects of av deletion (Acharya, Sokolovska et al. 2016, Raso, Sagadiev et al. 2018, Acharya, Raso et al. 2020), I used the congenic marker CD45 to selectively analyze the phenotype of B cells derived from the BDCA2-DTR transgenic bone marrow (Supp Fig 2A-B). I initially focused on transitional (T1 and T2), marginal zone (MZ) and follicular (Fo) B cells, based on expression of CD21, CD23 and CD24. In the absence of exogenous TLR stimulation, I observed no differences in total numbers of B cells, or in relative frequencies of T1, T2, MZ or Fo B cells between αv-DTR and WT-DTR chimeras (Fig 11D; Supp Fig 2C,D). As well as increasing total B cell numbers, R848 treatment caused changes in the proportions of B cell subsets. The frequency of MZ B cells was reduced, while T2 and Fo B cells were expanded relative to other B cell subsets (Fig 11D; Supp Fig 2C,D), consistent with previous studies showing that TLR and IFN-I stimulation cause activation and subsequent differentiation of MZ B cells (Giltiay, Chappell et al. 2013). αv-DTR chimeras showed a bigger drop in the frequency of MZ B cells after R848 treatment than control chimeras, and a smaller increase in total numbers, which was accompanied by an increase in the relative frequency of Fo B cells (Fig 11D; Supp Fig 2C,D). I also noted increased proportions of CD11c+ transitional B cells in αv-DTR chimeras (Supp Fig 2E), indicating that these populations may also be activated more strongly by dysregulated pDCs. Similar changes in MZ and CD11c+ transitional B cells were seen in both αv- and β3-DTR chimeras; to streamline experiments, further

studies were performed in β3-DTR chimeras only. IFN-I signaling in B cells promotes GC development, and I found that R848 treatment caused an increase in GC B cell frequency and total numbers in β3-DTR chimeras compared with control chimeras (Fig 11E).

Although our data are consistent with effects of αvb3-deletion in pDCs, it remained possible that loss of αvb3 from other immune cell types present in the chimeras were responsible for the effects on B cells. To completely exclude that possibility, I again used the 'reverse' bone marrow chimera approach described in Fig. 10, generating mice in which all pDCs are derived from WT bone marrow, but other cell types are derived from both WT and β3-ko bone marrow (Fig. 11F). In these chimeras, I observed no difference in frequencies of T2/MZ (Fig. 11G, Supp Fig 2I-J), or GC B cells (Fig. 11H) between β3-DTR or WT chimeras after R848 treatment. This result confirmed that the effects on B cell activation I observed were due solely to loss of avb3 from pDCs, and together, these data support a model in which dysregulated IFN-I production by αvβ3-knockout pDCs results in increased B cell activation and GC development.

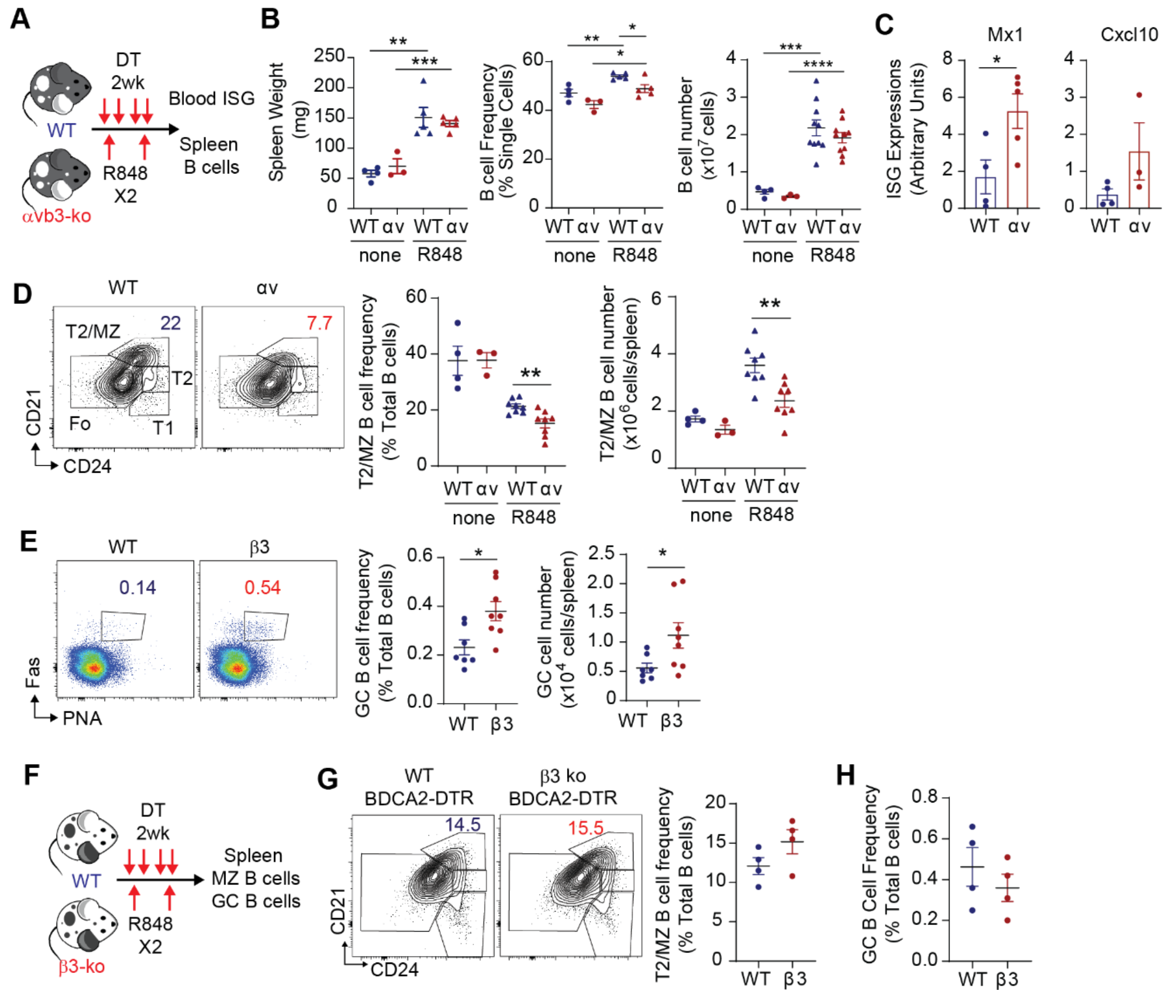

Figure 11: αvβ3 on pDCs impacts B cell activation: **(A)** Schematic of experimental design. av-tie2 and WT DTR chimeras were treated repeatedly with DTR to deplete pDCs and given two doses of R848 over three weeks. **(B)** Spleen weight (left), B cell (B220+ CD19+ cells) frequency (middle) and B cell number in spleens of WT and av-tie2 DTR chimeras treated with DT and R848 (R848) or DT with no R848 (none). **(C)** ISG expression measured by qPCR from whole blood RNA extracted from R848-treated αv-tie2 DTR and WT DTR chimeras. **(D)** Representative FACs analysis of T2/MZ B cells in αv-tie2 and WT DTR chimeras treated with DT and R848 (gated on CD45.1/ CD45.2+ CD19+ cells). Plots show relative proportions and total number of T2/ MZ B cells in αv-tie2 and WT DTR chimeras, treated with DT and R848 (R848) or DT with no R848 (none). **(E)** Representative FACs analysis of GC B cells in b3-ko and WT DTR chimeras treated with DT and R848 (gated on CD45.1/ CD45.2+ CD19+ cells). Plots show relative proportions and total number of GC B cells in b3-ko and WT DTR DTR chimeras, treated with DT and R848**. (F)** Schematic of experiment using 'reverse' control b3-ko and WT DTR chimeras. **(G-H)** Representative FACs analysis for T2/MZ, and frequencies of T2/MZ and GC B cells in reverse control b3-ko and WT DTR chimeras treated with DT and R848. Each data point represents a single mouse, with mean +/- SEM. *p* values are

4.3 Integrin αvβ3 limits autoimmune disease in a mouse model of SLE.

Although TLR7 signaling has been shown to be both necessary and sufficient for development of SLE-like autoimmunity in mouse models, acting in part through production of IFN-I and activation and expansion of autoreactive B cells (Soni, Perez et al. 2020), it is unclear whether dysregulated pDC TLR signaling alone is sufficient to promote autoimmunity. To test this, I used a model of repeated epicutaneous administration of the TLR7 ligand Imiquimod (IMQ) (Fig 12A), which models many aspects of SLE (Yokogawa, Takaishi et al. 2014, Liu, Seto et al. 2018), in αvβ3-knockout mice. To allow long term deletion of αvβ3 in pDCs without the confounding features of complete integrin knockouts, I used DC-specific αvβ3-conditional knockouts. Although the CD11c-CRE transgene deletes in approximately 50% of pDCs *in vivo* (Supp Fig. 3A-B), I verified that this model recapitulates many of the effects of pDC-specific αvβ3 deletion, including increased IFN-I production by pDCs *in vitro* and *in vivo*, increased pDC activation *in vivo* at baseline and in the pDC-specific chimera, as well as elevated ISGs in the blood (Supp Fig. 3C-J).

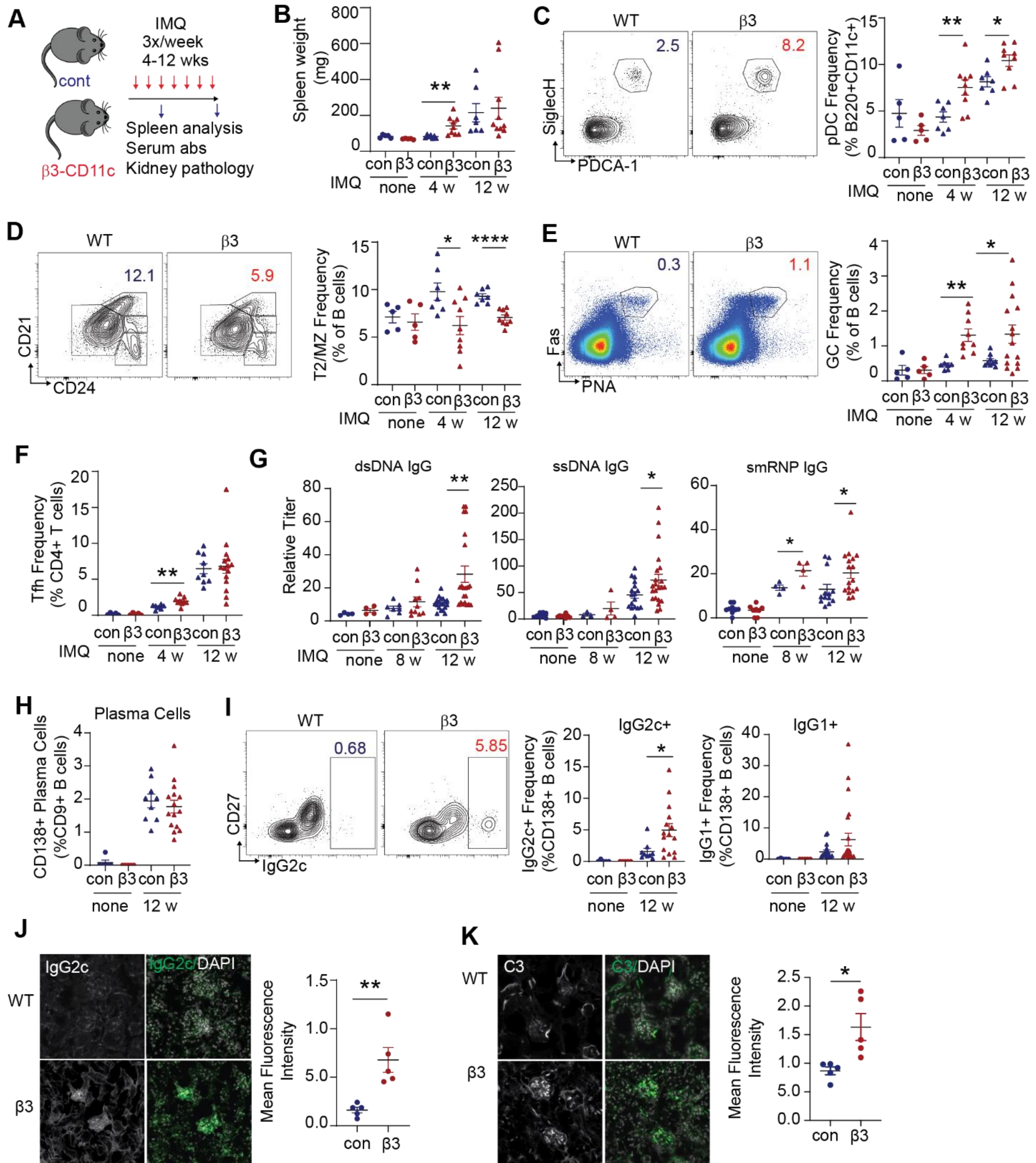

Figure 12: αvβ3 on DCs limits autoimmune disease: **(A)** Schematic of experimental design. β3-CD11c mice treated with Imiquimod (IMQ) TLR agonist epidermally 3 times a week for up to 12 weeks. **(B)** Spleen weight after treatment for indicated times. **(C-F)** FACs analysis of spleens from mice after treatment with IMQ. **(C)** Representative FACS plot of SiglecH and PDCA1 in gated B220+CD11c+ spleen cells from β3-CD11c and WT mice after 4 weeks treatment with IMQ. Gates and percentage of pDCs are indicated. Plot shows pDC frequency as percentage of B220+CD11c+ spleen cells.

Figure 12: αvβ3 on DCs limits autoimmune disease: **(D)** Representative FACS plot of CD21 and CD24 on B cells (B220+ CD19+ cells) in spleen of WT and β3-CD11c mice after 4 weeks treatment with IMQ. Gates and percentage of T2/MZ B cells are indicated. Plot shows T2/MZ frequency as percentage of B220+CD19+ B cells. **(E)** Representative FACS plot of Fas and PNA on B cells (B220+ CD19+ cells) in spleen of WT and β3-CD11c mice after 4 weeks treatment with IMQ. Gates and percentage of GC B cells are indicated. Plot shows GC frequency as percentage of B220+CD19+ B cells. **(F)** Frequency of Tfh cells (PD-1+CXCR5+ cells) as a percentage of total CD4+ T cells. **(G)** Serum IgG autoantibodies against dsDNA, ssDNA and smRNP in β3-CD11c and WT mice after IMQ treatment. **(H)** Frequency of CD138+ plasma cells in total CD19+ B cells for β3-CD11c and WT mice after 12 weeks of IMQ treatment. **(I)** Representative FACS plot of CD27 and IgG2c on plasma cells (CD19+ CD138+ cells) from spleen of WT and β3-CD11c mice after 12 weeks treatment with IMQ. Gates and percentage of IgG2c+ B cells are indicated. Plots show frequency of IgG2c+ and IgG1 cells as percentage of CD138+ plasma cells. **(J,K)** Representative images of kidney sections from WT and β3-CD11c mice after 12 weeks treatment with IMQ, stained for IgG2c (J) or Complement C3 (K) and cell nuclei (DAPI). Plots show quantification of IgG2c and C3 staining/ glomerulus based on analysis of 4 images per mouse. In all cases, data points are from individual mice, with mean +/- SEM. *p*-values are as shown or *$p<0.05$, **$p<0.01$, ***$p<0.001$, and ****$p<0.0001$ (Student's T test).

Although TLR7 signaling has been shown to be both necessary and sufficient for development of SLE-like autoimmunity in mouse models, acting in part through production of IFN-I and activation and expansion of autoreactive B cells (Soni, Perez et al. 2020), it is unclear whether dysregulated pDC TLR signaling alone is sufficient to promote autoimmunity. To test this, I used a model of repeated epicutaneous administration of the TLR7 ligand Imiquimod (IMQ) (Fig 12A), which models many aspects of SLE (Yokogawa, Takaishi et al. 2014, Liu, Seto et al. 2018), in αvβ3-knockout mice. To allow long term deletion of αvβ3 in pDCs without the confounding features of complete integrin knockouts, I used DC-specific αvβ3-conditional knockouts. Although the CD11c-CRE transgene deletes in approximately 50% of pDCs *in vivo* (Supp Fig. 3A-B), I verified that this model recapitulates many of the effects of pDC-specific αvβ3 deletion, including increased IFN-I production by pDCs *in vitro* and *in vivo*, increased

pDC activation *in vivo* at baseline and in the pDC-specific chimera, as well as elevated ISGs in the blood (Supp Fig. 3C-J).

β3-CD11c mice were treated with IMQ three times a week for up to 12 weeks (Fig. 12A). After 4 weeks of Imiquimod, β3-CD11c mice developed splenomegaly, which was not seen in imiquimod-treated littermate controls (Fig 12B). Spleen pDCs were also expanded in β3-CD11c mice after 4 weeks treatment, whereas pDC frequency did not change in imiquimod-treated controls (Fig 12C). In this model, Imiquimod treatment leads to an increase in the MZ/T2 B cell subset in control mice after 4 weeks, and this is maintained after 12 weeks treatment (Fig 12D). In addition, Imiquimod promoted significant expansion in GC B cell frequency in β3-CD11c mice compared with treated controls (Fig. 12E), associated with an increase in Tfh cells (Fig 12F). To test whether Imiquimod treatment resulted in increased autoreactive B cell activation in β3-CD11c mice, I measured autoantibody production. Anti-smRNP IgG was significantly increased in β3-CD11c mice compared to controls at both 8 and 12 weeks after Imiquimod treatment, and anti-dsDNA and ssDNA IgG were significantly increased after 12 weeks (Fig. 12G). SLE is associated with increased class-switching to IgG2c isotype, and while β3-CD11c mice had similar levels of CD138+ plasma cells (Fig. 12H), there was significantly increased proportions of IgG2c-switched spleen plasma cells compared with controls after 12 weeks of Imiquimod treatment (Fig. 12I). To determine whether these increased autoantibodies contribute to autoimmune pathology, I measured antibody deposition in the kidney of Imiquimod-treated mice. β3-CD11c mice had significantly higher levels of IgG2c (Fig. 12J) and complement C3 (Fig. 12K) in glomeruli than control mice. I therefore concluded that deletion of αvβ3 from pDCs resulted in increased

autoreactive B cell activation and autoimmunity in response to chronic TLR stimulation, particularly in female mice (Supp Fig 4A-H). Collectively, these data support a role for αvβ3 on pDCs in limiting IFN-I production which ameliorates many aspects of autoimmune disease, including lymphocyte activation and autoantibody production.

4.4 Discussion:

Here I examine how integrin αvβ3 limits pDC-derived cytokines to reduce B cell activation and prevent autoimmune disease. I have previously shown that αvβ3 integrin limits pDC IFN-I and proinflammatory cytokine production both *in vitro* and *in vivo*. This work demonstrates that this increased cytokine production in the absence of αvβ3 leads to a reduction in marginal zone (MZ) B cells and an expansion of germinal center B cells. Additionally, more autoantibodies are produced in the absence of αvβ3, as well as increased class-switched plasma cells. Finally, kidney damage, as evinced by deposition of immune complexes in glomeruli is increased without αvβ3, demonstrating the integrin's key role in preventing SLE disease.

A major consequence of dysregulated pDC IFN-I production in our studies was the activation and expansion of autoreactive B cells. Specifically, I saw a relative reduction in MZ B cells and increases in GC B cells and IgG2c-class switched plasma cells in pDC-specific αvβ3-$^{/-}$ mice after short or long term TLR stimulation. pDCs are positioned close to the spleen marginal zone, and IFN-I produced by pDCs promotes the activation of MZ and MZ-precursor B cells, which then migrate to the follicle and enter the GC or differentiate into extrafollicular plasmablasts (Asselin-Paturel, Brizard et al. , Swiecki,

Gilfillan et al. 2010, Sisirak, Ganguly et al. 2014, Li, Fu et al. 2015, Soni, Perez et al. 2020). I therefore conclude that increased IFN-I production by αvβ3-knockout pDCs drives increased MZ B cell activation and migration. IFNAR signaling in B cells is also reported to drive the production of IgG2c autoantibodies (Domeier, Chodisetti et al. 2018), and TLR-induced IFN-I has been shown to elicit antigen-specific IgG2c from follicular B cells (Swanson, Wilson et al. 2010), indicating that the increased IgG2c responses that I observe are also likely to be due to exposure of B cells to pDC-derived IFN-I. Our data are consistent with loss of αvβ3 in pDCs leading to increases in both extrafollicular and GC-dependent antibody producing cells, both of which have been implicated in production of pathologic autoantibodies (Li, Fu et al. 2015, Soni, Perez et al. 2020).

Previously, pDCs were shown to be required for development of SLE-like autoimmunity in mouse models, including those driven by increased TLR7 signaling or persistent DNA autoantigens (Rowland, Riggs et al. 2014, Sisirak, Ganguly et al. 2014, Soni, Perez et al. 2020). Our findings complement these previous studies by demonstrating that pDC-intrinsic increased IFN-I responses to TLR ligands are sufficient to exacerbate autoimmunity, and establish pDCs as key pathological effectors of lupus-like autoimmunity. The immunological consequences of deletion of αvβ3 from pDCs closely resemble the effects of deletion from B cells, which include increased GC B cell responses and generation of IgG2c plasma cells, and increased TLR-driven autoimmune pathology (Acharya, Raso et al. 2020), supporting a common role for αvβ3 in both cell types. Curiously, deletion of αvβ3 from B cells resulted in activation of MZ B cells and increases in GCs in the absence of exogenous TLR activation (Wang, Rodda et al. 2014,

Raso, Sagadiev et al. 2018), and development of autoantibodies with age (Acharya, Sokolovska et al. 2016, Acharya, Raso et al. 2020), indicating that αvβ3 prevents activation of autoreactive B cells at homeostasis. In contrast, pDC-specific deletion of αvβ3 resulted in relatively small increases in pDC activation and no major differences in MZ or GC B cell frequency in the absence of exogenous TLR stimulation. Hence this αvβ3-mediated pathway may not be essential for regulation of pDC responses to the low levels of exogenous TLR ligands seen at homeostasis, but rather provides effective regulation of autoimmune responses when nucleic acid TLR ligands are more abundant, such as during cellular damage, inflammation, or infection.

Chapter 5: αvβ3 promotes clustering of pDCs in response to TLR signaling

5.1 Introduction:

In response to pathogen-associated molecular patterns (PAMPs) recognized by TLR7/9, pDCs produce massive amounts of IFN-I. TLR stimulation has been shown to induce clustering of pDCs (Asselin-Paturel, Brizard et al. 2005), leading to large aggregates of self-adhesion in an IFN-I-dependent manner. Production of IFN-I after TLR7/9 stimulation has also been shown to require cell-cell contact through integrins (Saitoh, Abe et al. 2017), indicating the presence of a positive feedback loop between cell contact and IFN-I production. The loss of *RUNX2*, a transcription factor necessary for integrin-mediated adhesion machinery and expressed solely by pDCs, causes a pDC-specific loss of IFN-I production (Chopin, Preston et al. 2016), suggesting a role for integrins, such as LFA-1, in the TLR response and subsequent cytokine production.

Adhesion of pDCs by integrins is not limited just to self-priming, however, as pDCs can also form clusters with other cells. In response to certain viruses, IFN-I production by pDCs has been shown to be cell-cell contact-dependent (Assil, Coléon et al. 2019), requiring the formation of an interferogenic synapse for the proper transfer of PAMPs. It was later shown that this IFN-I production is contact-dependent in response to virally-infected cells, but not to cell-free viral particles (Yun, Igarashi et al. 2021). Severe SARS-CoV-2 infection has also been shown to require sustained integrin-dependent cell-cell contact between pDCs and infected cells for production of IFN-I (Venet, Ribeiro et al. 2023). Clustering of pDCs with other cell types, such as Tregs, has been linked to

suppression of activation (Onishi, Fehervari et al. 2008), suggesting cell-cell contact between pDCs and other cell types may be tolerogenic.

Certain other integrins, such as αvβ3, have been shown to modulate LFA-1 function to increase activation and motility (Weerasinghe, McHugh et al. 1998). Due to the effects I have previously shown of αvβ3 on pDC IFN-I production and interaction with B cells, I posited that αvβ3 may also affect pDC clustering and adhesion. Additionally, I sought to determine whether αvβ3 may play a role in the activation of LFA-1 for cell-cell adhesion.

5.2 αvβ3 is required for pDC clustering after TLR stimulation.

In response to TLR ligand stimulation, pDCs have been shown to cluster together in large aggregates, which is thought to promote production of type I IFN through paracrine signaling. To test whether αvβ3 affects this pDC clustering, pDCs grown from bone marrow were plated with TLR9 ligand, CpG-A, for two hours and clustering was enumerated by light microscopy (Fig. 13A-B). Clustering was defined as three or more cells adhered together and each point is one field of view, representative of three experiments. In response to TLR9 ligand, WT pDCs clustered together effectively compared to unstimulated cells. However, αv-ko pDCs failed to cluster more than unstimulated cells (Fig. 13A-B), indicating a failure of pDCs to aggregate in response to stimulation and a role for αvβ3 in self-adhesion of pDCs.

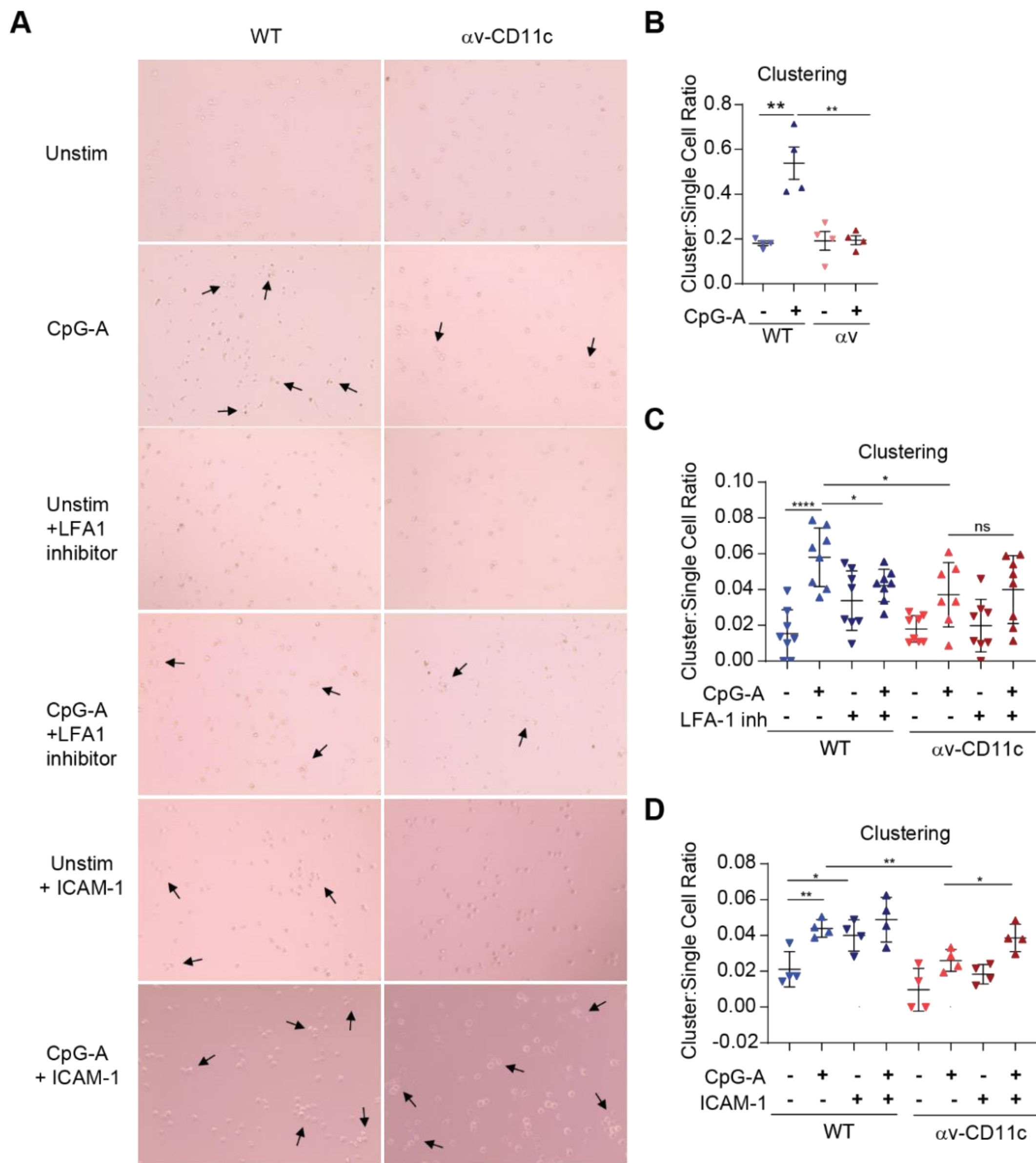

Figure 13: αvβ3 activates LFA-1 to promote clustering of pDCs: **(A)** Representative light microscope images of sorted pDCs stimulated with CpG-A and treated with LFA-1 inhibitor or supplemented with soluble ICAM-1. Arrows indicate 3 or more pDCs clustered together. **(B)** Hand counted enumeration of clustered cells from either WT or αv-CD11c mice stimulated with TLR9 ligand, CpG-A. **(C)** Clustered cells counted from WT or αv-CD11c mice stimulated with TLR9 ligand, CpG-A and treated with LFA-1 inhibitor. **(D)** Clustered cells from WT or αv-CD11c mice stimulated with CpG-A and supplemented with soluble LFA-1 ligand ICAM-1. Clustering was defined as three or more cells adhered together and each point indicates one field of view, with mean +/- SEM. Each graph is representative of three experiments. *p*-values are as shown or *$p<0.05$, **$p<0.01$, ***$p<0.001$, and ****$p<0.0001$ (Student's T test).

5.3 αvβ3 activates LFA-1 to promote clustering of pDCs.

Adhesion of pDCs to virally-infected cells has been shown to depend on interactions with integrin LFA-1, so we treated cells sorted from WT and αv-CD11c mice with an LFA-1 inhibitor to determine whether LFA-1 was responsible for the observed clustering and whether αvβ3 interacted with the other integrin. Similar to the previous experiment, increased clustering was observed in response to TLR9 stimulation in the WT mice, while treatment with LFA-1 inhibitor abolished that increase (Fig. 13C). This demonstrates that TLR-induced clustering is dependent on LFA-1. In the absence of αvβ3, clustering in response to TLR9 stimulation with or without LFA-1 inhibitor showed no increase over unstimulated cells (Fig. 13C), suggesting αvβ3 is involved in the same pathway as LFA-1, but that some clustering is independent of either integrin and rather a consequence of TLR signaling.

ICAM-1 is a ligand for LFA-1, so to further explore the mechanism of integrin-mediated clustering, I stimulated pDCs from WT and αv-CD11c mice in plates coated with ICAM-1 with TLR9 ligand CpG-A and measured clustering. WT pDCs treated with ICAM-1 demonstrated increased clustering even without TLR stimulation (Fig. 13D), further indicating a dependence on LFA-1 for cell adhesion. In unstimulated αv-ko pDCs treated with ICAM-1 there was no such increase in clustering. However, the addition of ICAM-1 restores the clustering of pDCs in response to TLR9 stimulation in αv-CD11c mice, suggesting that αvβ3 activates LFA-1 to lead to the self-adhesion, or clustering, of pDCs.

5.4 Discussion:

Here we have shown that in response to TLR stimulation, pDCs form large aggregates, or clusters, of cells. This clustering has been shown to contribute to production of IFN-I, as well as the efficient transfer of viral PAMPs for clearance of virally-infected cells. Furthermore, we have shown that this clustering depends partially on activation of LFA-1 by the integrin αvβ3. In the absence of αvβ3, very little clustering occurs in response to TLR9 stimulation, but the addition of LFA-1 ligand, ICAM-1, partially restores this clustering effect. These two integrins have been known to interact previously, as αvβ3 is necessary for LFA-1-mediated transmigration of monocytes (Weerasinghe, McHugh et al. 1998). Crosstalk between αvβ3 integrin and LFA-1 promotes activation and translocation of monocytes (Weerasinghe, McHugh et al. 1998), with αvβ3 being necessary for LFA-1 adhesion. Our work supports this, as the addition of LFA-1 ligand, ICAM-1, restores the clustering which is reduced in the absence of αvβ3.

In this study, we see decreased clustering in the absence of αvβ3, yet increased IFN-I in response to TLR stimulation. This could reflect the ability of αvβ3 to negatively regulate IFN-I production through cell-cell contact, forming a tolerogenic synapse in response to self-ligand, as opposed to the interferogenic synapse formation (Assil, Coléon et al. 2019) observed in response to viral infection. The primary mechanism of regulation of IFN-I production is likely through endosomal trafficking, however, the adhesion of αvβ3 through cell-cell contact and activation of LFA-1 is another possible contributor. Our work further supports the role of αvβ3 in activation of LFA-1 to promote self-adhesion of pDCs and raises many questions about αvβ3 integrin's role in adhesion of pDCs to other cell types for both viral infection as well as other immune responses, such as autoimmunity.

Chapter 6: Discussion

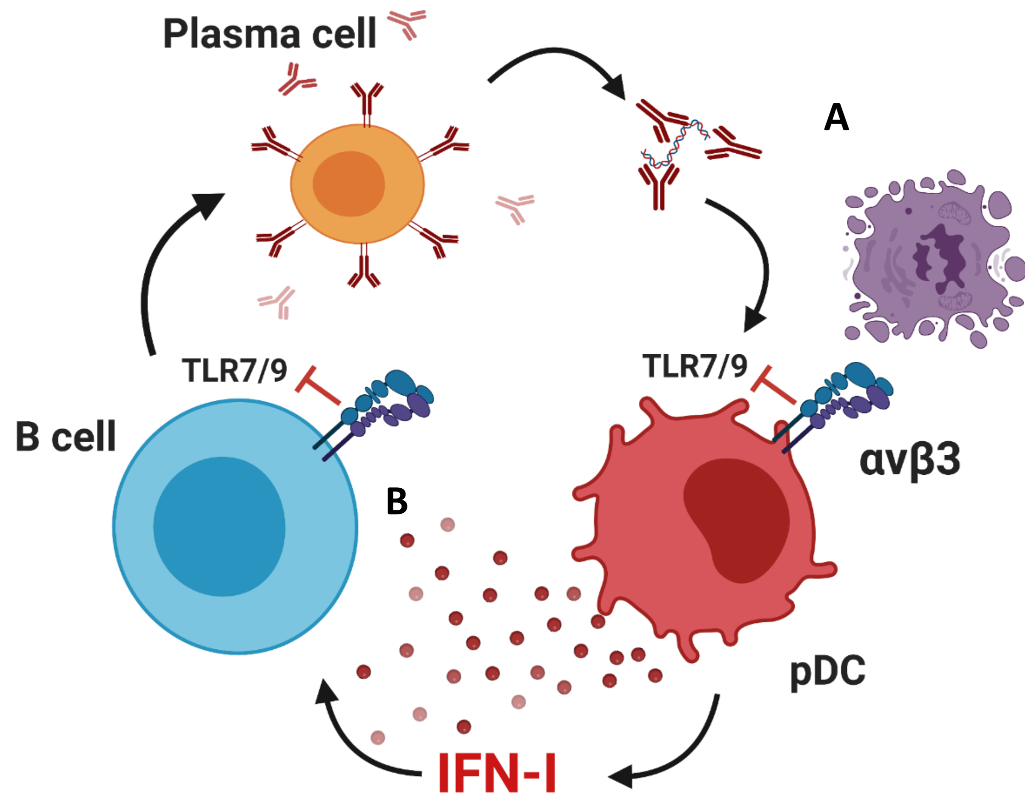

Figure 14: Model of αvβ3 action on TLR signaling in pDCs to reduce autoinflammatory loop seen during lupus: **(A)** In response to self-NA, integrin αvβ3 limits TLR7/9 signaling in pDCs, decreasing IFN-I production which limits B cell activation and autoantibody production. **(B)** Integrin αvβ3 also limits TLR7/9 signaling in B cells to reduce formation of autoantibody complexes, reducing the ICs available to further stimulate pDCs

Here I identify integrin αvβ3 as a negative regulator of IFN-I production in pDCs. I show that deletion of αv or β3 from pDCs results in elevated IFN-I secretion in response to stimulation through TLR7 and TLR9. *In vivo* this leads to increased activation of B cells, production of autoantibodies and autoimmune organ damage in a TLR-driven model of

autoimmunity. I propose that this αvβ3 regulatory pathway exists to limit immune responses to self TLR-ligands, and to prevent development of autoimmunity (Fig. 14).

Additional endosomal and cell-surface proteins are implicated in promoting signaling to IRF7 in pDCs, including AP3 (Sasai, Linehan et al. 2010) and Dock 2 (Gotoh, Tanaka et al. 2010) respectively. Additionally, prior studies have concluded that LC3 may not be required for IFN-I production in response to soluble TLR ligands such as R848 or CpG DNA (Henault, Martinez et al. 2012). Hence it remains possible that αvβ3 may affect additional non-autophagy-related mechanisms of TLR-endosome traffic in pDCs. Together, these results highlight the importance of the rate and route of endosomal trafficking of TLR-ligand complexes in determining the cellular response and suggest that immune cell subsets that are specialized to efficiently activate IRF7 downstream of TLRs, such as pDCs or MZ and GC B cells (Acharya, Sokolovska et al. 2016, Muir, Sagadiev et al. 2022), also possess regulatory mechanisms to control these responses and prevent autoimmunity.

Although our data are consistent with αvβ3 promoting uptake and traffic of TLR ligands, I cannot exclude additional roles for αvβ3 such as in cell-matrix and cell-cell interactions. Integrin αLβ2 (LFA-1) mediates cell-cell contact between pDCs and infected cells, leading to PAMP transfer and the induction of an antiviral response (Saitoh, Abe et al. 2017, Assil, Coléon et al. 2019, Yun, Igarashi et al. 2021). Furthermore, cell-to-cell contact between pDCs and B cells, through LFA-1, has been shown to contribute to autoreactivity and autoantibody production after TLR stimulation (Ding, Cai et al. 2009), suggesting adhesion between pDCs and B cells can directly impact development of SLE.

It is therefore possible αvβ3 may exert contact-dependent regulation of B cells to prevent autoimmune disease.

Integrin αvβ3 and the related integrin αvβ5 are involved in the recognition and clearance of apoptotic cells and cellular debris (Savill, Dransfield et al. 1990, Savill, Hogg et al. 1992, Rubartelli, Poggi et al. 1997, Albert, Pearce et al. 1998, Lucas, Stuart et al. 2006, Lacy-Hulbert, Smith et al. 2007), which serve as a rich source of autoantigens and nucleic acid TLR ligands. In macrophages and DCs, exposure to apoptotic cells downregulates responses to TLR ligands (Stuart, Lucas et al. 2002, Lucas, Stuart et al. 2003, Lucas, Stuart et al. 2006), and promotes immune tolerance. I propose that αvβ3 on pDCs may serve as a coreceptor for apoptotic cell-derived TLR ligands to regulate signaling and prevent autoimmunity. Supporting this idea, pDCs express the scavenger receptor CD36 (Parcina, Schiller et al. 2009), which cooperates with αv integrins to bind apoptotic material, and have been shown to internalize apoptotic cell-derived microparticles and respond to associated nucleic acids (Schiller, Parcina et al. 2012, Simpson, Miles et al. 2016, Ainola, Porola et al. 2018). Furthermore, pDCs are implicated in promoting tolerance induced by apoptotic cells *in vivo* in a mouse model (Bonnefoy, Perruche et al. 2011), although direct regulatory effects of apoptotic material on pDCs have not yet been demonstrated.

In response to dysregulated pDC IFN-I production, I observed a reduction in MZ B cells and increases in GC B cells and class-switched plasma cells in a pDC-specific model of αvβ3 deletion after either acute or chronic exposure to TLR stimulation. This leads me to the conclusion that αvβ3 limits the migration of B cells from the marginal zone to a more

activated state in the germinal center and prevents the production of autoreactive antibodies. Our findings complement previous studies indicating the important role of pDC-derived IFN-I in the development and early stages of SLE. In our study, αvβ3 regulates pDC responses only after TLR stimulation, unlike our findings in B cells where regulation in the absence of TLR ligands was observed. This suggests that pDCs may not play a role in the response to exogenous ligands, but rather in response to large quantities of self-nucleic acid ligands.

pDCs play a major role in promoting immunity to viral infections, through recognition of viral nucleic acids by TLRs and other nucleic acid receptors, and by production of IFN-I. Our data therefore raise the possibility that αvβ3 may downregulate antiviral immunity. Supporting this, we have previously shown that deletion of αvβ3 from B cells increases antiviral antibody responses (Raso, Sagadiev et al. 2018), and these reduced immune responses to viral antigens may therefore reflect a tradeoff between increased immune protection and potential autoimmunity. Curiously, several viruses, including herpes simplex virus (HSV), foot and mouth virus and SARS-CoV-2, utilize αvβ3 as a co-receptor to facilitate cell entry (Berinstein, Roivainen et al. 1995, Gianni, Leoni et al. 2012, Liu, Lu et al. 2022). Engagement of αvβ3 by HSV is reported to engage antiviral innate immune signaling through TLR2 and SYK-CARD9-TRIF pathways to promote IFN-I production (Gianni, Leoni et al. 2012, Gianni, Leoni et al. 2013, Gianni and Campadelli-Fiume 2014). Several key differences may contribute to these apparently opposing roles of αvβ3 in promoting or reducing IFN-I production. Studies of HSV were performed in non-immune cell lines, which express TLR2 but not the endosomal nucleic acid sensors TLR7,8 or 9. Furthermore, we have shown that the αvβ3-regulatory pathway

requires activation of autophagy through a non-canonical mechanism that is not present in all cell types (Muir, Sagadiev et al. 2022). Finally, I would emphasize that our studies in pDCs, and previous work from B cells, show that αvβ3 does not completely prevent TLR7/9 responses, but accelerates TLR7/9 trafficking and signaling, allowing limited production of inflammatory cytokines and IFN-I. Our data therefore do not exclude a role for αvβ3 in promoting TLR signaling in other cell types or downstream of TLRs other than TLR7 and TLR9.

It is increasingly apparent that dysregulated innate immune recognition of nucleic acids, particularly TLR7, is a major driver of systemic autoimmune disease. Studies in mouse models using genetic or antibody-mediated deletion of pDCs or disruption of IFN-I signaling have demonstrated that pDCs are necessary for development of lupus-like autoimmunity through IFN-I production. Our data show that pDC-intrinsic dysregulation of TLR signaling and IFN-I production is sufficient to promote B cell activation and autoimmunity, and underscore the importance of regulation of TLR signaling in pDCs to maintain tolerance to self-TLR ligands. Polymorphisms in the autophagy genes *ATG5, ATG16L1,* and *ATG16L2* are associated with autoimmune disease, including SLE, but the mechanisms by which disruption of autophagy affects autoimmune disease remain unclear. Our new findings reported here, together with our previous work in B cells, strongly support a role for αvβ3 integrins and non-canonical autophagy in regulation of pDC activation, IFN-I production and activation of autoreactive B cell responses, leading to prevention of autoimmunity to nuclear antigens.

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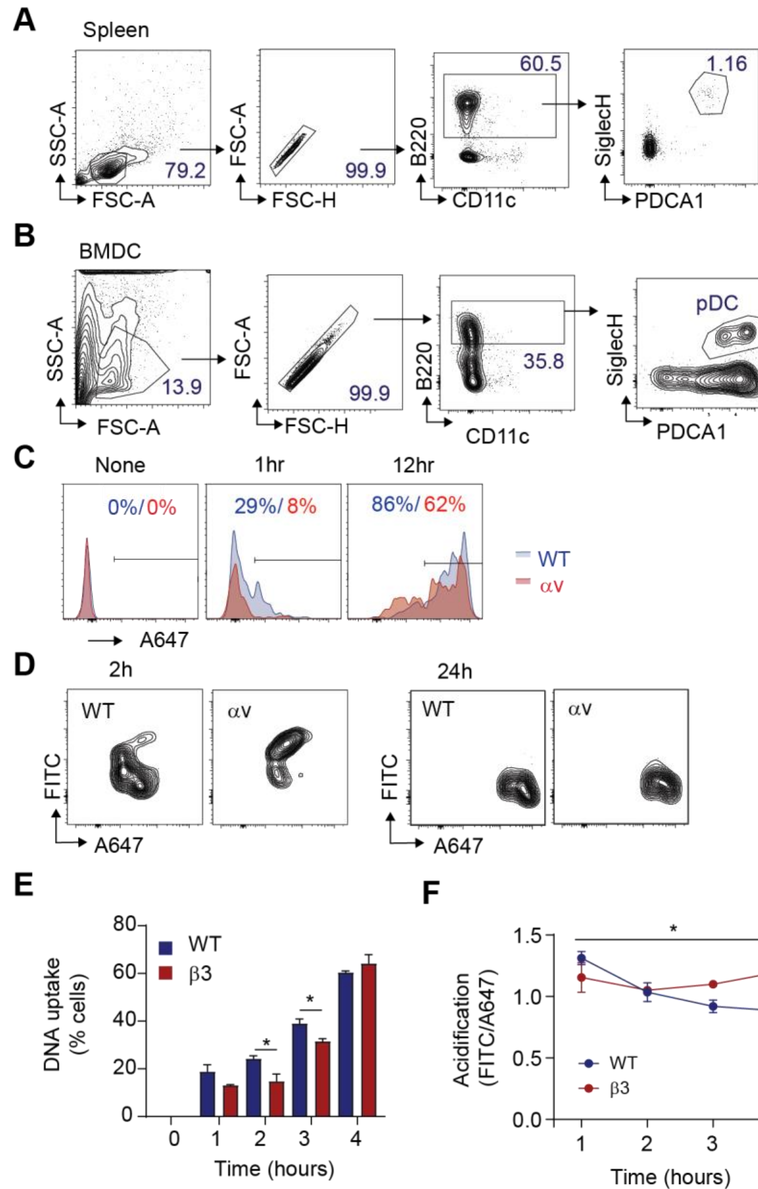
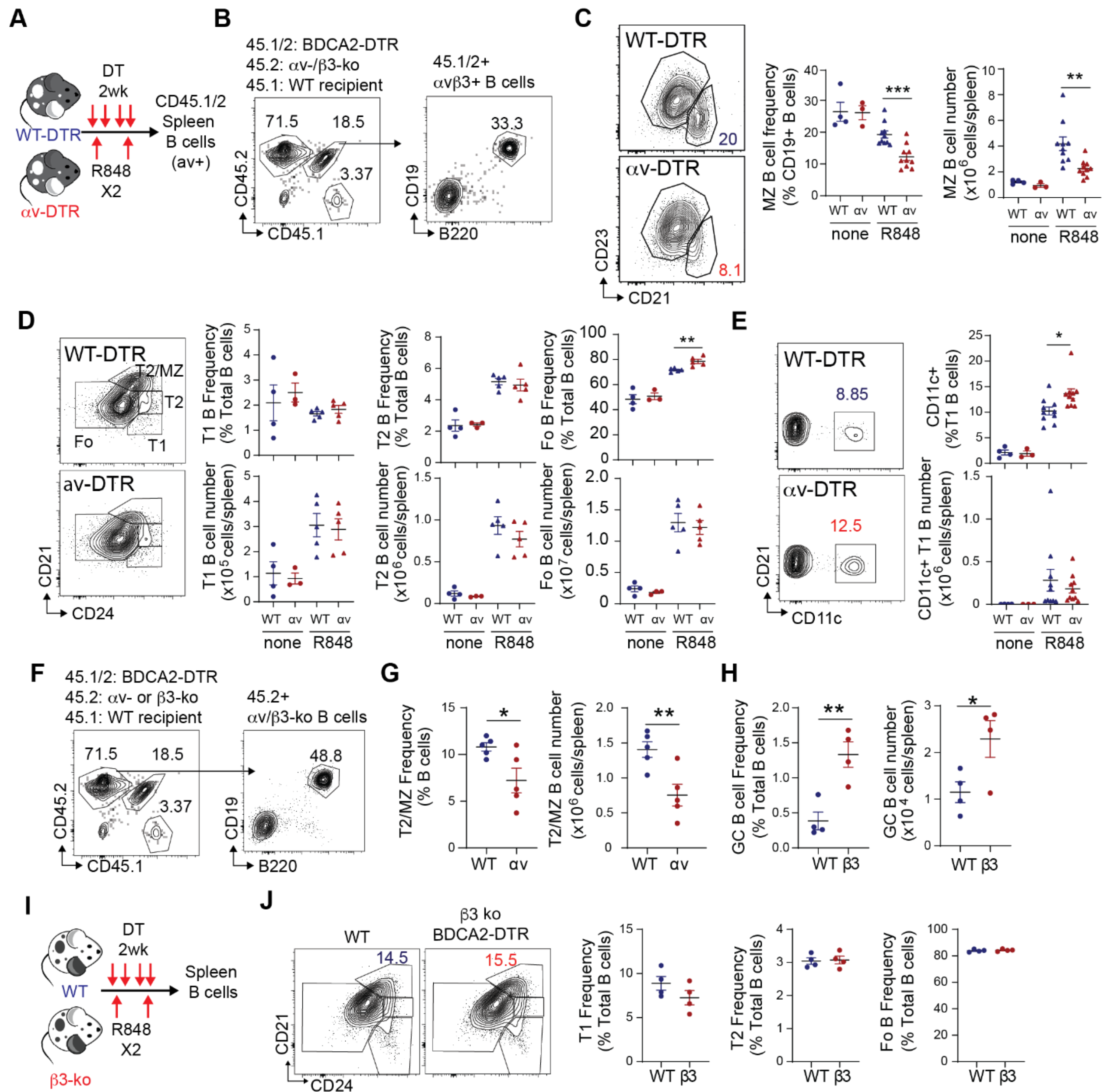
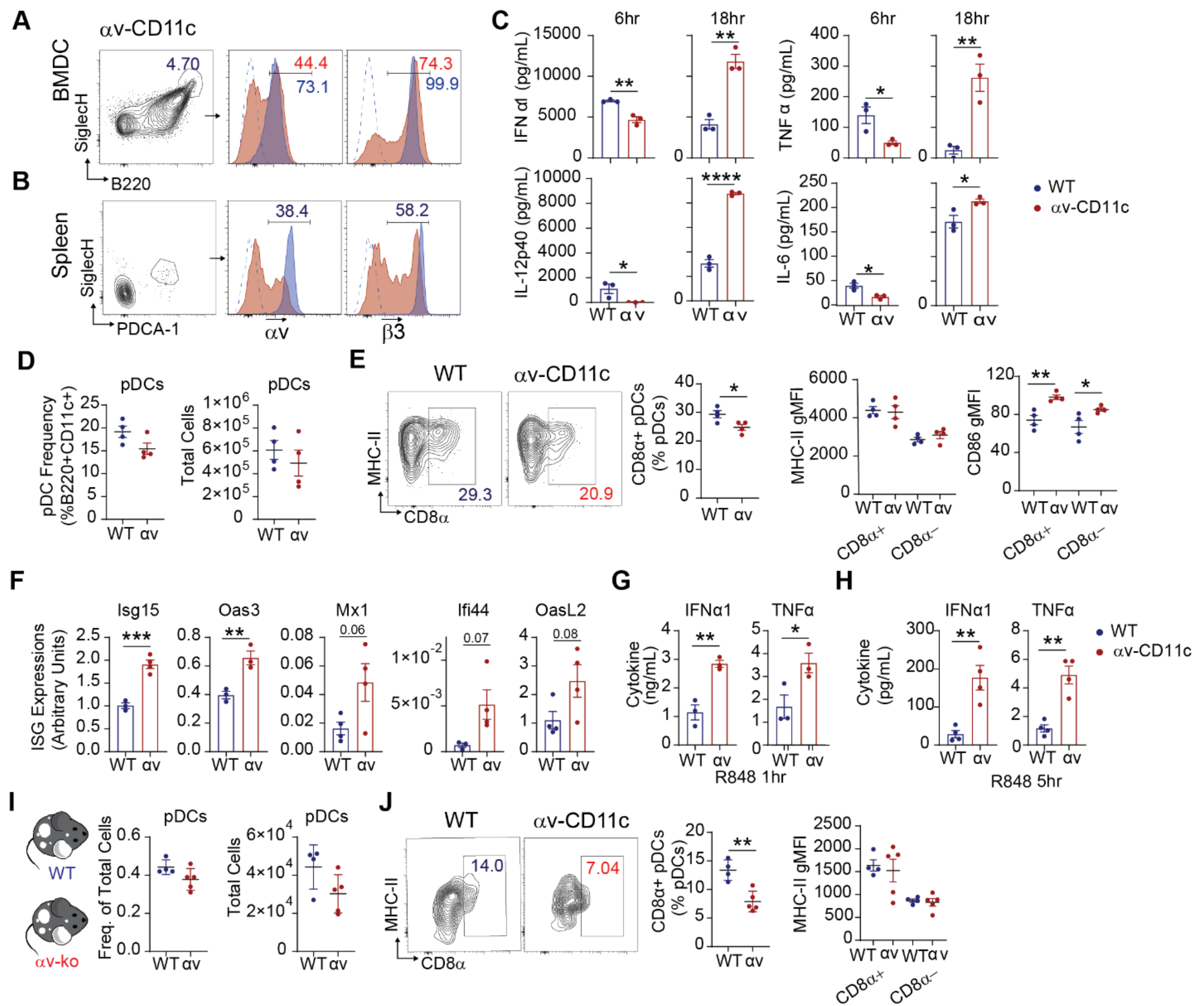
Supplementary Material:



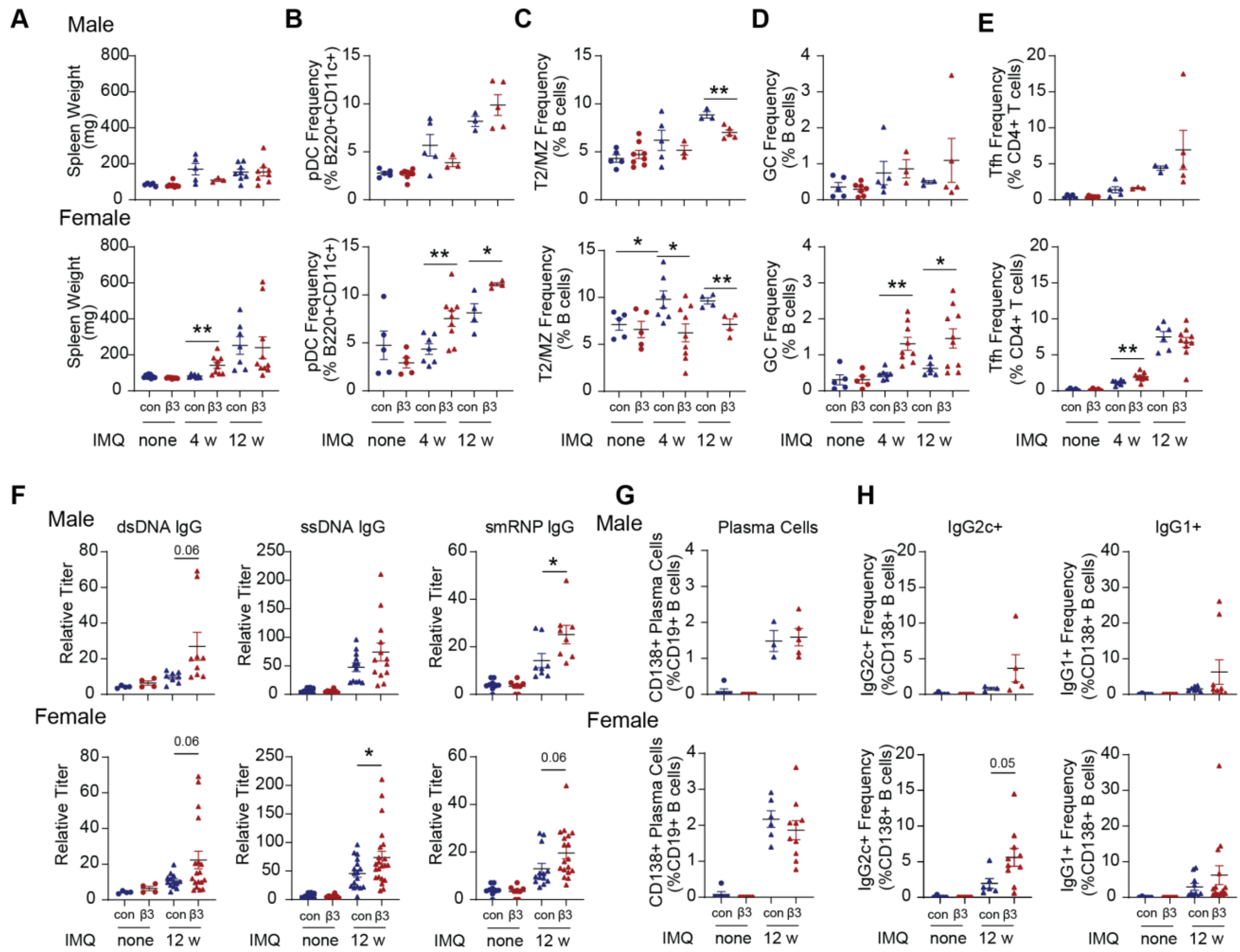
Supplemental Figure 1: FACS gating and uptake/acidification of ODN by pDCs: **(A)** Single lymphocytes are identified based on FSC-A, SSC-A and FSC-H. B220+ cells are then selected and pDCs gated as B220+ PDCA1+ SiglecH+ cells. **(B)** Gating strategy to identify pDCs in total spleen DC preparations (sorted by CD11c+ positive selection; STEMCELL Technologies). Cells are gated for single lymphocytes and singlets, then B220+ cells are gated for PDCA1+ SiglecH+ pDCs. **(C)** Representative histograms for assays of uptake of CpG-A ODN-A647 by pDCs from WT (blue) and αv-tie2 (red) mice at indicated times after CpG addition. **(D)** Representative FACS plots FITC and A647-derived fluorescence in A647+ pDCs from WT and αv-tie2 mice, 2 and 24 hours after addition of labelled CpG-A ODN. **(E)** Uptake of labelled CpG-A ODN by pDCs from β3-ko and WT mice at indicated timepoints. Data are mean +/-SD of replicate pDC preparations from one experiment. *$p<0.05$ and **$p<0.01$ from data analyzed with Student's T test. **(F)** Relative FITC and A647-derived fluorescence in A647+ pDCs from β3-ko and WT mice incubated with CpG-A FITC and CpG-A A647 for indicated times. Data are mean +/-SD of replicate pDC preparations from one experiment analyzed by Two-Way ANOVA.



Supplemental Figure 2: Analysis of B cell subsets in avβ3-DTR chimeras**:** **(A)** Schematic of experimental design. av-tie2 and WT DTR chimeras were treated repeatedly with DTR to deplete pDCs and given two doses of R848 over three weeks**.** **(B)** Gating strategy for analysis of WT spleen B cells. Splenocytes are gated on CD45.1/ CD45.2 double positive cells, which are derived from DTR mice and therefore have intact av expression, and then for B cells based on B220 and CD19. **(C-E)** Representative FACS analysis, frequency and total number of MZ B cells (C) and T1, T2, and Fo B cells (D), and CD11c+ Age-associated B cells from the T1 B cell compartment, in αv-tie2 and WT DTR chimeras, treated with DT and R848 (R848) or DT with no R848 (none). **(F)** Gating strategy for analysis of αvβ3-knockout spleen B cells in αv-tie2 DTR, β3-DTR and WT DTR chimeras. Splenocytes are gated on CD45.2 positive cells which are derived from αv or β3-ko mice, or from WT mice in control chimeras), and then for B cells based on B220 and CD19. **(G,H)** Frequency (left) and number (right) of T2/MZ (G) and GC (H) αv or β3-ko B cells from DTR chimera. **(I)** Experimental scheme for 'reverse' control b3-ko and WT DTR chimeras. **(J)** Representative FACs analysis and frequencies of T1, T2, and Fo B cells in reverse control b3-ko and WT DTR chimeras. Each data point represents a mouse and *p* values are as shown or **p*<0.05, ***p*<0.01, and ****p*<0.001. All analyses were done with Student's T test, with values shown as mean +/- SEM. Similar results were seen in 4 (B-G) or 2 (J) independent experiments.



Supplemental Figure 3: αv-CD11c pDCs correspond to αv-tie2 and $\beta3^{-/-}$: **(A)** Surface expression of integrin αv and β3 on pDCs grown in culture from bone marrow of αv-CD11c and WT mice. Histograms show staining for αv and β3 in pDCs from αv-CD11c mice (red) and WT mice (blue). Streptavidin control staining is shown with a dashed line. Numbers indicate the percentage of pDCs that express αv and β3 (αv-CD11c mice in red, WT mice in blue). **(B)** Surface expression of αv and β3 integrin on B220+ CD11c+ SiglecH+ PDCA-1+ pDCs from αv-CD11c (red) and matched wild type (WT) mice (blue). Numbers indicate the percentage of αv and β3-positive cells in αv-CD11c mice. **(C)** Concentration of cytokines measured in cell culture supernatant after CpG-A stimulation of pDCs from αv-CD11c mice. Data are mean +/-SD of replicate cultures from one experiment. For all data shown, similar results were seen in at least three independent experiments. p values are as shown or *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001 (Student's T test). **(D-E)** Analysis of pDCs from spleen of αv-CD11c mice and matched wild-type (WT) mice at 10-12 weeks of age. (D) pDC (PDCA1+SiglecH+) frequency shown relative to all B220+CD11c+ cells in spleen determined by flow cytometry. (E) Representative FACS plots and combined analysis of CD8α+ pDCs (% of total pDCs) and levels of surface MHCII and CD86 (gMFI) on CD8α+ and CD8α- spleen pDCs. **(F)** ISG expression measured by qPCR from whole blood RNA extracted from 10–12 week-old αv-CD11c mice. **(G-H)** Serum cytokine concentration 1 hour (G) or 5 hours (H) post-injection with R848 in αv-CD11c mice and matching wild type (WT) mice. **(I-J)** av-CD11c-DTR and WT-DTR chimeras were treated repeatedly with DTR to deplete pDCs over three weeks. Frequency of pDCs (I) and CD8a+ pDCs (J) in av-CD11c BDCA2-DTR chimera with MHC-II expression by gMFI. Each data point represents an individual mouse in a single experiment, and mean +/- SEM are shown. *p*-values are as indicated or *p<0.05 (Student's T test). In all cases, similar results were seen in 3 independent experiments.



Supplemental Figure 4: Sex-specific differences in a TLR7-driven SLE model Effects of epicutaneous IMQ treatment in WT and β3-CD11c mice as described in Figure 6 of the main paper, separated by sex. **(A-E)** Data show Spleen weight (A), and frequencies of pDCs (B), T2/MZ B cells (C), GC B cells (D), and Tfh cells in spleen (E). **(F)** Serum IgG autoantibodies against dsDNA, ssDNA and smRNP. **(G-H)** Frequencies of CD138+ plasma cells in total CD19+ B cells (G) and frequencies of IgG2c+ and IgG1 cells (H) expressed as a percentage of CD138+ plasma cells. Each data point represents a single mouse and *p* values are as shown or **p*<0.05, ***p*<0.01, and ****p*<0.001. In all cases, data points are from individual mice, with mean +/- SEM. *p*-values are as shown or **p*<0.05, ***p*<0.01, (Student's T test). Similar results were seen in 4 (B-G) or 2 (J) independent experiments.