JARID1c and JARID1d as putative genetic mediators of sexually dimorphic dendritic cell function

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December 2019

A thesis submitted to McGill University in partial fulfilment of the requirements of the degree of Master of Science.

ABSTRACT

Sex differences in immunity are well-documented; females typically mount a more potent response than males. One consequence of a highly potent immune response is the risk of bypassing self-tolerance mechanisms to promote autoimmune pathologies. Indeed, 80% of autoimmune patients are female. Although hormonal and genetic factors have been implicated in sex-biased immunity, the molecular mechanisms that account for these differences remain poorly understood. Dendritic cells (DCs) are crucial for controlling immune responses; upon stimulus recognition, DCs transition from an actively restrained "steady-state" to an activated state capable of instructing antigen-specific adaptive immune responses. Although much is known about the molecules that promote DC activation, the mechanisms involved in actively maintaining the steady-state remain poorly understood. Our lab has identified an epigenetic axis in DC activation, whereby the transcriptional repressors PCGF6 and JARID1c (KDM5c) restrain DC activation by altering the DC epigenome. JARID1c is an X-linked H3K4 histone demethylase that escapes X-inactivation; females express two copies of *Jarid1c*, whereas males express one copy of *Jarid1c* and its Y-linked paralogue *Jarid1d*. Here, **we hypothesize that JARID1c and JARID1d contribute to sex-specific immune responses of DCs**. Using bone marrow-derived dendritic cells (BMDCs) from C57BL/6 age-matched mice as an *in vitro* model, we found female-derived BMDCs to mount a more potent immune response than male-derived BMDCs. We also demonstrate that JARID1c-deficient BMDCs from both sexes mount a less robust immune response compared to sex-matched wildtype BMDCs. *In vivo* studies revealed that cDC1s, cDC2s and pDCs from male and female mice differ in number and in activation. Finally, we found that JARID1c-deficient DCs exert a decreased pro-inflammatory phenotype compared to sex-matched wildtype controls. This trend was true both at steady-state and following infection with LCMV-Armstrong. Together, our study investigates sex-biased immune response and provides evidence to support epigenetic regulation by JARID1c/d as a factor implicated in sexually dimorphic immunity.

RÉSUMÉ

Les différences d'immunité entre les sexes sont bien documentées; les femmes ont généralement une réponse plus puissante que les hommes. L'une des conséquences d'une réponse immunitaire extrêmement puissante est le risque de contourner les mécanismes d'auto-tolérance pour favoriser les pathologies auto-immunes. En effet, 80% des patients auto-immuns sont des femmes. Bien que des facteurs hormonaux et génétiques aient été impliqués dans l'immunité sexiste, les mécanismes moléculaires à l'origine de ces différences restent mal compris. Les cellules dendritiques (CDs) jouent un rôle crucial dans le contrôle de la réponse immunitaire. Une fois le stimulus reconnu, les CDs passent d'un «état stable» activement restreint à un état activé capable d'instruire les réponses immunitaires adaptatives. Bien que l'on en sache beaucoup sur les molécules qui favorisent l'activation des CDs, les mécanismes impliqués dans le maintien actif de l'état d'équilibre restent mal compris. Notre laboratoire a identifié un axe épigénétique dans l'activation des CDs, selon lequel les répresseurs transcriptionnels PCGF6 et JARID1c (KDM5c) restreignent l'activation des CDs en modifiant l'épigénome des CDs. JARID1c est une histone déméthylase H3K4 liée à l'X qui échappe à l'inactivation de l'X; les femelles expriment deux copies de *Jarid1c*, tandis que les mâles expriment une copie de *Jarid1c* et de son paralogue lié à Y, *Jarid1d*. Ici, **nous émettons l'hypothèse que JARID1c et JARID1d contribuent aux réponses immunitaires spécifiques du sexe des CDs**. En utilisant des CDs dérivées de la moelle osseuse (CDMO) de souris C57BL/6 appariées selon l'âge comme modèle *in vitro*, nous avons constaté que les CDMOs dérivées de femmes développaient une réponse immunitaire plus puissante que les CDMOs dérivées de mâles. Nous démontrons également que les CDMOs déficients en JARID1c des deux sexes génèrent une réponse immunitaire moins robuste que les CDMOs de type sauvage. Des études *in vivo* ont révélé que les CDc1, les CDc2 et les CDp de souris mâles et femelles diffèrent en nombre et en activation. Enfin, nous avons constaté que les CDs déficientes en JARID1c exerçaient un phénotype pro-inflammatoire diminué par rapport aux témoins de type sauvage. Cette tendance s'est vérifiée à la fois à l'état d'équilibre et après une infection par le virus murin LCMV-Armstrong. Ensemble, notre étude examine la réponse immunitaire biaisée par le sexe et fournit des preuves pour soutenir la régulation épigénétique par JARID1c/d en tant que facteur impliqué dans l'immunité dimorphique sexuelle.

ACKNOWLEDGEMENTS

I would like to first and foremost extend my deepest gratitude to Professor Connie Krawczyk for granting me the opportunity to complete not only a Master's degree, but also an undergraduate honors thesis in her laboratory over the past 3 years. I struggle to think of anyone else who has taught me more about leadership. Her calm, understanding, logical and enthusiastic approach to science and life has left an impression on me that will pay great dividends in my future career. I would also like to thank Dr. Krawczyk for inviting me to complete my Master's work at the Van Andel Institute, which has provided a challenging yet exceptionally rewarding experience. I would also like to thank all of my current and past lab members: Hannah Guak, Giselle Boukhaled, Brendan Cordeiro, So-Yoon Won, Benedeta Hasaj, Jacky Tung, Ryan Yang, Peter Jeon, Orsolya Lapohos, Colleen Russett and Frank Telfer from McGill, as well as Alex Vander Ark, Paula Davidson and Lukai Zhai from Van Andel for their constant support and for creating a proactive and welcoming learning environment that spurs intellectual curiosity. I would like to specifically thank Hannah Guak for guiding me throughout the past year and showing me how outstanding scientists should conduct themselves. I would further like to thank the members of my supervisory committee, Dr. Vincent Giguère, Dr. Ana Nijnik and Dr. John White for their frequent and helpful advice during the course of my degree. This project would not have been possible without the funding provided by CIHR, in particular the Sex as a Biological Variable Catalyst funding opportunity, FRQS and NSERC. I would like to thank both the Department of Physiology at McGill, as well as the Van Andel Institute for welcoming me with open arms and providing me with the resources to carry out this project. Last, but certainly not least, a special thanks goes out to my family and friends for their unconditional love and support, and to Janna for our stimulating discussions, for pushing me back on track when I lean off course, and for seeing me through the inevitable ups and downs of scientific research. Their contributions to my growth as a scientist cannot be overstated.

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LIST OF ABBREVIATIONS

5mC – 5-methylcytosine

5hmC – 5-hydroxymethylcytosine

AFA – Adaptive focused acoustics

AIRE – Autoimmune regulator

ANOVA – Analysis of variance

APC – Antigen-presenting cell

ARE – Androgen response element

ASD – Autism spectrum disorder

β-ME – Beta-mercaptoethanol

Batf3 – Basic leucine zipper transcriptional factor ATF-like 3

BCR – B-cell receptor

BMDC – Bone marrow-derived dendritic cell

BSA – Bovine serum albumin

BST2 – Bone marrow stromal antigen 2

CCL – C-C motif chemokine

CCR – C-C motif chemokine receptor

CD – Cluster of differentiation

cDC – Classical/conventional dendritic cell

CDCM – Complete dendritic cell media

CDP – Common dendritic cell precursor/progenitor

ChIP – Chromatin immunoprecipitation

CIITA – Class II major histocompatibility complex transactivator

CLP – Common lymphoid precursor/progenitor

CMP – Common myeloid precursor/progenitor

CTL – Cytotoxic T lymphocyte

CXCL – C-X-C motif chemokine ligand

CXXC – C-X-X-C finger protein

CyTOF – Mass cytometry by time-of-flight

DAPI – 4′,6-diamidino-2-phenylindole

DC – Dendritic cell

DHT – Dihydrotestosterone

DNA – Deoxyribonucleic acid

DNMT – DNA methyltransferase

ds – Double-stranded

DTT – 1,4-dithiothreitol

E2 – 17-beta-estradiol

EAE – Experimental autoimmune encephalomyelitis

ECL – Enhanced chemiluminescence

EDTA – Ethylenediaminetetraacetic acid

EGTA – ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid

EGFR – Epidermal growth factor receptor

EHMT2 – Euchromatic histone-lysine N-methyltransferase

ELISA – Enzyme-linked immunosorbent assay

ERE – Estrogen response element

FCS – Fetal calf serum

FDC – Follicular dendritic cells

FH – Follicle stimulating hormone

FL – Farnhab lab

Flt3L – FMS-like tyrosine kinase 3 ligand

FoxP3 – Forkhead box P3

GATA3 – GATA binding protein 3

GM-CSF – Granulocyte-macrophage colony-stimulating factor

GMP – Granulocyte-monocyte precursor/progenitor

H3K4 – Histone 3 Lysine 4

HAT – Histone acetyltransferase

HBSS – Hanks' balanced salt solution

HDAC – Histone deacetylase

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HGFR – Hepatocyte growth factor receptor

HIV – Human immunodeficiency virus

HMT – Histone methyltransferase

HPRT – Hypoxanthine phosphoribosyltransferase

HPV – Human papillomavirus

HSC – Hematopoietic stem cell

ICOSL – Inducible costimulator-ligand

IFN – Interferon

Ig – Immunoglobulin

IGEPAL – Octylphenoxypolyethoxyethanol

ILC – Innate lymphoid cell

IMD – Immune deficiency

IRF – Interferon regulatory factor

iTreg – Induced regulatory T cell

JARID1c – Jumonji AT-rich interactive domain 1c

JARID1d – Jumonji AT-rich interactive domain 1d

JMJD2D – Jumonji domain-containing protein 2D

JMJD3 – Jumonji domain-containing protein D3

KDM – Lysine-specific demethylase

KDM4D – Lysine-specific demethylase 4D

KDM5B – Lysine-specific demethylase 5B

KDM5c – Lysine-specific demethylase 5c

KDM6B – Lysine-specific demethylase 6B

LCMV – Lymphocytic choriomeningitis virus

LH – Luteinizing hormone

LPS – Lipopolysaccharide

LTα – Lymphotoxin alpha

MAPK – Mitogen-activated protein kinase

MHC – Major histocompatibility complex

mi – Micro

MLL – Mixed-lineage leukemia

moDC – Monocyte-derived dendritic cell

MS – Multiple sclerosis

MSCV – Murine stem cell virus

Mtb – *Mycobacterium tuberculosis*

mTEC – Medullary thymic epithelial cell

NEXSON – Nuclei extraction by sonication

NF-κB – nuclear factor kappa-light-chain-enhancer of activated B cells

NK – Natural killer

NOD – Non-obese diabetic

nTreg – Natural regulatory T cell

NuRD – Nucleosome remodeling deacetylase

P4 – Progesterone

PCGF6 – Polycomb Group Ring Finger 6

PAMP – Pathogen-associated molecular pattern

PBMC – Peripheral blood mononuclear cell

PBS – Phosphate-buffered saline

PCR – Polymerase chain reaction

pDC – Plasmacytoid dendritic cell

PFU – Plaque-forming unit

PHA – Phytohaemagglutinin

PIPES – Piperazine-N,N′-bis(2-ethanesulfonic acid)

PMA – Phorbol 12-myristate 13-acetate

pMHC – peptide:MHC

PMSF – phenylmethylsulfonyl fluoride

PRC1 – Polycomb repressive complex 1

PRR – Pattern recognition receptor

PVDF – Polyvinylidene fluoride

qPCR – Quantitative polymerase chain reaction

RING – Really interesting new gene

RNA – Ribonucleic acid

RORγT – Retinoid-related orphan receptor gamma t

RSV – Respiratory syncytial virus

SDS-PAGE – Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SETDB1 – SET domain bifurcated histone lysine methyltransferase 1

SIRPα – Signal regulatory protein alpha

SLE – Systemic lupus erythematosus

SLO – Secondary lymphoid organ

ss – Single-stranded

STAT6 – Signal transducer and activator of transcription 6

STING – Stimulator of interferon genes

SUV39H1 – Suppressor of variegation 3-9 homolog 1

T-bet – T-box protein expressed in T cells

TBS – Tris-buffered saline

Tcf4 – Transcription factor 4

TCR – T-cell receptor

TDG – Thymine-DNA glycosylase

TET – Ten-eleven translocation

T_{CM} – Central memory T cells

Tfh – Follicular helper T cells

TGF – Transforming growth factor

Th – T helper cell

TLR – Toll-like receptor

TNF – Tumor necrosis factor

Treg – Regulatory T cell

WDR5 – WD repeat-containing protein 5

XCR1 – X-C motif chemokine receptor 1

XLID – X-linked intellectual disability

Zeb2 – Zinc finger E-box binding homeobox 2

PREFACE AND CONTRIBUTIONS

My thesis work is focused on JARID1c and its Y-linked paralog JARID1d in the context of DC function. Since DCs play a significant role in guiding immune responses to various pathogens and in certain cases to self, an extensive review of DC function, activation and ontology will first be discussed in the introduction. In addition, the work presented in this study focuses on sex differences in immune response, specifically in DCs. Phenotypic differences in male and female immune response have been well-documented, however DCs have been poorly characterized in the context of sex-biased immunity, as well as the molecular mechanisms that contribute to the sex disparity. Hormonal and genetic factors have both been shown to contribute to sex-specific immunity. To study the molecular factors that contribute to male and female differences in immunity, known hormonal and genetic contributions to phenotypic differences between the sexes will be reviewed in the introduction. Since sex hormones vary extensively during the estrus cycle in females, the estrus cycle will also be briefly reviewed. Since JARID1c and JARID1d are H3K4 demethylases, an overview of epigenetics will be provided with a focus on documented epigenetic contributions to DC function. Finally, our current understanding of the biological and physiological roles of JARID1c and JARID1d will be discussed. This will be followed by the rationale for my M.Sc. project, a detailed presentation of the results of this study and a thorough discussion on relevant implications. It should be noted that the study was conceptualized with the help of Dr. Connie Krawczyk, and the experimental work was carried out by myself, with technical assistance provided by lab members under certain circumstances. I also performed all data analysis, and interpreted experiment results

1. INTRODUCTION

1.1 The mammalian immune system: innate immunity

The mammalian immune system has evolved to protect the host organism from a broad spectrum of infectious and non-infectious agents including microbes (bacteria, viruses, fungi and parasites), allergens and toxic chemicals. In mammals, the immune system includes two levels of organization that cooperate at the molecular and cellular levels to confer protection: innate immunity and adaptive immunity. Innate immunity is provided by (i) anatomical and physiological barriers and (ii) innate immune cells. Though primitive, innate immunity provides the host with an indiscriminate defense system against immunological threats. Adaptive immunity first evolved among jawed vertebrates (1) and allows for an immune response tailored to the infectious or non-infectious agent, as well as immunological memory against that agent. Failure in any of these systems threatens homeostatic function in the host and increases susceptibility to infection.

External structures, including the skin and mucosa, evolved as primary physical barriers against foreign challenges by preventing pathogen access and colonization. Most of the defensive functions of the skin, which is built from epithelial cells, are localized to the stratum corneum through its microflora, low water content and antimicrobial lipids (2, 3). Found adjacent to epithelial cells is a mucus layer consisting mainly of a complex web of mucin and antimicrobial peptides that cover epithelial surfaces and serves to impede microbial entry (4). However, loss of physical barrier integrity, either through injury or through pathogen-mediated processes, can facilitate infection.

Exposure to inflammatory foreign stimuli initiates the innate immune response, a germline-encoded program that consists of both chemical and cellular elements. The chemical components include soluble, bioactive molecules that are constitutively or inducibly present in biological fluids (such as complement proteins (5), defensins (6) and ficolins (7)) or are released by activated innate cells (including chemokines that attract inflammatory leukocytes to the infection site, and cytokines that mediate that inflammation (8, 9)). The cellular component consists of an arsenal of host cells that survey the microenvironment at barrier sites and are hard-wired to detect foreign antigens and orchestrate an immune response.

Innate immune cells include mast cells, macrophages, natural killer (NK) cells, monocytes, neutrophils, basophils, gamma delta T cells, innate lymphoid cells (ILCs), eosinophils and dendritic cells (DCs). Each of these cells possess at least one intrinsic property that contributes to pathogen clearance. For example, basophils can be directly activated by parasite-derived factors to produce large quantities of effector molecules that promote type II immune responses conducive to parasite clearance (10). Neutrophils produce neutrophil extracellular traps (NETs), which are composed of a meshwork of chromatin fibers decorated with antimicrobial peptides and enzymes that represent an important strategy to immobilize and kill invading microbes (11). Macrophages are highly phagocytic cells that kill microbes, engulf apoptotic cells and heavily contribute to wound healing by clearing cellular debris left over from an immune response or from injury (12, 13).

Several key features of innate immunity differentiate it from adaptive immunity. First, innate immune responses are immediate and germline-encoded; they are fully functional prior to, or rapidly following microbial challenge, and thus do not require prior exposure to the microbe. Second, the innate response is initiated by the recognition of patterns of molecular structures rather than microbe-specific antigens. Third, it has traditionally been thought that the innate system lacks immunological memory; repeated exposure to antigen will not enhance the magnitude or effectiveness of the innate response. However, recent studies have suggested that several innate cell types can be conditioned by their environment to either increase their sensitivity to antigen (termed “trained immunity”) or decrease their sensitivity (tolerance). A number of proteins have been implicated in this process, including β-glucan (14, 15) and the interleukin (IL)-1 family of cytokines (16). Trained immunity and tolerance can thus be viewed as a *de facto* innate immune memory that instructs innate immune responses to subsequent antigen exposure (17).

1.1.1 Dendritic cells

In certain circumstances, the first line of defense provided by innate immunity can be sufficient to clear a small dose of pathogen. For example, innate immunity is sufficient to clear *Chlamydia trachomatis* infection in the female mouse genital tract (18). However, elevated doses of low-grade immunogenic microbes, or low doses of high-grade immunogenic pathogens, may establish an infection that fails to be adequately cleared by the innate system alone. Under these circumstances,

the highly robust, versatile and antigen-specific adaptive immune response must be engaged to efficiently clear the pathogen.

DCs act as the antigenic messengers that link innate and adaptive immunity. Initially described by Ralph Steinman and Zanvil Cohn, DCs were originally identified as stellate cells with the capacity to stimulate naïve T cells (19–21). DCs can be classified into two functionally distinct branches; classical/conventional DCs (cDCs) and plasmacytoid DCs (pDCs). cDCs were originally identified by Steinman and Cohn and are specialized in antigen presentation. pDCs were isolated two decades later, and though they can also present antigen, they are better equipped for type I interferon production following viral infection (22–25). cDCs can be further divided into two major subsets, cDC1s and cDC2s, that differ in several ways including the cytokines they produce and the adaptive response they are likely to initiate (26) (reviewed below).

DCs are located at barrier sites (including the skin, gut and respiratory mucosa), and recent studies have begun to classify and characterize DC subsets and function in several tissues (27–29). In the absence of inflammatory antigen, resident DCs at barrier sites assume a highly dendritic morphology that is used to sample the microenvironment. Termed the steady-state, DCs in this context possess an enhanced capacity for antigen uptake and processing but poor ability to present antigen and activate naïve T cells (30). Upon exposure to inflammatory antigens, DCs transition to the activated/mature state capable of priming and sustaining naïve T cell expansion (31).

DCs are equipped with a family of membrane-bound and cytoplasmic pattern-recognition receptors (PRRs), of which the family of Toll-like receptors (TLRs) has been studied most extensively (32). PRRs possess the unique capacity to bind to pathogen-associated molecular patterns (PAMPs), which are characterized as foreign invariant molecular compounds common among entire groups of microbes and essential to pathogen survival (33). Ten germline-encoded TLRs have been identified in humans; each of which is specialized in recognizing distinct PAMPs from various intracellular and extracellular microbial sources (reviewed in (34, 35)). TLR-1 and TLR-2 recognize bacterial lipopeptides, lipoproteins and glycolipids. TLR-2 also binds to fungi-derived zymosan. TLR-3 and TLR-7/8 recognize viral dsRNA and ssRNA respectively. TLR-4 binds to lipopolysaccharide (LPS) from gram-negative bacteria, while TLR-5 recognizes bacterial flagellin. TLR-6 is specialized in the recognition of diacylated lipopeptides from mycoplasma, and

TLR-9 binds to unmethylated CpG DNA of bacterial, viral and protozoan origin. In contrast to other TLRs, TLR-10 has recently been shown to suppress inflammatory signaling by acting as a negative regulator of the MyD88 adaptor protein (36).

Cognate PAMP/PRR interaction by DCs triggers intracellular signaling cascades that ultimately prompts an irreversible differentiation program conducive to DC activation. For example, TLR-4 interaction with LPS at the cell surface results in downstream activation of the pro-inflammatory NF-κB, MAPK and IRF-3 pathways (37, 38). In order for robust T cell activation to occur, DCs must deliver three signals to the naïve T cell: antigen presentation, costimulation and cytokine production (39) (**Figure 1**). The pro-inflammatory pathways activated by PAMP/PRR interaction mediate the molecular adaptations made by DCs to deliver these three signals to T cells. Molecular adaptations include the loss of endocytic activity (40) and a reduction in antigen processing and presentation by MHC I and MHC II peptide complexes at the cell surface (Signal 1) (41, 42). Antigen presentation by steady-state DCs is transient; peptide:MHC II (pMHC) complexes assembled in intracellular compartments during antigen processing are unstable on the cell surface. Stable pMHC formation necessitates a decrease in the expression of MHC II genes following activation (43). MHC II regulation is provided by the transcriptional coactivator CIITA (encoded by the *Ciita* gene) (44). CIITA functions by nucleating the formation of an enhancesome upstream of MHC II gene loci, inducing their *de novo* transcription (45), as well as enhancing the constitutive expression of MHC I (18). Pro-inflammatory transcriptional programs induced by PAMP/PRR interaction downregulate *Ciita*, promoting stable pMHC complexes at the cell surface.

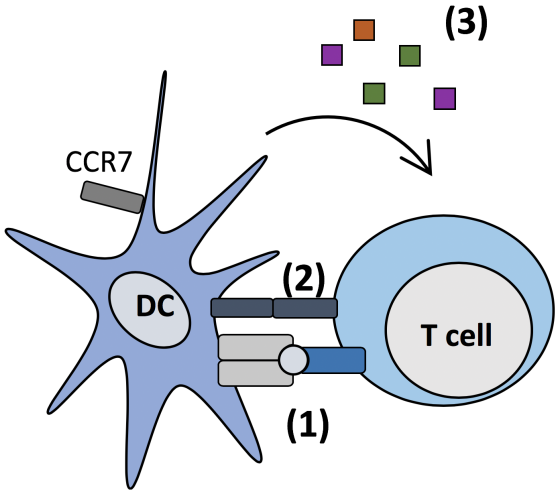

Figure 1. Three signal model of T cell activation by DCs. Activated DCs must deliver three signals to efficiently stimulate naïve T cells: (1) antigen presentation via pMHC-TCR interaction; (2) costimulation (e.g. CD80/86-CD28 interaction); (3) pro-inflammatory cytokines. Activated DCs also express CCR7 to allow for migration to T-cell zones of secondary lymphoid organs.

In addition to stable antigen presentation, co-stimulatory molecules (CD80, CD86, CD40) are upregulated (Signal 2) (46), and pro-inflammatory cytokines (IFN, IL-6, TNF, IL-12) are secreted to instruct naïve T cell differentiation into effector T cells (Signal 3) (47). Lastly, activated DCs will upregulate the expression of specific chemokine receptors, such as CCR7 (48). This allows for DC migration to T-cell zones of secondary lymphoid organs (SLOs) where they will encounter naïve, antigen-specific T cells and deliver the three molecular signals required for robust T cell activation (49, 50).

1.1.2 DC ontogeny

DC development in mammals initiates in the bone marrow, where hematopoietic stem cells (HSCs) differentiate into either the common lymphoid progenitor (CLP) or the common myeloid progenitor (CMP) (51). The CMP further differentiates into the granulocyte-monocyte progenitor (GMP), which, as the name implies, will give rise to granulocytes and monocytes, as well as the monocyte-macrophage DC progenitor (MDP) (52). MDPs differentiate into the common DC progenitor (CDP). CDPs then mature into pre-cDCs that migrate out of the bone marrow to populate almost every tissue, and further mature into cDCs (53) **(Figure 2)**.

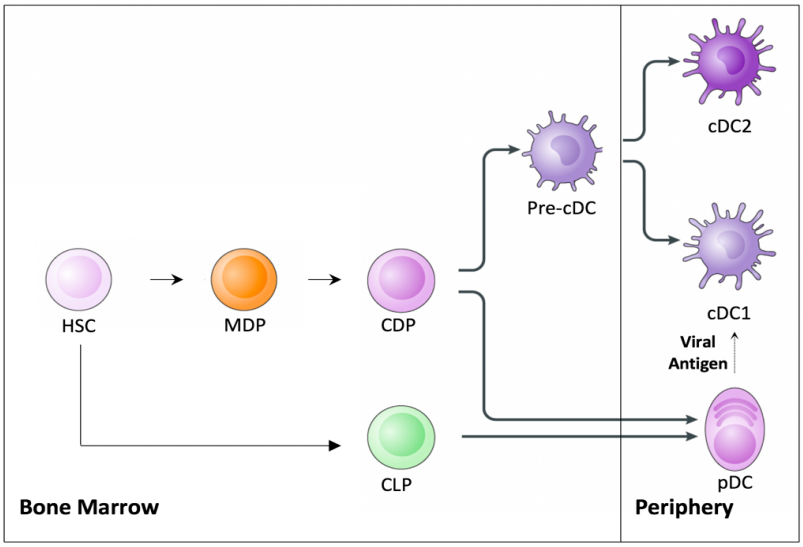

Figure 2. DCs are derived from hematopoietic precursors in the bone marrow. Hematopoietic stem cells (HSCs) in the bone marrow differentiate into macrophage-dendritic cell progenitors (MDPs) that then differentiate into common DC progenitors (CDPs). CDPs can differentiate directly into pDCs or into pre-cDCs, which mature into cDC1s or cDC2s in the periphery. The common lymphoid progenitor (CLP) also matures into pDCs. Under certain conditions such as stimulation with viral antigen, pDCs can differentiate into cDC1s. Adapted from Eisenbarth, *Nat. Rev. Immunol.*, 2019 (53).

1.1.3 Classical/conventional dendritic cells (cDCs)

The use of lineage markers to identify DC subsets *in vivo* has proven challenging in both human and murine models. In mouse, DC identification historically relied on the surface expression of MHC II and CD11c (54, 55), which can also be expressed by macrophages in a tissue-specific context (56), thus resulting in poor DC identification. Indeed, advancements in flow cytometry and mass cytometry (CyTOF) have confirmed that reliance on MHC II and CD11c alone is insufficient for proper *in vivo* cDC identification (57). Instead, newer technologies have revealed that pre-DCs will differentiate into cDC1s and cDC2s (58–60). Pre-DCs are committed to the cDC1 or cDC2 lineage prior to migrating out of the bone marrow (61).

cDC1s and cDC2s are subdivided according to the expression of surface markers and specific transcription factors required for maturation and lineage commitment. At the transcriptional level, Batf3 and Irf8 commit pre-DCs to the cDC1 lineage (62, 63), whereas Irf4 commits pre-DCs to the cDC2 lineage (64). Further, an unsupervised, high-dimensional approach revealed that cDC1s and cDC2s express unique surface markers that can inform gating strategies that effectively excludes macrophages and can be used to study each subset individually (28). These markers include XCR1 on cDC1s and CD172a (SIRPα) on cDC2s (28, 65).

cDC1s and cDC2s possess non-redundant but complementary functions that work together to launch adaptive immunity (reviewed in (66)) **(Figure 3)**. cDC1s are highly specialized in cross-presentation of exogenous antigen on MHC I and are thus efficient at both presenting antigen to, and activating, $CD8^+$ T cells (63, 65). cDC1s also initiate type I immune responses by activating type 1 ILCs (ILC1s) and NK cells as well as promoting T_H1 polarization (67). cDC2s, on the other hand, are specialized in presenting antigen on MHC II molecules and are thus considered to be more efficient at differentiating $CD4^+$ T cells (51). For example, cDC2s have been shown to initiate type III immune responses via ILC3 activation and stimulate T_H17 differentiation to clear extracellular bacteria (68, 69). In this way, each cDC subset is specialized in recognizing a particular threat and mounting the innate and adaptive defenses best suited to clear it. However, several studies suggest that cDC1 and cDC2 functionality is not so dichotomous; cDC1s have been found to present antigen to $CD4^+$ T cells to induce regulatory T cell (Treg) differentiation (70), and there is also evidence to suggest that cDC2s are capable of cross-presentation (71).

1.1.4 Plasmacytoid DCs

HSCs in the bone marrow will give rise to both the CLP and CMP. While the CMP will differentiate into cDCs, the CLP will mature into pDCs. However, there is evidence to suggest that immature pDCs in the bone marrow are capable of differentiating into $CD8^+$ cDCs (cDC1s) following exposure to viral antigen (72, 73). pDCs are also believed to be long-lived cells, but may exist in a dormant state for most of their lifespan (72).

At the cellular level, pDCs derive their name from their round morphology similar to that of a plasma cell (74). Steady-state pDCs reside primarily in lymphoid organs, are identified by the expression of CD317/BST2 and Ly6C (75, 76), express low levels of CD11c, MHC II and costimulatory molecules, and are positive for the B cell marker B220 (77). However, pDCs express high levels of TLR7 and TLR9, which respond to ssRNA and unmethylated CpG islands respectively. pDCs are specialized in high-level secretion of type 1 interferon (IFN-α/β) in response to viruses and/or virus-derived nucleic acids (24, 78), implicating them as key effectors of antiviral immunity **(Figure 3)**. This distinguishing pDC feature can be attributed to a baseline expression of *Irf7* (the master regulator of IFN expression) (79, 80) and to post-translational mechanisms that prime pDCs for rapid IFN secretion following viral challenge (81). In response to influenza infection, pDCs will also secrete chemokines including CXCL8, CXCL10, CCL3 and CCL4 to attract immune cells to the site of inflammation (82).

Under certain conditions, pDCs secrete TGF-β and ICOSL to promote Treg expansion (83, 84). Further, pDC activation includes the secretion of other pro-inflammatory cytokines such as TNFα (85) and IL-12 (86), as well as the acquisition of antigen presentation capabilities (87). Together, these factors promote pDC-mediated $CD8^+$ T cell responses (88). Overall, steady-state pDCs resemble lymphocytes, but adopt a more cDC-like phenotype following activation by viral antigen (reviewed in (89)). pDCs are especially important for inducing plasma cell differentiation (90), establishing pDCs as a key link between innate and adaptive immunity.

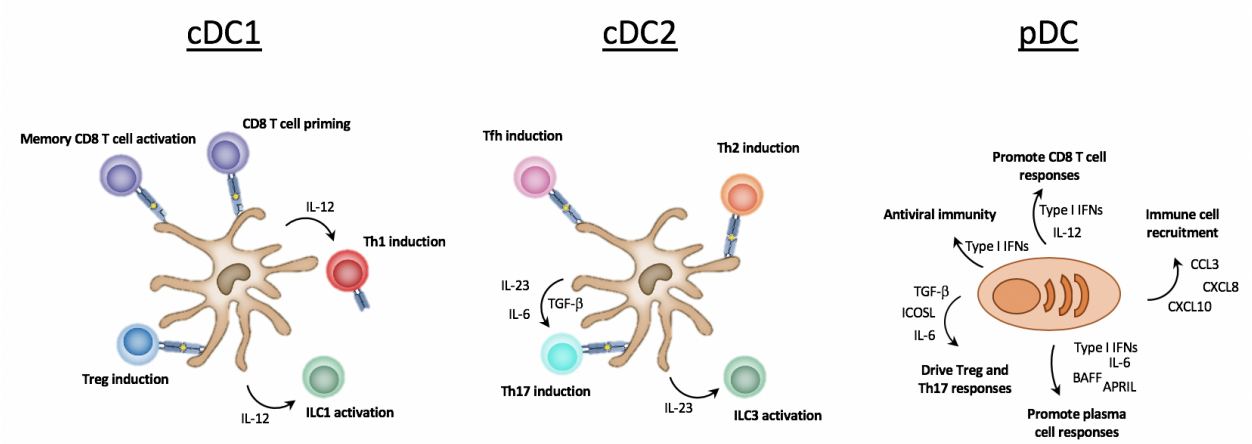

Figure 3. Functions of dendritic cell subsets. cDC1s and cDC2s are specialized in promoting type I and type II immune responses respectively, however cDC1s can also mediate type II responses and cDC2s can also mediate type I responses. pDCs are specialized in antiviral immune responses, but can also promote $CD8^+$ T cell and plasma cell activation. Adapted from Durai and Murphy, *Immunity*, 2016 (66) and Swiecki and Colonna, *Nat. Rev. Immunol*., 2015 (89).

1.1.5 BMDCs as a model for DC biology

DCs represent a relatively rare cell population *in vivo.* for example, DCs make up roughly 1-3% of a healthy human adult spleen (91). For this reason, much of our understanding of DC biology has been gleaned from *in vitro* models. In particular, treatment of murine hematopoietic precursors with granulocyte-macrophage colony-stimulating factor (GM-CSF) or with FMS-like tyrosine kinase 3 ligand (Flt3L) has been used to differentiate $CD11c^+MHCII^+$ cells that resemble *in vivo* tissue DCs (reviewed in (51)). These cells, termed bone marrow-derived dendritic cells (BMDCs), have been used extensively to characterize the biochemical and cellular features of DCs. GM-CSF and Flt3L-derived BMDCs are phenotypically different. Whereas GM-CSF-driven cultures do not give rise to pDCs (92), cultures supplemented with Flt3L give rise to three unique DC subsets including $CD172a^{high}$ and $CD172a^{low}$ cDC-like DCs and $B220^+$ pDCs (60, 93, 94). These cells have been shown to resemble their splenic counterparts at a phenotypic and functional level as measured by antigen presentation, cytokine production and TLR expression (60).

However, the resemblance between tissue DCs and BMDCs has been controversial. For example, GM-CSF-derived BMDC cultures have been shown to be heterogeneous with regards to hematopoietic origin and gene expression profiles (95). It has been suggested that $CD11c^+MHCII^+$ cells derived from BMDC cultures actually encompass at least two distinct cell populations that

are derived from either a monocytic or DC myelopoietic branch, and that both cell types display unique immune phenotypes following LPS stimulation (95). In an effort to inhibit monocyte differentiation, GM-CSF is often used in conjunction with IL-4 (96). Nonetheless, cell types derived from BMDC cultures remain heterogeneous and are pooled for subsequent analysis, resulting in experimental findings that can be difficult to interpret. Efforts have been made to discriminate the heterogeneity of BMDC cultures. For example, one group characterized adherent, loosely-adherent and adherent BMDC culture cells and found that non-adherent BMDCs were mostly homogenous and cDC-like based on their expression of costimulatory molecules (97). Though there are limitations associated with GM-CSF and Flt3L-driven BMDC culture systems, they nonetheless provide a means to generate large cell numbers that are phenotypically similar to tissue DCs. Still, further research is necessary to isolate BMDC subsets and to optimize the GM-CSF and Flt3L culture systems to produce homogenous DCs that resemble *in vivo* subsets.

1.2 The mammalian immune system: adaptive immunity

The innate immune system provides a fast-acting mechanism to protect the host from invading pathogens. However, complete pathogen clearance may necessitate a robust immune response provided by the adaptive immune system. For example, intracellular pathogens (such as viruses) exert their pathology within non-immune cells and in doing so are effectively hidden from the innate response.

Adaptive immunity is highly specific; while the germline-encoded membrane-bound receptors of innate cells limit their specificity, membrane-bound receptors on adaptive cells can distinguish subtle differences in antigen structure, enabling an immune response tailored to the invading pathogen (98). Robust immunological memory is also a distinguishing feature of adaptive immunity; due to the differentiation of memory lymphocytes following initial antigen exposure, recurrent exposure of the immune system to an antigen enhances the ability of the immune system to respond to that antigen in the future.

Importantly, two complementary mechanisms, central and peripheral tolerance, are in place to help ensure that the adaptive immune system responds to foreign antigens while remaining immunologically unresponsive to self-antigens (reviewed in (99)). Central tolerance occurs during

lymphocyte development in the thymus and bone marrow and refers to the processes of positive and negative selection that promote the death of self-reactive lymphocytes. Though efficient, central tolerance mechanisms are insufficient to delete all self-reactive lymphocytes, in part because not all self-antigens are expressed to a high-enough degree in the thymus and bone marrow (100, 101). Therefore, peripheral tolerance mechanisms are utilized to promote tolerance of lymphocytes that first encounter their cognate self-antigens outside of the thymus and bone marrow (e.g. antigens uniquely expressed during chronic infection). For example, anergy is a peripheral tolerance mechanism whereby T cells activated in the absence of costimulation enter a long-term hyporesponsive state (reviewed in (102)). A break in tolerance, whereby an adaptive immune response is mounted toward self-molecules (such as myelin) can induce severe autoimmune pathology that greatly endangers the host (103).

1.2.1 T cell development

Adaptive immunity is mediated by two major lymphocyte populations that coordinate distinct immunological programs; T cells (which express the T cell receptor - TCR) drive cell-mediated adaptive immune responses, while B cells (which express the B cell receptor - BCR) direct humoral immunity. During development, both T and B lymphocytes undergo VDJ recombination, whereby germline-encoded genes are re-assembled to produce a receptor with unique antigen specificity (104, 105). Since the assembled genes are randomly selected from a vast pool of germline-encoded genes, millions of receptor combinations are possible, enabling the specificity of adaptive immunity.

T cells develop in the thymus where they undergo the process of selection in addition to VDJ recombination (106). Briefly, medullary thymic epithelial cells (mTECs) express a transcriptional activator named autoimmune regulator (AIRE), which will stimulate the expression of numerous self-molecules (107, 108). T cells equipped with TCRs that react too strongly to these self-molecules will be deleted (negative selection), and remaining T cells enter the periphery (109). In addition to the TCR, T cells also express either CD4 or CD8 coreceptors, which determine the T cells' interaction with MHC molecules on antigen-presenting cells (APCs); $CD4^+$ T cells can bind to antigen presented by MHC II, while $CD8^+$ T cells can respond to MHC I. Since T cell activation requires TCR interaction with MHC molecules (which are self-molecules), T cell development in

the thymus also includes a process known as positive selection, whereby TCRs lacking affinity for self-molecules are deleted (110).

1.2.2 T cell activation and differentiation

T cell activation requires antigen presentation by professional APCs. There are three APCs, including DCs, macrophages, and to a smaller extent, activated B cells. However, DCs are unrivaled in their ability to activate T cells (20). Following PAMP/PRR interaction, DCs express CCR7 that is used to follow a chemokine gradient of CCL19 and CCL21 to the T cell zones of the draining lymph node (48). There, DCs communicate the three immunological signals (antigen presentation, costimulation and cytokine secretion) to T cells whose TCR is specific for the presented antigen (**Figure 1**). Once the pMHC:TCR interaction is stabilized, the DC conveys costimulation that activates the T cell. For example, CD80 and CD86 on DCs will bind to the costimulatory molecule CD28 on T cells, triggering IL-2 production by the T cell that acts in an autocrine fashion to promote expansion and survival (111). Finally, the cytokine profile produced by the DC, along with the strength of costimulation, will instruct $CD4^+$ T cell differentiation into one of several specialized effector T cell subsets. These subtypes include T_H1, T_H2, T_H17, Treg, T_H9 and T_{fh}, and each play a major role in mediating cell-mediated immunity through the secretion of specific cytokines (112). In addition to polarizing $CD4^+$ T cells, DCs also re-activate memory T cells following secondary antigen exposure (113).

T_H1 differentiation is initiated by IFNγ and IL-12, which will activate T-box transcription factor (T-bet), the master regulator of T_H1 differentiation. T_H1 cells are important for the elimination of intracellular pathogens, but are also associated with organ specific autoimmunity (114). They secrete IFNγ, IL-2 and lymphotoxin-α (LTα). IFNγ enhances phagocytic activity by activating macrophages and other phagocytes (115), while IL-2 promotes $CD8^+$ T cell proliferation required to kill infected cells (116). LFα is associated with autoimmune disease.

IL-4 and IL-2 initiate T_H2 differentiation by activating the master regulator GATA3, (117). T_H2 cells secrete a variety of cytokines, including IL-4, IL-5, IL-9, IL-13, IL-10 and IL-25. T_H2 immunity promotes the clearance of extracellular parasites (such as helminths), but also drive asthma pathology and other allergic disease (114, 118).

Several key cytokines promote T_H17 differentiation, including IL-6, IL-21, IL-23 and TGF-β. Together, these factors drive the expression of RORγt, the master regulator of T_H17 immunity (119). T_H17 cells secrete IL-17A, IL-21 and IL-22, which direct an immune response against extracellular bacteria and fungi, but have also been shown to exacerbate autoimmune pathology (120).

Tregs can be further classified into two subtypes depending on their lineage. Induced Tregs (iTregs) are defined as $FOXP3^+CD4^+CD25^+$ cells that develop in peripheral lymphoid organs following APC-mediated naive $CD4^+$ cell activation, which differ from natural Tregs (nTregs) that are released directly from the thymus already expressing FOXP3, the major Treg transcription factor (121). FOXP3 expression in iTregs is driven by TGF-β, and both iTregs and nTregs contribute to immunological tolerance by negatively regulating the immune response. Tregs secrete large amounts of IL-10, a potent inhibitory cytokine that suppresses the proinflammatory response and attenuates IgE production, thus limiting tissue damage incurred by an active immune response (122, 123), as well as IgE-driven allergic inflammation (124).

APCs presenting antigen on MHC I complexes can also activate and differentiate $CD8^+$ T cells into cytotoxic T lymphocytes (CTLs) (125, 126). CTLs function mainly to recognize infected host cells presenting the same antigen and to subsequently kill them by delivering cytotoxic proteins (mainly granzymes and perforin) into the host cell to trigger apoptosis (127).

1.2.3 B cell development

B cells develop in the bone marrow, where they undergo VDJ recombination and a selection process similar to T cells in the thymus (128). Immature B cells then migrate to the spleen where they differentiate into mature, naïve B cells. Mature B cells remain in the spleen or migrate to the B cell zones of lymph nodes where they encounter soluble antigen or larger antigens attached to the surface of neighboring cells including macrophages and follicular dendritic cells (FDCs) (129–131).

1.2.4 B cell activation

B cell activation can occur through a T-cell-dependent or T-cell-independent mechanism. Antigens

expressed in an organized, highly repetitive fashion can activate B cells independently of T cells by crosslinking BCRs in a multivalent fashion (132). Otherwise, robust B cell activation requires $CD4^+$ T cell help. In T-cell-dependent B cell activation, naïve $CD4^+$ T cells are activated in the T cell zones of lymph nodes by APCs and upregulate CD40L. The same antigen (in its native conformation) will be engulfed by B cells in the B cell zone, processed, and presented on MHC II complexes. Similar to DCs at barrier sites, B cells upregulate CD40 and CCR7 and migrate to the T cell zone by a gradient of CCL19 and CCL21. This enables T:B cell interaction that will activate the B cell through CD40 stimulation by CD40L on the activated $CD4^+$ T cell (133).

Activated B cells proliferate rapidly to form a "germinal center". Here, activated B cells undergo isotype switching and somatic hypermutation, during which the BCR is heavily mutated to improve specificity with its cognate antigen (134). Only B cells that bind their respective antigens with high affinity are selected to survive and differentiate into antibody-producing plasma cells that neutralize foreign antigen.

1.3 Sex differences in immunity

A substantial body of literature suggests sexual dimorphism in immune response; females typically mount a more potent response than males. An individual's biological sex (i.e. male, female) is defined by chromosomal content, reproductive organs and sex steroid levels, and is distinct from gender (i.e. man, woman), which refers to behaviors and activities defined by a society's cultural values. Although gender may dictate behaviors that influence the course of infection, sex as a biological variable has been studied more extensively. Sex-mediated differences are seen in both the innate and adaptive branches of immunity, and may be explained by both genetic and hormonal mediators (135).

1.3.1 Sex differences in innate immunity

Key features of innate immunity including PRRs, cellular composition and cytokine production differ between the sexes. For example, female mice are more sensitive to viral antigens; the X-linked PRR *Tlr7*, which detects ssRNA, escapes X-inactivation, resulting in higher *Tlr7* expression and increased sensitivity to viral stimuli (136). Indeed, human female peripheral blood

mononuclear cells (PBMCs) and pDCs stimulated *in vitro* with TLR-7 ligands secrete more IFNα than human males (137, 138). Interestingly, no sex bias is observed for TLR-9 stimulation (137), which is not X-linked. Moreover, TLR pathway and pro-inflammatory gene expression (including *MyD88*, *Irf7*, *Ifnb*, *Nfkb Ifng* and *Tnf*) are higher in female mice than in male mice (139, 140).

The composition and activity of innate cells differ between the sexes in mice. Notably, APCs from female mice have been shown to be more efficient at presenting peptides than APCs from male mice (141). Both neutrophils and macrophages from females are more phagocytic than their male counterparts (142). In addition, the draining lymph nodes of female SJL mice have been found to have a reduced number of ILC2s, which is thought to contribute to increased autoimmune pathology in mouse models of multiple sclerosis (MS) (143). Interestingly, androgens have been shown to negatively regulate ILC2 development (144), and the lungs of female C57BL/6 mice have been shown to harbor significantly greater ILC2 numbers than the lungs of male mice (145). The increased ILC2 population in female lungs may render females more susceptible to potent allergic respiratory inflammation (144, 145).

Several studies have shown a prominent sex-bias in cytokine and chemokine production, which represent major functions of innate immunity. For example, TLR-9 stimulation on PBMCs from human males results in greater IL-10 production, and thus an enhanced anti-inflammatory phenotype compared to human females (146). Although females are generally more pro-inflammatory than males, certain exceptions exist, particularly in the context of TLR-4. For example, murine *Tlr4* expression is greater on male cells than on female cells, thus LPS-activated neutrophils and peritoneal macrophages from mouse and human male mice are more pro-inflammatory than mouse and human female cells as measured by the production of TNF (147) and CXCL10 (148). Therefore, whereas females express more *Tlr7*/*TLR7* pathway genes than males, *Tlr4*/*TLR4* is more highly expressed on male cells. Since *Tlr7* and *Tlr4* respond to viral and bacterial stimuli respectively, the observed sex-bias in PRR expression may underlie fundamental differences in male and female innate immunity, and may seed downstream bias in adaptive immunity.

1.3.2 Sex differences in adaptive immunity

Multiple studies have confirmed sex bias among $CD4^+$ T cells, $CD8^+$ T cells and B cells. For

example, adult females have more $CD4^+$ T cells and a higher CD4/CD8 ratio compared to age-matched males (149), and this finding has been described in multiple ethnic groups (150–152). The same studies found males to have a higher $CD8^+$ T cell frequency. However, females have more activated $CD4^+$ and $CD8^+$ T cells compared to males following PBMC stimulation *in vitro* (149, 153). Transcriptional analyses corroborate these findings; T cells from females stimulated with PMA:ionomycin upregulate both antiviral and pro-inflammatory genes to a higher degree than T cells from males (154).

As previously discussed, the cytokine profile secreted by APCs instruct $CD4^+$ T cell polarization into one of several T cell subsets. The activity and distribution of these subsets differ significantly between the sexes. For example, females produce more T_H1-type cytokines (especially IFNγ) following parasitic infection (155), resulting in enhanced protection among females. PBMCs from females stimulated with mitogen phytohaemagglutinin (PHA) secrete elevated levels of T_H2-type cytokines (especially IL-4) compared to male PBMCs (156). Importantly, human studies suggest higher numbers of Tregs in adult males compared to females, contributing to a less inflammatory environment (157).

Sex as a biological variable has perhaps been most well characterized in the context of B cell immunity. Females have been shown to mount greater antibody responses and possess higher basal immunoglobulin levels and higher B cell numbers compared to males (149, 158, 159). Further, the majority of genes that show sexually dimorphic expression patterns are significantly upregulated in B cells from females compared to males (160).

1.3.3 Genetic mediators of sex differences in immunity

Males and females differ in their chromosomal content; males are XY, while females are XX. Many immune genes are encoded on the X chromosome. X-linked immune genes include those involved in both innate (*Tlr7*, *Tlr8*) and adaptive (*Il12rg*, *Foxp3*) immunity. Since numerous genes have been shown to escape X-inactivation, X-linked immune genes may be more highly expressed in XX females, contributing to sex differences in immune function (161). The Y chromosome encodes a limited set of genes, but polymorphisms in the Y chromosome have been shown to contribute to susceptibility to viral infection (162). The X chromosome also encodes 10% of the 800 miRNAs in the mammalian genome, whereas the Y chromosome only encodes two miRNAs

(163). miRNAs are short RNA transcripts with regulatory function. Few studies have looked at the role of miRNAs in mediating sex-biased immunity, however at least two X-linked miRNAs (miRNA-18 and miRNA-19) have been shown to influence sex differences in immune response (reviewed in (164, 165)). For example, miRNA-18 was found to be upregulated during relapse in MS patients and thus may contribute to the inflammatory processes that induce relapse (166). As an X-linked miRNA, miRNA-18 is more highly expressed in females, which may partially explain the higher incidence of MS and MS relapse among females. In addition, miRNA-19 has been found to promote T_H1, T_H2 and T_H17 differentiation (167–169), which may explain the enhanced T cell responses observed in females.

Genetic disorders implicating the X chromosome have been informative in describing the effects of the X chromosome on immune response. For instance, Klinefelter syndrome occurs when males acquire an extra X chromosome (XXY males). Men with Klinefelter syndrome respond more like XX females in the context of immune challenge; they have higher immunoglobulin concentrations, $CD4^+$ T cell numbers, CD4/CD8 T cell ratios and B cell numbers than XY males (170). In contrast, females with Turner syndrome (that is, females with only one X chromosome or with major X chromosome deletions) have lower IgG and IgM levels and fewer T and B cells compared to XX females (171). Interestingly, Klinefelter males develop autoimmune disorders at a rate similar to XX females, while Turner females are less likely than XX females to develop autoimmune pathologies (172, 173). Together, these findings implicate the X chromosome as a driver of autoimmune pathology.

1.3.4 Hormonal mediators of sex differences in immunity

Sex hormones include 17β-estradiol (E2), progesterone (P4) and androgens, and have been well studied in the context of inflammation. For example, androgen response elements (AREs) and estrogen response elements (EREs) are present in the promoters of several innate immunity genes (140), and half of the activated genes in female-derived T cells contain EREs in their promoters (154), suggesting that sex hormones heavily influence both innate and adaptive immunity differently between the sexes.

E2 has been shown to be a major mediator of innate immunity and thus adaptive responses downstream. Exogenous E2 treatment promotes neutrophil expansion (174), and enhances NK cell

cytotoxicity by promoting IFNγ production (175). However, E2 also reduces the expression of NK cell surface activation markers and their secretion of granzyme B (176). E2's effect on monocytes and macrophages is concentration-dependent; a low dose of E2 promotes a pro-inflammatory response characterized by elevated pro-inflammatory cytokine production (including IL-1, IL-6 and TNF), while a high dose of E2 instead promotes an anti-inflammatory response characterized by a reduction in these cytokines (177). E2 has also been found to enhance the expression of *Tlr4* on the surface of murine peritoneal macrophages (178).

E2 is especially important in driving DC development and function. E2 exposure *in vitro* facilitates HSC differentiation into functional $CD11c^+$ DCs (179, 180) and promotes pro-inflammatory cytokine and chemokine production *in vivo* (181–183). Together with GM-CSF, E2 promotes monocyte differentiation into inflammatory DCs that show enhanced pro-inflammatory cytokine expression, TLR-9 signaling, antigen processing and antigen presentation to naïve T cells (184).

E2 contributes to both cell-mediated and humoral immune responses. Briefly, low-dose E2 promotes T_H1-type responses and cell-mediated immunity by upregulating T-bet and IFNγ (185), whereas high-dose E2 favors T_H2-type responses and humoral immunity (186). E2 also supports Treg expansion *in vivo* (187) and *in vitro* (188), and also decreases IL-17 secretion by T_H17 cells (189). Importantly, physiological levels of E2 stimulate humoral responses to infection (190) in part by inducing somatic hypermutation and isotype switching (191).

P4 has an overall anti-inflammatory effect and signals through progesterone receptors expressed by several immune subtypes including NK cells, macrophages, DCs and T cells (192). Macrophages and DCs treated with P4 are less activated and produce lower amounts of pro-inflammatory cytokines (193, 194). TLR and NF-κB pathways are also antagonized by P4 (193, 195). Progesterone has also been shown to skew $CD4^+$ T cells towards T_H2 immunity as characterized by increased expression of T_H2-type cytokines (196). Finally, there is evidence to suggest that P4 can expand $FoxP3^+$ Tregs while preventing T_H17 differentiation (197).

Similar to progesterone, dihydrotestosterone (DHT), testosterone and other androgens generally suppress immune cell activity (155). Testosterone reduces NK cell activity *in vivo* (198) and reduces surface expression of TLR-4 on macrophages both *in vitro* and *in vivo* (199). Testosterone reduces pro-inflammatory cytokine secretion (mainly TNFα) by macrophages (200, 201) while

promoting IL-10 and TGF-β, thus reducing inflammation. The immune response of males with androgen deficiencies resemble those of females; low-androgen males produce more pro-inflammatory cytokines and have higher antibody titers and a higher CD4/CD8 T cell ratio (202–205). In contrast, female mice dosed with testosterone become less inflammatory (206). Taken together, these studies indicate that sex hormones greatly influence immune activity and therefore represent a major mediator of sex-biased immunity.

1.3.5 Sex differences in disease pathogenesis

Several pathologies including autoimmune disease, malignancy and infectious diseases possess a strong immune component. Since genetic and hormonal mediators dictate a considerable sex-bias in immune response, a strong sex-bias can also be found in disease pathogenesis **(Figure 4)**. 80% of all autoimmune patients in the US are female (207), with the female bias being most pronounced in Sjögren's syndrome, systemic lupus erythematosus (SLE) and thyroid diseases. Animal models have complemented epidemiological studies; the nonobese diabetic (NOD) model of spontaneous type 1 diabetes as well as certain murine strains of experimental autoimmune encephalomyelitis (EAE) show a similar female bias (208, 209). Female EAE mice have more activated T_H1 cells and express higher levels of IFNγ, whereas males show greater T_H17-type immunity (209, 210). Interestingly, castration of male mice exacerbates pathology while ovariectomy of female mice attenuates disease progression (211), implicating sex hormones in autoimmunity.

Sex is also an important factor in the pathogenesis and prognosis of several cancers. In general, males are at a high risk of malignancy (212) and a twofold greater risk of mortality from malignant cancers compared to females (213). Cancer treatments also show sex-specific outcomes; immune checkpoint inhibitors are more efficacious in females than in males (214). The cause of the observed male-bias is unknown, but it is hypothesized to be in part the result of a relatively suppressed immune system with a reduced capacity for tumor immunosurveillance (213).

Males are generally more susceptible to infectious diseases caused by bacteria, viruses, parasites and fungi (215). In particular, pre-pubescent males are at highest risk; newborn males are more susceptible to terminal infection (216), and school-aged males in several countries have higher rates of protozoan, trematode and nematode infections (217, 218). In addition, HIV-1-infected females often have reduced viral load and enhanced T cell activation compared to males (218).

Together, epidemiological studies parallel mechanistic findings and highlight important sex differences that contribute to pathology. In spite of this growing body of evidence, immunology ranks the lowest of ten biological disciplines for reporting the biological sex of the animal or human subject, with fewer than 10% of original research articles analyzing data by sex (219).

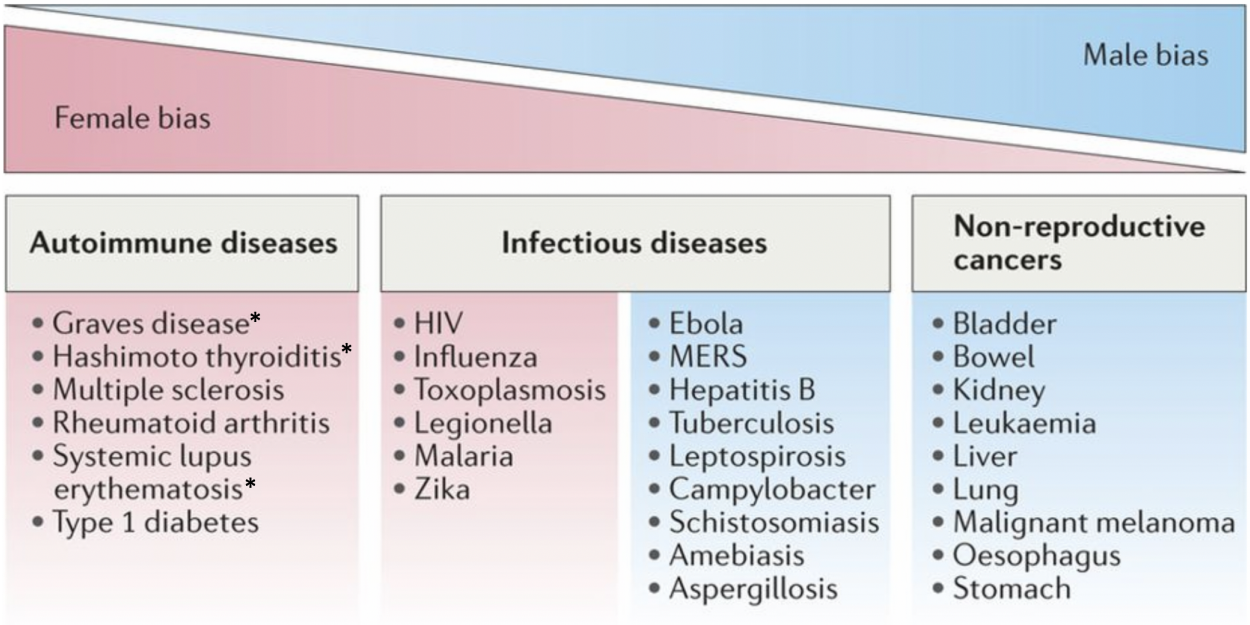

Figure 4. Sex bias in inflammatory disorders, infectious diseases and non-reproductive cancers. Females are at a higher risk of developing autoimmune pathologies, but men are at a higher risk of developing non-reproductive cancers. An asterix (*) identifies autoimmune diseases that females are at least four-times at higher risk of developing compared to males. Adapted from Klein and Flanagan, *Nat. Rev. Immunol*., 2016 (135).

1.3.6 The murine estrus cycle

In response to systemic changes in E2 and P4, the endometrium of the uterus undergoes extensive remodeling in preparation for embryo implantation, placenta formation and gestation. If fertilization fails to occur, the uterine lining is again remodeled. This process repeats itself until a successful fertilization event occurs (220, 221). Since the process is both cyclical and mediated mainly by female sex hormones (although non-steroid hormones such as pituitary gonadotrophins and prolactin also influence uterine remodeling events), it is referred to as the estrus cycle.

The murine estrus cycle requires 4-5 days to complete and includes four distinct stages: proestrus,

estrus, metestrus and diestrus (222, 223). Identifying the stage of the estrus cycle can be useful for mating purposes and for research purposes. As a result, a variety of methods have been developed to determine estrus stage and include vaginal cytology (222–224) , electrical impedance (225), biochemical analysis of urine (226) and visual observation of the external genitalia (227).

Vaginal cytology is one of the most widespread and practical approaches for identifying all four estrus cycle stages (228) **(Figure 5)**. Proestrus vaginal smears are characterized by the presence of clustered nucleated epithelial cells among anucleated cornified epithelial cells. The estrus stage consists almost entirely of cornified epithelial cells. Vaginal smears with a mix of cornified epithelial cells and leukocytes are indicative of metestrus, while a near-homogenous population of leukocytes correlates with the diestrus stage (223). In mice, systemic E2 increases gradually during metestrus and diestrus, and peaks in proestrus before declining rapidly prior to the estrus stage. In contrast, P4 peaks in diestrus (229, 230).

Similar to other mucosal tissues, the uterus harbors a diverse immune cell population, of which certain subsets have been shown to increase or decrease during different stages of the estrus cycle (231, 232). Interestingly, most immune cell populations in the uterus, particularly granulocytes, peak in number during the estrus stage (233). However, it is unknown whether similar fluctuations in immune cell number occur in other tissues, including in the spleen and lymph nodes.

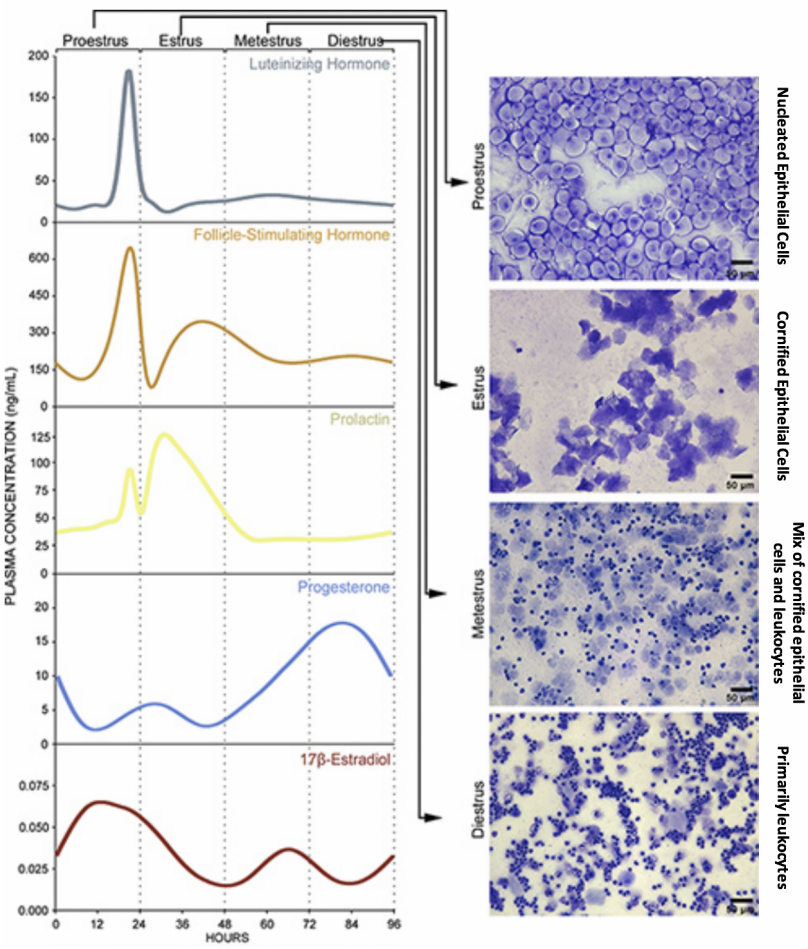

Figure 5. The murine estrus cycle comprises four distinct stages that can be identified by vaginal cytology. The proestrus stage is identified by the presence of clusters of nucleated epithelial cells as well as high serum concentrations of luteinizing hormone (LH), follicle-stimulating hormone (FH) and E2. Cornified epithelial cells and elevated serum prolactin are characteristic of the estrus stage. A mix of cornified epithelial cells and leukocytes identifies the metestrus stage, which is generally low in LH, FH and prolactin, but possesses increasing levels of E2 and P4. P4 peaks during the diestrus stage, which is characterized by the presence of primarily leukocytes. Adapted from McLean *et al. J. Vis. Exp.,* 2012 (323).

1.4 Epigenetic regulation of gene expression

Broadly defined, epigenetics refers to the regulation of gene expression by mechanisms other than changes in DNA sequence (234, 235). Epigenetic modifications regulate transcription factor accessibility to chromatin and include DNA methylation (236) and histone modifications (237, 238) (**Tables 1 & 2**). Epigenetic mechanisms have also emerged as an area of interest in the study of immune cell development and function (239–242), with a comprehensive atlas of chromatin accessibility in eighty-six immune cell types recently reported (243).

Enzyme Family	Examples	Catalyzed Residue(s)*	Transcriptional Response
DNA Methyltransferase (DNMT)	DNMT1 DNMT3a DNMT3b	Cytosine	Activation/Repression
DNA Demethylase	TET1-3	5-methylcytosine (5mC)**	Activation
Histone Acetyltransferase (HAT)	HAT1	H2AK5 ; H4K5, H4K12	Activation
	p300	H2AK5 ; H2BK5, K12, K15, K20; H3K9, K14, K18, K23, K27 ; H4K5	
	CBP	H2AK5 ; H2BK12, K15 ; H3K18, K23, K27	
	hGCN5	H3K9, K14, K18, K23	
	Tip60	H2AK5, H3K14, H4K5	
	PCAF	H3K14	
	SRC-1	H3K9, K14	
	OGA	H3K14	
	CLOCK	H3K14	
	hMOF	H4K16	
	ATF2	H2BK5, K12, K15 ; H4K5	
Histone Methyltransferase (HMT)	KMT2A-G	H3K4	Activation
	KMT2H	H3K4, H3K36	
	KMT3A	H3K36	
	KMT3B	H3K36, H4K20	
	KMT3C	H3K4, H3K36	
	KMT4	H3K79	
	KMT7	H3K4	
	CARM1	H3R2, R17, R26	
	PRMT1	H4R3	
Lysine Demethylase (KDM)	KDM3A-B	H3K9	Activation
	JMJD1C	H3K9	
	KDM4A	H3K9, H3K36, H1.4K26	Activation/Repression
	KDM4B	H3K9, H3K36, H1.4K26	
	KDM4C	H3K9, H3K36, H1.4K26	
	KDM4D	H3K9	Activation
	KDM6A	H3K27	
	KDM6B	H3K27	
	KDM7A	H3K9, H3K27	
	PHF8	H3K9	
	PHF2	H3K9	

Table 1. Enzymes mediating transcriptional activation. *Lysine (K), arginine (R). ** TET catalyzes 5mC to 5hmC, which will be repaired by thymine-DNA glycosylase (TDG) to yield non-methylated cytosine. Adapted from Boukhaled *et. al., Front. Immunol.*, 2019 (235).

Enzyme Family	Examples	Catalyzed Residue(s)*	Transcriptional Response
DNA Methyltransferase (DNMT)	DNMT1 DNMT3a DNMT3b	Cytosine	Activation/Repression
Histone Deacetylase (HDAC)	HDAC1-11 SIRT1 SIRT2 SIRT3 SIRT4-5 SIRT6 SIRT7	K residues, specificity unknown H1K26 ; H3K9, K14, K56 ; H4K16 H3K56 ; H4K16 H4K16 None H3K9, K56 H3K18	Repression
Histone Methyltransferase (HMT)	KMT1A-B KMT1C KMT1D KMT1E-F KMT5A-C KMT6 PRMT5 PRMT6	H3K9 H3K9, H3K27, H3K56 H3K9, H3K27 H3K9 H4K20 H3K9, H3K27 H3R8 H3R2	Repression
Lysine Demethylase (KDM)	KDM1A KDM1B KDM2A KDM2B	H3K4, H3K9 H3K4 H3K36 H3K36, H3K4	Repression
	KDM4A KDM4B KDM4C	H3K9, H3K36, H1.4K26 H3K9, H3K36, H1.4K26 H3K9, H3K36, H1.4K26	Activation/Repression
	KDM5A-D KDM8 NO66	H3K4 H3K36 H3K4, H3K36	Repression
Really Interesting New Gene (RING)	RING1A RING1B	H2AK119ub1 (E3 Ligase activity)	Repression

Table 2. Enzymes mediating transcriptional repression. *Lysine (K), arginine (R). Adapted from Boukhaled *et. al., Front. Immunol.*, 2019 (235).

1.4.1 DNA methylation

DNA methylation of cytosine residues (5-methylcytosine; 5mC) is mediated by the family of DNA methyltransferases (DNMTs) (236, 244, 245). The propagation of methylated DNA can occur in two contexts; DNMT1 at the replication fork mediates the inheritance of methylation signatures from a mother cell to daughter cells, while *de novo* methylation is driven primarily by DMNT3A/B (246). The relationship between a gene's methylation profile and its activation is complex. For example, DNA methylation in promoter regions antagonizes gene expression, whereas methylation within the gene body generally promotes gene activation (236).

DNA methylation is a reversible process; loss of 5mC can occur passively through cell division (where methylation is not copied) or can be actively mediated in a replication- independent manner

by Ten eleven translocation (TET) hydroxylases (244). TET hydroxylases catalyze the oxidation of 5mC to 5-hydroxymethylcytosine (5hmC) in an $Fe2^{+}$- and α-ketoglutarate-dependent manner (247). 5hmC can be iteratively oxidized by TET enzymes to other oxidized cytosines that are recognized and excised by thymine DNA glycosylase and replaced with an unmodified cytosine by base-excision repair. 5hmC is found in promoter gene bodies of actively transcribed genes, suggesting that it may have functions other than mediating DNA demethylation (244, 248, 249).

1.4.2 Histone modification

The enzymatic addition or removal of chemical groups to the four core histones (H2A, H2B, H3 and H4) modulate nucleosome structure and thus influence chromatin dynamics and gene activity by determining the location and activity of regulatory factors that control transcription (250). The most widely studied histone modifications are acetylation, methylation and ubiquitylation (251–256). The complete set of these marks constitute the histone code and ultimately determines the activation status of a gene (237).

In general, the acetylation of lysine residues provides a relaxed chromatin landscape amenable to transcriptional activation. Acetylation and deacetylation activities are mediated by histone acetyltransferases (HATs) and histone deacetylases (HDACs) respectively. Histone methylation is more complex and its regulatory effect on gene expression is dependent on context. For example, H3K4 trimethylation (H3K4me3) is considered a euchromatin mark generally associated with transcriptional activation, but H3K9 trimethylation (H3K9me3) is enriched in transcriptionally inactive loci and is thus associated with heterochromatin. Further, the two marks are thought to be incapable of co-localizing, since the H3K9 methyltransferases SETDB1, EHMT1, EHMT2 and SUV39H1 can only function in the absence of H3K4me3 (257, 258). There are many described histone methyltransferases (HMTs) and lysine demethylases (KDM) that target a wide range of lysine and arginine residues. Finally, ubiquitination has been mostly studied in the context of Really Interesting New Gene (RING) proteins that are associated with polycomb repressive complex 1 (PRC1) and deposit ubiquitin on H2A.

Profiling a set of well-studied histone marks can give an overall picture of the activity of a given gene or regulatory region. H3K36me3, H3K27Ac, and H3K4me3 are commonly enriched at active genes, whereas H3K27me3 and H3K9me3 are enriched at silenced genes. H3K4me1 is often found

at enhancers while H3K4me3 is enriched at active promoters (259). An enhancer is considered "poised" if it carries H3K4me1 alone or in combination with H3K27me3, and is considered active if H3K4me1 is in combination with H3K27ac (260–263). The genome-wide histone modification profile helps determine cellular identity in part by instructing binding events at specific chromosomal loci; histone modifications can alter the accessibility of transcriptional machinery at underlying genes, or can serve as beacons to recruit chromatin remodelers to either detect, deposit, or remove these histone marks (264). Any irregularities in this system can thus threaten cellular identity, potentially initiating disease (265, 266).

1.4.3 Epigenetic regulation of DC function

Expanding evidence suggests that epigenetic modifications contribute significantly to the regulation of DC function **(Table 3)**. Epigenetic mechanisms are implicated in the maintenance of the steady-state and responses to activating stimuli. For example, Polycomb group factor 6 (PCGF6) is a member of Polycomb repressive complex 1 (PRC1.6), which is known to promote gene silencing by catalyzing the monoubiquitylation of histone H2A by a RING E3 ligase (267, 268). In DCs, PCGF6 is necessary to maintain the steady-state (269). More specifically, PCGF6 functions to antagonize genes important for DC activation by promoting H3K4 demethylation.

As previously discussed, DCs must provide at least three signals to activate T cells: antigen presentation (signal 1), co-stimulation (signal 2), and lineage-specifying cytokine production (signal 3). The expression of proteins that constitute these signals are regulated transcriptionally, and increasing evidence suggests they are also regulated epigenetically. In steady-state splenic DCs, the expression of costimulatory molecules *Cd80* and *Cd86* is repressed by H3K27me3, which is relieved by the H3K27 demethylase KDM6B (JMJD3) during LPS stimulation (270). Furthermore, the repressive mark H3K9me3 was found to be enriched at the promoters of proinflammatory cytokines *Il12a, Il12b*, and *Il23* in steady-state BMDCs. Upregulation of these cytokines in LPS-activated BMDCs is largely governed by the recruitment of Trabid, a deubiquitinase that stabilizes the H3K9 demethylase KDM4D (JMJD2D) (271). Nucleosome Remodeling Deacetylase complex (NuRD) also reinforces DC activation by suppressing antigen uptake and processing (*Cd68, Slc11a*) and stimulating antigen presentation (*Ciita, H2-Aa*) (272). This occurs by stabilizing antigen-loaded MHC and by upregulation of specific costimulatory molecules and cytokines.

Immune mediators in the inflammatory microenvironment such as cytokines, chemokines and lipids can temper DC responses to activating stimuli. IL-10 has long been known to potently downregulate IL-12 production (273). HDAC11 represses *IL10* and in doing so, promotes the activation and IL-12 production of primary human DCs, which is required for efficient $CD4^+$ T cell differentiation (274). STAT6, a downstream effector of IL-4 signaling, also antagonizes histone acetylation at the *Il10* promoter following LPS stimulation (275). Lipid mediators, such as prostaglandins, can also be sculptors of the epigenome in DCs. Prostaglandin I2 suppresses H3K4me3 enrichment at the *TNFA* promoter by inhibiting components of a methyltransferase complex, MLL and WDR5, from translocating into the nucleus (276). A further study by the same group found that antagonism of the cysteinyl leukotriene receptor promotes an anti-inflammatory phenotype in human moDCs by enhancing H3 acetylation at the *IL10* promoter (277). Inhibiting chromatin remodelers could be an effective therapeutic avenue for inflammatory conditions, in particular those driven by TNFα or controlled by IL-10. Together these studies demonstrate that epigenetic mechanisms significantly contribute to the activation of DCs, and importantly, that factors in the inflammatory environment that modify the epigenome may have lasting effects on DC responsiveness.

	Enzyme	Function	Known target genes in DCs	Notes	Reference
Promotes DC activation	**KDM6B (JMJD3)**	H3K27 demethylase	*Cd80*, *Cd86*, *CD103*		(263)
	WDR5	H3K4 methyltransferase	*TNFA*		(269)
	KDM4D (JMJD2D)	H3K9 demethylase	*Il12*, *Il23*	Recruited by Trabid	(264)
	NuRD complex (HDAC1, HDAC2)	Histone deacetylation complex	*Tnfrsf9*, *Cd40*, *Cd80*, *Cd86*, *Cd68*, *Slc11a*, *Ciita*. *H2-Aa*	Recruited by Mbd2	(265)
	HDAC11	Histone deacetylase	*IL10*		(267)
Promotes DC steady-state	**PCGF6**	Transcriptional repressor	*Ciita*, *H2-Ab1*, *Il12a*, *Il12b*	Forms complex with KDM5C	(262)
	KDM5B	H3K4 demethylase	*Ifnb*, *Il6*, *Tnfa*	Upregulated by RSV	(271)
	HDAC2	Histone deacetylase	*Il6*	Recruited by Tet2	(273)

Table 3. Epigenetic factors that influence DC activity. Adapted from Boukhaled *et. al., Front. Immunol.*, 2019 (235).

1.4.4 DC epigenetics in inflammatory diseases

The study of antiviral immunity has provided key insights into the contribution of epigenetic mechanisms to DC activation. For example, respiratory syncytial virus (RSV) infection can be cleared by a T_H1 cytokine profile, but RSV-infected patients often mount a T_H2 cytokine response non-conducive to efficient RSV clearance. One group found aberrant T_H2 responses to be driven by an RSV-mediated upregulation of endogenous H3K4 demethylase KDM5B in several DC types, a transcriptional repressor of T_H1-associated cytokines important for RSV clearance (278). Furthermore, during viral infection in mice, TET2 is recruited by CXXC5 to the *Irf7* promoter to induce *Irf7* hypomethylation and expression in pDCs, resulting in the onset of an antiviral response (279). Given the role of TET2 in stabilizing HDAC2 at the *Il6* promoter (280), TET2 drives dichotomous DC functions; while TET2 can recruit HDAC2 to help repress *Il6* and resolve IL-6-driven inflammation, it can also initiate an inflammatory antiviral response by hypomethylating and upregulating *Irf7* expression.

DCs are an important driver of the inflammation associated with autoimmune disease. In particular, histone demethylases and hydroxylases containing the JmjC domain, including KDM5C (JARID1c), JMJD2D, and JMJD3, play a significant role in DC-mediated pathogenesis. KDM5C is an important regulator of the steady-state and activation of murine DCs (269). Trabid promotes experimental autoimmune encephalitis (EAE) by stabilizing JMJD2D at the *Il12* promoter, enhancing IL-12 production and immunopathology (271). However, JMJD3 inhibition limits EAE pathology and promotes a tolerogenic DC profile characterized by the reduced expression of CD80/86, and reduced secretion of proinflammatory cytokines IL-6, IFN-γ, and TNFα (270). Several diseases have been linked to aberrant DC methylation profiles in DCs. DNA hypermethylation at the *IRF8* promoter has been noted in Ocular Behcet's Disease (281) and Koyanagi-Harada Disease (282). In both cases, pharmacological DNA demethylation suppressed proinflammatory cytokine production by patient-derived DCs *ex vivo*. In contrast, genome-wide DNA demethylation was observed in the pDCs of patients with SLE, resulting in increased *IFNA* expression which could contribute to SLE onset (283).

1.4.5 Jumonji AT-rich interactive domain 1c (JARID1c)

JARID1c, also named lysine demethylase 5c (KDM5C) was first identified as an X-linked protein that escapes X-inactivation (284). It's Y-linked paralogue, JARID1d (KDM5D), was discovered as the protein product of the H-Y antigen, which mediates the rejection of male skin grafts by female recipients of the same inbred strain of rodents (285). JARID1 proteins are transcriptional repressors that act by demethylating H3K4 at gene promoters (286). JARID1c encodes 26 exons and six functional domains whose quaternary structure has been determined (287) (**Table 4**). The JmjC domain possesses the enzymatic activity of JARID1c (288–293). The JmjN domain is important for maintaining the structural integrity of the JmjC domain (288). JARID1c also encodes the ARID/BRIGHT domain, which can bind DNA in a sequence-specific and nonsequence specific manner (294, 295). However it is currently unknown whether JARID1c binds to DNA or to adaptor proteins. The PHD domain has been shown to bind H3K9me3 (296, 297). The function of the C5HC2 zinc finger and PHD2 domains are currently unknown, but the structurally similar C2HC4 zinc finger has been shown to be important for the demethylase activity of KDM3A, a JmjC-containing protein (293). In humans, *JARID1c* is most strongly expressed in the brain and skeletal muscle and lowest in the heart and liver (298).

Domain Name	Protein Location	Function	References
JmjN	aa. 13-59	Interacts with JmjC. The interaction is critical for JARID1c's overall stability and the catalytic function of JmjC	(287)
ARID/BRIGHT	aa. 76-184	A known DNA-binding domain that can bind DNA in a sequence-specific and non-sequence specific manner	(293, 294)
PHD1	aa. 326-374	Binds H3K9me3. Also interacts with JmjC to stabilize both itself and the histone peptide substrate. This interaction also positions H3K4me2/3 into the JmjC catalytic core.	(295, 296)
JmjC	aa. 501-617	Possesses the H3K4me3 and H3K4me2 demethylase catalytic activity. JmjC is also bound to two cofactors that are necessary for its proper function: Ferrous ion (Fe2+) and alpha-ketogluterate (2-oxoglutaric acid)	(287-292)
C5HC2 Zn finger	aa. 707-760	Required for the catalytic/demethylase activity of all JARID proteins	(292)
PHD2	aa. 1187-1250	Unknown	N/A

Table 4. Mouse and human JARID1c encode six domains. JARID1c encodes the JmjJ, ARID/BRIGHT, PHD1, JmjC, C5HC2 Zn finger and PHD2 domains. JmjC possesses JARID1c's H3K4 demethylase activity. The function of the PHD2 domain is unknown.

As they constitute an X-Y pair, JARID1c and JARID1d have been associated with sex-specific diseases; JARID1c in X-Linked Intellectual Disability (XLID) (298) and JARID1d in prostate cancer (299). However, JARID1c has been better characterized. Numerous studies have reported mutations in *JARID1c* among XLID patients (298, 300–308), and two studies reported *JARID1c* mutations in patients with autism spectrum disorder (ASD) (309, 310) (**Figure 6**). Many reported missense mutations in *JARID1c* have been shown to reduce demethylase activity *in vitro* (301). Interestingly, JARID1c functions are highly evolutionarily conserved; JARID1c-deficient mice exhibit abnormal learning and social behavior (311–313), *Drosophila* carrying an allele analogous to a disease-causing mutation in humans show learning and memory defects (314, 315), and *Jarid1c*-knockdown in zebrafish resulted in significant neurodevelopmental defects (316). Mechanistic work in zebrafish demonstrated that JARID1c is implicated in dendritic morphogenesis in neurons by regulating Netrin G2, which mediates neurite growth (317).

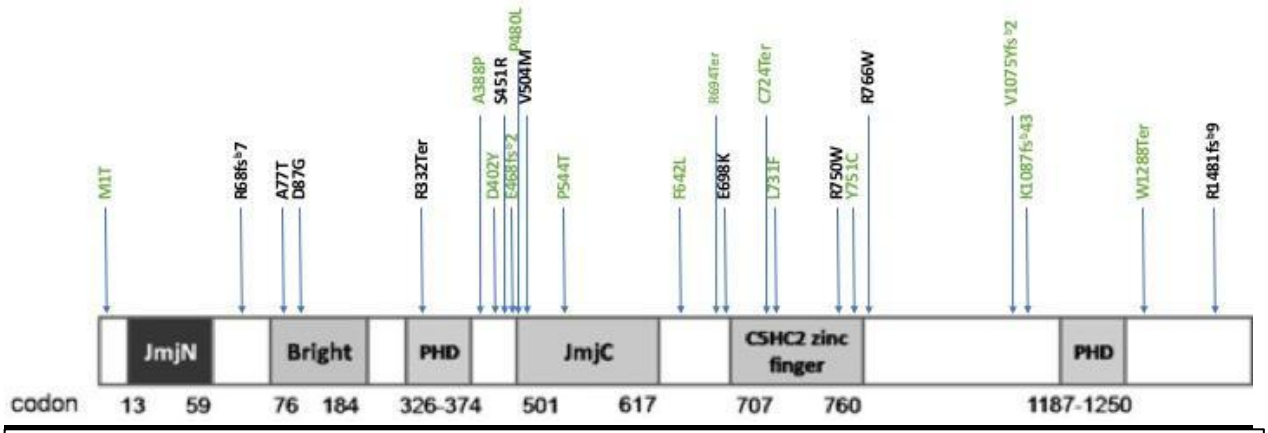

Figure 6. Reported JARID1c mutations. Mutations in green indicate mutations with a reported functional decrease in JARID1c enzymatic activity. Mutations in black have been reported among XLID patients, but have not been shown to decrease JARID1c enzymatic activity. Adapted from Jensen et. al., Am. J. Hum. Genet., 2005 (298).

More recently, studies have focused on JARID1c in the context of infection, immunity and cancer. For example, high-risk human papillomavirus (HPV) encodes the E6 protein that is required for cellular transformation following infection. E6 was found to function as an oncoprotein in part by destabilizing JARID1c to allow for the unregulated expression of *EGFR* and *HGFR* oncogenes (318). Furthermore, upon recognition of pathogen-derived or damaged self-DNA, TLR-9 signals through Stimulator of interferon genes (STING) to mount an interferon response. JARID1c was

found to bind to the *STING* promoter in multiple breast cancer cell lines thereby suppressing *STING* (319). Pharmacological inhibition of JARID1c also stimulated a robust interferon response. In addition, *Drosophila* encode the immune deficiency (IMD) pathway, which activates NF-κB to promote the production of antimicrobial peptides that constitute innate immunity in *Drosophila (320)*. A recent study found that KDM5 regulates component genes of the IMD pathway in order to promote host-commensal bacterial homeostasis in the gut (315). Interestingly, a reduction in KDM5 causes intestinal barrier dysfunction and changes in social behavior, suggesting a role for KDM5 in the gut-brain axis. Taken together, JARID1c has been well characterized in neuronal function, but its role in modulating the immune response is only beginning to be understood.

1.5 Rationale and goal of M.Sc. project

Biological sex is increasingly appreciated as an important variable in immune function. Females mount a more robust response than males that can be both beneficial and life-threatening; although an active immune system better protects females from terminal cancers and infectious disease, females are at a much higher risk of developing autoimmune pathology. Several studies have reported on the role of sex hormones and genetic factors in sex-biased immunity. However, the molecular mechanisms that underpin sex-specific immunity remain poorly characterized.

DCs bridge innate and adaptive immunity by presenting antigen in the context of self to antigen-specific T cells. Due to their direct influence on the outcome of an adaptive immune response, inappropriate DC activation can promote a misguided immune response to self-tissue, provoking autoimmune pathology. Studying the mechanisms involved in maintaining the DC steady-state in the absence of pro-inflammatory stimuli and the molecular players that drive activation therefore greatly contribute to our growing understanding of immune-mediated response to infection and pathology.

Our lab has shown that DCs are actively maintained in the steady-state by epigenetic factors such as PCGF6, and that PCGF6 is found in complex with the transcriptional repressor JARID1c. Epigenetic factors in activated DCs have also been reported to drive the expression of pro-inflammatory cytokines. Sex differences in immunity is well-documented. To better understand the genetic mediators that underpin these differences, we studied the importance of JARID1c and

JARID1d in DCs. Since X- and Y-linked transcriptional repressors are thought to exert non-redundant functions, **we hypothesize that JARID1c and JARID1d contribute to sex-specific immune responses of DCs.** We addressed this hypothesis in the following four objectives:

Objective 1: To characterize similarities and differences in DC activation and function between male and female mice both *in vitro* and *in vivo*. BMDCs were cultured in the presence of LPS, and DC activation was measured by pro-inflammatory gene transcription, expression of surface activation markers and pro-inflammatory cytokine secretion. Spleen and lymph nodes from age-matched male and female mice were also compared to characterize DC populations and DC activation at steady-state *in vivo*.

Objective 2: To determine whether estrus stage is a variable influencing immune cell function *in vivo*. Since sex hormones play a significant role in modulating the immune response, and since E2 and P4 levels both vary throughout the estrus cycle, we next aimed to determine whether estrus stage is a variable influencing immune cell function *in vivo*. Immune cells from the spleen and lymph nodes of age-matched female mice in two stages of the estrus cycle (estrus or met/diestrus) were compared to characterize variation among several immune cell types.

Objective 3: To determine whether JARID1c contributes to sex-biased immunity by regulating DC function *in vitro* and *in vivo*. JARID1c-deficient and wildtype BMDCs were cultured in the presence of LPS. DC activation was measured by pro-inflammatory gene transcription, expression of surface activation markers and pro-inflammatory cytokine secretion. Spleen and lymph nodes from age-matched *Jarid1c*$^{fl/fl}$ CD11c-Cre females, *Jarid1c*$^{y/fl}$ CD11c-Cre males and wildtype controls were also compared to characterize DC populations and DC activation both at steady-state and following infection with lymphocytic choriomeningitis virus (LCMV)-Armstrong (Arm).

Objective 4: To examine whether JARID1c localizes to the promoter region of a specific, distinct set of genes at both steady-state and during activation. We performed chromatin immunoprecipitation (ChIP)-qPCR on female-derived, wildtype BMDCs cultured with LPS following the recently-described NEXSON protocol.

Knowledge acquired through this study will contribute to our growing understanding of sex as a

biological variable of immune function and will provide key insights into the epigenetic mechanisms that drive DC activation. To the best of our knowledge, our work also represents the first study to separate females by estrus stage to study immune cell populations in SLOs, and thus delineate the short-term effects of E2 and P4 on immune cell function.

2. MATERIALS AND METHODS

2.1 Mice

Male and female C57BL/6 mice were either purchased from Charles River Laboratories at 6-8 weeks of age (Montreal, QC, Canada) or bred in-house under pathogen-free conditions at McGill University or at Van Andel Research Institute (Grand Rapids, Michigan, USA). *Jarid1c*$^{fl/wt}$ mice of the C57BL/6 background were obtained from Dr. Art Arnold (University of California, Los Angeles) (311), bred in-house and crossed with CD11c-Cre mice. All procedures conducted at McGill University were carried out in accordance with guidelines of the Canadian Council on Animal Care, as approved by the Animal Care Committee of McGill University. All procedures carried out at Van Andel Research Institute were done in compliance with the *Guidelines for the Care and Use of Animals for Scientific Research*, as approved by the Institutional Animal Care and Use Committee (IACUC) of the Van Andel Research Institute.

2.2 Murine BMDC culture

Bone marrow cells were cultured and differentiated into BMDCs as described (321). Briefly, bone marrow flushed from the tibias and femurs of C57BL/6 mice are seeded at $7.5x10^5$ - $1x10^6$ cells per well in 6-well non-tissue culture treated plates containing 3mL/well complete DC media (CDCM): 10% fetal calf serum (FCS, Corning), 2mM L-glutamine (Gibco), 100U/mL Penicillin/Streptomycin (Gibco) and 1:1000 β-ME (Gibco) supplemented with 20ng/mL GM-CSF (Peprotech). Cells are cultured at 37℃ for 8-9 days under 5% CO2 with CDCM+GM-CSF replenished on days 3,6 and 8. On day 8-9, cells were collected and stimulated with 100 ng/mL LPS (*Escherichia coli* serotype 0111:B4) (Invivogen) for the indicated amount of time.

2.3 RNA Extraction and qPCR

RNA was extracted from BMDCs following the Trizol reagent (Life Technologies) extraction protocol with one modification: RNA in Trizol is incubated at -80℃ overnight. RNA purity was increased by re-precipitation with ammonium acetate. RNA concentration and quality were determined using NanoDrop One technology (Thermo Scientific). RNA with a 260/280 and 260/230 > 2.00 were selected as input to synthesize cDNA using All-in-One Reverse Transcriptase MasterMix (ABM). qPCR was then performed with SensiFast SYBR (Bioline) using the CFX96

Real-Time PCR system (Bio-Rad). Relative fold change was calculated using the ΔΔCq method normalized to hypoxanthine guanine phosphoribosyltransferase (*Hprt*). qPCR graphs represent the average fold change from pooled biological replicates and error bars indicate the standard error of the mean. Primer sequences are listed below:

Target	**Forward Primer**	**Reverse Primer**
Hprt	AGG ACC TCT CGA AGT GTT GG	GGC TTT GTA TTT GGC TTT TCC
Birc2	TGG TGG CTT GAG ATG TTG GG	GCA CCA CTG TCT CTG TAG GG
Cd274	CTC GCC TGC AGA TAG TTC CC	GGG AAT CTG CAC TCC ATC GT
Cd40	GAG CCC TGT GAT TTG GCT CT	AGA TGG ACC GCT GTC AAC AA
Il12a	GTT CCA GGC CAT CAA CGC AG	TCC CAC AGG AGG TTT CTG GC
Il6	AAG CCA GAG TCC TTC AGA GAG	TGG TCC TTA GCC ACT CCT TCT
Cd80	CCT CGC TTC TCT TGG TTG GA	GGA GGG TCT TCT GGG GGT TT
Jarid1c	AGA AGG AGC TGG GGT TGT AC	CCA CAC ACG CAG ATA GAA GC
Jarid1d	AGT GAG CTG CTT CAG CGA TT	GGG TGA TTT GCG GTG TTT GT
Il12b	CTG GAG CAC TCC CCA TTC CT	CGC CTT TGC ATT GGA CTT CG
Cd86	CTT ACG GAA GCA CCC ACG AT	TCC ACG GAA ACA GCA TCT GAG
Ifnb	AGC AAG AGG AAA GAT TGA CGT GG	CCT GAA GAT CTC TGC TCG GAC
H2-Ab1	CGG CTT GAA CAG CCC AAT GT	CGC ACT TTG ATC TTG GCT GG

2.4 Flow cytometric analysis

BMDC surface staining: Following LPS stimulation, live BMDCs were washed once with flow wash (PBS, 2% FCS, 2mM EDTA, 0.5% sodium azide) and stained with fluorochrome-conjugated antibodies at their appropriate dilution. Typically, $2x10^5$ cells/well in 96-well plates are stained first with 50 ⎧l viability dye solution in PBS for 30 min at 4°C protected from light, followed by 50 ⎧l of antibody mix in flow wash for 30-45 min at 4°C protected from light. Cells are washed again with flow wash, and $4x10^4$ cells are acquired on a CytoFLEX (Beckman Coulter). Analysis was performed using FlowJo (Tree Star, Oregon, USA). See figure legends for analysis gates.

Splenocyte/lymph node surface staining: Following splenic and lymph node digestion (see below), splenocytes and lymph node cells are washed once with flow wash and stained with fluorochrome-conjugated antibodies at their appropriate dilution. Typically, $5x10^6$ splenocytes and $3x10^6$ lymph node cells in 96-well plates are stained first with 75 ⌠l viability dye solution in PBS for 30 min at 4°C protected from light, followed by 75 ⌠l of antibody mix in flow wash for 1h at 4°C protected from light. Cells are washed again in flow wash and fixed in 75 ⌠l IC fixation buffer (eBioscience) for 30 min at 4°C. Cells are washed again in flow wash, acquired on an Aurora (Cytek) and analyzed with FlowJo. See figure legends for analysis gates.

Splenocyte/lymph node intracellular staining: Cells are stained as described above with a few key modifications. Following staining with viability dye and the antibody mix, cells are washed again in flow wash and stained with 75 ⌠l FoxP3 fixation/Permeabilization buffer (eBioscience) for 30 min at 4°C protected from light. Cells are then resuspended in Permeabilization buffer and left at 4°C overnight. The next morning, cells are stained with 75 ⌠l FoxP3 antibody in Permeabilization buffer for 45-60 min. Cells are washed again in Permeabilization buffer, resuspended in flow wash and acquired on an Aurora (Cytek). Analysis was performed on FlowJo. All conjugated antibodies were anti-mouse and are listed below:

Antigen	Clone	Fluorophore(s)	Company
CD103	2E7	AF700	eBioscience
CD4	RM4-5	AF700	eBioscience
CD172a (SIRPα)	P84	APC	eBioscience
CD226	10E5	APC	eBioscience
CD40	1C10	APC	eBioscience
CD44	IM7	APC	eBioscience
CD62L	MEL-14	APC-Cy7	eBioscience
MHC II	M5/114.15.2	APC-Cy7	eBioscience
CD49b	DX5	APC-Cy7	eBioscience
CD80	16-10A1	BV421, PE	BioLegend
CD11b	M1/70	BV570	BioLegend

CD64	X54-5/7.1	BV605	BioLegend
CD279 (PD-1)	29F.1A12	BV605	BioLegend
NK1.1	PK136	BV605, FITC	BioLegend
CD45R (B220)	RA3-6B2	BV650	BioLegend
XCR1	ZET	BV650, FITC	BioLegend
CD69	H1.2F3	BV711	BioLegend
Ly-6C	HK1.4	BV711	BioLegend
CD40	1C10	SB780	eBioscience
CD11c	N418	eFluor-450, PerCP Cy5.5	eBioscience
CD223 (LAG-3)	C9B7W	eFluor-450	eBioscience
CD8α	53-6.7	eFluor-450, PE Cy7	eBioscience
Ly-6G (Gr-1)	1A8-Ly6g	eFluor450, FITC	eBioscience
CD19	1D3	FITC, PE/Dazzle 594	eBioscience
FOXP3	FJK-16s	eFluor-488	eBioscience
CD3	17A2	FITC	eBioscience
CD86	GL1	FITC	eBioscience
KLRG-1	2F1	FITC	eBioscience
Siglec-H	551	FITC	BioLegend
CD25	PC61.5	PE	eBioscience
F4/80	BM8	PE/Dazzle 594	BioLegend
CD317 (PDCA-1)	927	PE	eBioscience
CD127	A7R34	PE Cy7	eBioscience
CD274 (PD-L1)	10F.9G2	PE Cy7	BioLegend
CD26	H194-112	PerCP Cy5.5	eBioscience
CD3ε	145-2C11	PerCP Cy5.5	eBioscience

2.5 ELISA

Cell culture supernatants from male- and female-derived JARID1c-deficient BMDCs and sex-matched wildtype BMDCs were collected without stimulation or following 6h and 18h LPS

treatment. Supernatants were assayed for IL-12p40, IL-6, TNFα and IL-12p70 by enzyme-linked immunosorbent assay (ELISA) using Ready-SET-Go! Kits (eBioscience) according to the manufacturer's instructions.

2.6 Western blot

BMDCs were harvested with lysis buffer (see below). Cell lysates were sonicated using the CPX1800 sonicator (Fisherbrand) for 10 min on the high-power setting. Protein content in sonicated lysates was quantified as described (322). 30ug of whole cell lysate was diluted in 6X SDS sample buffer and electrophoresed on 8% polyacrylamide gels. Samples were typically electrophoresed at 70V for 30 min followed by 120V for 60 min in running buffer. Proteins resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) were transferred to polyvinylidene fluoride (PVDF) membranes by using the "Standard" program on the Trans-Blot Turbo Transfer System (Bio-Rad). Transferred membranes were stained with Ponceau S staining solution (CST), washed and blocked for 45 min with 5% (w/v) skim milk powder dissolved in TBS-T. Membranes were then washed with TBS-T and incubated overnight at 4°C with rabbit anti-mouse primary antibodies diluted in TBS-T with 5% bovine serum albumin (BSA). Membranes were washed three times with TBS-T and incubated with peroxidase-labeled goat anti-rabbit IgG (CST) for 45-60 min in 5% (w/v) skim milk powder dissolved in TBS-T. The antigen-antibody complex was visualized on the ChemiDoc MP Imaging System (Bio-Rad) using the SuperSignal West Dura Extended Duration enhanced chemiluminescence (ECL) detection reagent (Thermo Scientific), following manufacturer instructions. Quantitative western blot analysis was performed with Image Lab software (Bio-Rad)

Reagent	Composition
Lysis buffer	50mM Tris-HCl pH 7.4, 110mM NaCl, 5mM ethylenediaminetetraacetic acid (EDTA), 1% Chaps: 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
1x loading buffer	1.5% SDS (v/v), 10% glycerol (v/v), 62.5mM Tris-HCl (pH 6.8), 0.0025% bromophenol blue (v/v), 2% β-ME.
Resolving gel buffer	1.5M Tris-HCl pH 8.8
Stacking gel buffer	0.5M Tris-HCl pH 6.8
Running buffer	25mM Tris, 192mM glycine, 0.1% SDS (v/v), pH 8.3
Transfer buffer	25mM Tris, 192mM glycine, 0.1% SDS (v/v)
TBS-T	Tris-HCl buffered saline (TBS) with 0.1% Tween-20 (v/v)
Primary antibodies	JARID1c (Bethyl A301-034A): 1:1000 β-Actin (CST 4970): 1:1000
Secondary antibodies	Anti-rabbit, HRP-linked antibody (CST 7074). Dilute 1:5000 for JARID1c, 1:20000 for β-Actin.

2.7 Vaginal lavage

Vaginal lavage and estrus stage typing were performed as described (323). Briefly, the vaginal canal was flushed with sterile dH_2O and the collected vaginal cells were placed on a microscope slide and allowed to dry. The dry slide was immersed in 0.1% crystal violet solution for 2-3 min and washed twice in sterile dH_20 for 2-3 min. Excess water was blotted away with tissue paper. Glycerol and a coverslip were added to the smear, and the cells were visualized using light microscopy. Estrus stage was determined as follows: during proestrus, clusters of well-formed nucleated epithelial cells predominate. The presence of cornified squamous epithelial cells indicate the estrus stage, metestrus was defined by the presence of leukocytes, and the presence of both leukocytes and nucleated epithelial cells indicated the diestrus stage.

2.8 Splenic and lymph node DC preparation

Spleen: Spleens from C57BL/6 mice were injected with digestion buffer (1mg/mL Collagenase D (Sigma-Aldrich), 10ug/mL DNase I (Sigma-Aldrich) in HBSS+/+ (Gibco)) and digested for 20 min at 37°C. Spleens were then sliced and digested for an additional 20 min at 37°C. Digested splenocytes were homogenized through a 70µm filter and red blood cells were lysed with

ammonium chloride solution (150mM NH_4Cl, 10mM Tris, pH 7.0). Splenocytes were resuspended in flow wash and stained for flow cytometry (described above).

Lymph nodes: Lymph nodes from C57BL/6 mice were sliced and digested in digestion buffer for 20 min at 37°C. Digested cells were homogenized through a 70µm filter, resuspended in flow wash and stained for flow cytometry (described above).

2.9 *In vivo* LCMV injections

C57BL/6 mice were injected i.p. with $2x10^5$ plaque-forming units (PFU) of LCMV- Armstrong provided by the lab of Russell Jones. After 24h, spleens and lymph nodes (brachial, inguinal and mesenteric) were harvested, digested and stained with antibodies for flow cytometry (see above).

2.10 JARID1c chromatin immunoprecipitation (ChIP)

Following LPS stimulation, $5x10^6$ BMDCs were washed with PBS and crosslinked in fixation buffer (see below) with 0.6% formaldehyde (Covaris) for 5 min at RT on a shaking platform. The crosslinking reaction was quenched for 5 min with 125mM glycine. Cells were washed in PBS and cell pellets were flash-frozen and stored at -80°C for storage. Once thawed, nuclei were isolated as described (324). Briefly, cell pellets were resuspended in 1mL Farnhab Lab (FL) buffer and transferred to 1mL adaptive focused acoustics (AFA) tubes (Covaris). Nuclei were isolated by sonication for 2 min on an E220 Focused Ultrasonicator (Covaris) with the following settings: peak power 75W; duty factor 2% and 200 cycles/burst at 4°C. Sonication efficiency was tested by staining a sonicated aliquot 1:5000 with DAPI (4',6-diamidino-2-phenylindole). Stained nuclei were visualized using the EVOS FL Cell Imaging System (AMG). Isolated nuclei were collected by centrifugation (1000xg, 5min, 4°C) washed once in FL buffer, and sheared in shearing buffer for 9 min using the E220 Focused Ultrasonicator (peak power 140W, duty factor 5%, 200 cycles, 4°C). Shearing efficiency was assessed with an Agilent 2100 Bioanalyzer as per the manufacturer's instructions. Sonicated chromatin was quantified with a Lowry assay, and 30ug/ChIP was incubated in ChIP cocktail buffer overnight with 2.8ug anti-mouse JARID1c (Bethyl A301-034A). The antigen:antibody complex was incubated for 3h with Protein G Dynabeads (Invitrogen) pre-cleared overnight with 20µg of Herring Sperm DNA (Sigma-Aldrich). Bead-antibody-antigen complexes were then washed at 4°C for 5 min as follows: 3x WB1, 3x WB2, 2x WB1, 1X low-

salt TE. The antibody:antigen complex was then eluted from the beads by incubating beads in elution buffer for 15 min at 65℃ with gentle shaking. Eluents are reverse-crosslinked at 65℃ overnight, followed by successive treatments with DNase-free RNase (37℃, 1h) (Invitrogen) and Proteinase K (37℃, 2h) (Invitrogen). DNA was then purified on Qiagen MiniElute columns as per manufacturer instructions and used as a template for qPCR. qPCR data was analyzed using percent input. ChIP-qPCR primers align to the promoter regions of the genes listed below.

Reagent	Composition
Fixation buffer	50mM HEPES-KOH pH 7.6, 100mM NaCl, 1mM EDTA pH 8.0, 0.5mM EGTA pH 8.0
Farnham Lab buffer	5mM PIPES pH 8.0, 85mM KCl, 0.5% IGEPAL, supplemented with EDTA-free protease-inhibitor cocktail (Roche)
Shearing buffer	10mM Tris-HCl pH 8.0, 0.1% SDS, 1mM EDTA, supplemented with EDTA-free protease-inhibitor cocktail (Roche)
ChIP cocktail buffer	40mM Tris-HCl pH 7.6, 150mM NaCl, 1mM EDTA pH 8.0, 1% Triton X-100, 0.5% NP-40, supplemented with EDTA-free protease-inhibitor cocktail (Roche)
WB1 (Wash buffer 1)	50mM Tris HCl pH 7.6, 150mM NaCl, 5mM EDTA, 1% Triton X-100, 0.5% NP-40
WB2 (Wash buffer 2)	50mM Tris HCl pH 7.6, 500mM NaCl, 5mM EDTA, 1% Triton X-100, 0.5% NP-40
Low-salt TE	10mM Tris HCl pH 8.0, 50mM NaCl, 1mM EDTA, 0.5mM phenylmethylsulfonyl fluoride (PMSF) (added just prior to use)
Elution buffer	10mM Tris-HCl pH 8.0, 150mM NaCl, 10mM EDTA, 5mM 1,4-dithiothreitol (DTT), 1% SDS

Target	Forward Primer	Reverse Primer
Ciita	CCT TTG AGT CAA GGC AAC AA	GGA TGC TCT GAT CAA TGT GG
Il12a	ACC TGG ATG GCA GGA ACT AC	CTT GCC CAG GAG GTT ACA AT
Actb	TAG GCG TAA AGT TGG CTG TG	TCG CTC TCT CGT GGC TAG TA

2.11 Statistical analysis

Statistical analyses were performed on GraphPad Prism 8.0 using a paired two-tailed t-test or one-way ANOVA as indicated. Lines between bars indicate where direct comparisons were drawn between groups. Stars above lines indicate a statistically significant difference between groups. Differences were considered significant at $p<0.05$ (*=$p<0.05$, **=$p<0.01$, ***=$p<0.001$, ****=$p<0.0001$).

3. RESULTS

3.1 Male- and female-derived DCs manifest disparate inflammatory phenotypes *in vivo* and *in vitro*

Although numerous sex differences in immunity have been reported, sex-based differences in DC function and composition are not well described. To determine whether male- and female-derived BMDCs differ in their inflammatory potential, BMDCs from young (6-8 weeks) male and female C57BL/6 wildtype mice were stimulated with LPS (6h or 18h) or left unstimulated. DC activation was examined by analyzing transcript levels of pro-inflammatory genes by RT-qPCR, surface expression of activation markers by flow cytometry and cytokine secretion by ELISA. Female-derived BMDCs manifested a more pro-inflammatory phenotype following 6h LPS stimulation relative to male-derived BMDCs (**Figure 1**). At the transcript level, the expression of pro-inflammatory cytokine genes (*Ifnb, Il6, Il12b, Il12a*), surface activation markers (*Cd40*, *Cd80*) and the immune-suppressor *Cd274* were all significantly higher in BMDCs from females than BMDCs from males (**Figure 1A)**. The anti-inflammatory gene *Il10* was also more highly expressed in male-derived BMDCs compared to females (**Figure 1A**).

To evaluate surface marker expression by flow cytometry, BMDCs were first defined as $CD11c^{+}Gr1^{-}$ cells (**Figure 1B**). Following 6h LPS stimulation, a trend for increased MHC II, CD40 and CD86 expression was observed among female-derived BMDCs compared to male-derived BMDCs (**Figure 1C**). With the exception of CD40, this trend towards female-bias was no longer apparent after 18h LPS stimulation (**Figure 1C**). In contrast, a trend for increased PD-L1 expression was observed among female-derived BMDCs compared to male-derived BMDCs following 18h, but not 6h LPS stimulation (**Figure 1C**). A similar trend was also observed at the level of cytokine secretion; a 30-50% decrease was observed in the secretion of pro-inflammatory cytokines IL-12p40, IL12-p70, IL-6 and TNFα by male-derived BMDCs relative to female-derived BMDCs following 6h LPS stimulation (**Figure 1D**). Similar to co-stimulatory surface markers, the sex-bias was not present following 18h LPS stimulation, with the exception of IL-12p40 secretion, which remained ~40% lower in BMDCs from males relative to BMDCs from females (**Figure 1D**).

3.2 The murine estrus cycle influences immune cell subset composition in the spleen and lymph nodes

Hormonal effects on immune cell function, particularly the impact of estrogen, have been well-characterized in both innate and adaptive immunity (135). Further, previous studies have shown that immune cell populations in the uterus vary during the murine estrus cycle. Since serum concentrations of E2 and P4 cycle throughout the estrus cycle (325), the influence of estrus stage on immune cell populations of the innate and adaptive systems was determined. *In vivo* dendritic cell subsets were investigated first by flow cytometry. cDCs were defined as $MHCII^{+}Lin^{-}CD26^{+}CD11c^{+}$ cells; cDC1s were further identified as $XCR1^{+}$ cells and cDC2s as $CD172a^{+}$ cells (**Figure 2A)**. pDCs were identified as $MHCII^{+}Lin^{+}CD11b^{-}CD11c^{+}Ly6C^{+}PDCA1^{+}$ cells **(Figure 2A**). Vaginal lavages and cytology were also performed on a cohort of female C57BL/6 mice at steady-state as outlined in (323). Females were determined to be in the estrus or met/diestrus stage of the estrus cycle (**Figure 2B**). Proestrus mice were difficult to identify and hence excluded from this study. Mice were immediately sacrificed, and the spleen and lymph nodes (brachial and inguinal) were harvested, digested and stained for flow cytometry. Male mice were included to allow for comparison by biological sex.

The cDC1 subset was investigated first, and a trend for more cDC1s in the lymph nodes of females in estrus compared to the lymph nodes of females in met/diestrus was observed. Significantly more cDC1s were found in the spleens of females in estrus compared to the spleens of females in met/diestrus (**Figure 2C**). Data from females in estrus and met/diestrus were pooled to allow for comparison by sex. cDC1s in the spleen and lymph nodes were more abundant in male mice compared to female mice (**Figure 2C**). Analysis of the activation phenotype between female mice in estrus and met/diestrus revealed a trend for increased surface CD80 expression but decreased surface CD40 expression on splenic cDC1s from females in met/diestrus compared to females in estrus (**Figure 2D**). CD40 was also found to be more highly expressed on cDC1s from females in estrus compared to male-derived cDC1s (**Figure 2D**). Finally, data from females in estrus and met/diestrus were pooled to allow for comparison by biological sex. cDC1s from the lymph nodes of female mice expressed significantly higher levels of MHC II compared to cDC1s from the lymph nodes of males (**Figure 2E**). A trend for increased expression of MHC II by splenic cDC1s from females compared to splenic cDC1s from males was observed (**Figure 2E)**.

The cDC2 and pDC subsets in the spleens and lymph nodes of males, females in estrus and females in met/diestrus were then investigated. No significant differences were observed between females in estrus and females in met/diestrus with regards to absolute cDC2 count in the spleens and lymph nodes (**Figure 3A**). However, when females in estrus and females in met/diestrus were pooled to allow for comparison by biological sex, cDC2s were found to be significantly more abundant in the spleens and lymph nodes of male mice compared to female mice (**Figure 3A**). The surface expression of MHC II on male and female splenic and lymph node cDC2s was next investigated to determine activation status. A significant increase in MHC II expression on the surface of cDC2s in the spleen was observed, but no significant differences were found in lymph nodes (**Figure 3B**). Similar to the cDC2 subset, there were no significant differences between females in estrus and females in met/diestrus with regards to absolute pDC count in the spleens and lymph nodes (**Figure 4A**). However, when data from females in estrus and females in met/diestrus were pooled to allow for comparison by biological sex, pDCs were observed to be significantly more abundant in the lymph nodes, but not the spleens of male mice compared to female mice (**Figure 4A**). MHC II surface expression was then investigated on pDCs from male and female spleens and lymph nodes, and no significant differences between the sexes in either tissue were observed (**Figure 4B**).

Adaptive immune cells were next examined to determine whether they were similarly influenced by the estrus cycle in mice. B cells drive the humoral immune response by differentiating into antibody-producing plasma cells upon activation. Since females have been shown to mount more potent antibody responses than males (159), splenic B cells were studied in the context of the estrus cycle. A trend for elevated splenic B cell counts was observed among females in estrus compared to females in met/diestrus (**Figure 5A**). However, a greater proportion of splenic B cells from females in met/diestrus compared to splenic B cells from females in estrus expressed the B cell surface activation marker CD80 (**Figure 5A**). Splenic B cells from females in met/diestrus also expressed CD80 to a higher degree compared to splenic B cells from females in estrus (**Figure 5A**).

Splenic $CD4^+$ and $CD8^+$ T cells at different stages of the estrus cycle were then examined to study the influence of the estrus cycle on T cell activation and cell-mediated immunity. Similar to B cells, a trend for fewer splenic $CD4^+$ T cells was observed in females in met/diestrus compared to females in estrus (**Figure 5B**). However, splenic $CD4^+$ T cells from females in met/diestrus

expressed significantly more of the activation marker CD69 compared to splenic $CD4^+$ T cells from males and females in estrus (**Figure 5B**). A trend for increased numbers of anti-inflammatory Tregs in the spleen of females in met/diestrus relative to the spleens of males and females in estrus was also observed (**Figure 5B**). Further, a trend for a greater proportion of $CD69^+$ and $CD25^+$ splenic $CD8^+$ T cells from females in met/diestrus compared to females in estrus was observed (**Figure 5C**). A significantly greater proportion of $CD69^+$ and $CD25^+$ splenic $CD8^+$ T cells from females in met/diestrus compared to males was also observed (**Figure 5C**).

Naïve, effector and central memory (T_{CM}) $CD4^+$ T cell subsets were then studied. Naïve, effector and T_{CM} $CD4^+$ T cells were defined as $CD62L^+CD44^-$, $CD62L^-CD44^+$ and $CD62L^+CD44^+$ cells respectively, and each $CD4^+$ T cell population was investigated in the spleens of males, females in estrus and females in met/diestrus (**Figure 6A**). No significant differences were found in the relative proportions of naïve and effector splenic $CD4^+$ T cells between males, females in estrus and females in met/diestrus (**Figure 6B**). However, the relative proportion of T_{CM} cells in the spleen were found to be significantly greater among females in estrus and females in met/diestrus compared to males **(Figure 6B)**. Overall, these findings demonstrate estrus stage to be an important variable in immune cell function and may contribute to innate immunity as well as to both the humoral and cell-mediated branches of adaptive immunity.

3.3 JARID1c and JARID1d are differentially expressed following LPS stimulation and show sex-specific regulation

Although vital for proper immune function, the molecular mechanisms that underlie DC activation remain unclear. Our lab has shown that the transcriptional repressor PCGF6 suppresses DC activation *in vitro* (269). Since we also found PCGF6 to complex with X-linked JARID1c, JARID1c's expression dynamics were evaluated. Similar to many X-linked immune genes, *Jarid1c* was found to be more highly expressed in female-derived steady-state BMDCs than in male-derived steady-state BMDCs (**Figure 7A**), suggesting that *Jarid1c* partially escapes X-inactivation in female-derived BMDCs. The effect that LPS stimulation would have on *Jarid1c* and *Jarid1d* mRNA expression was then evaluated. Interestingly, *Jarid1c* and *Jarid1d* were differentially regulated following LPS stimulation; while *Jarid1c* was significantly upregulated with LPS stimulation in female-derived BMDCs, *Jarid1d* was significantly downregulated in

male-derived BMDCs (**Figure 7B**). In addition, *Jarid1c* expression was not significantly altered in male-derived LPS-activated BMDCs (**Figure 7B**).

Earlier studies have observed that LPS-driven DC activation is not a progressive process, but instead consists of precise functional stages characterized by the regulation of a specific gene subset (326). Since a wave-like model of DC activation would likely involve an epigenetic input, *Jarid1c* transcript and JARID1c protein expression in female-derived BMDCs at different timepoints following LPS activation were investigated. At the level of transcription, a trend for increased *Jarid1c* expression following 2h LPS stimulation was observed, and *Jarid1c* was significantly more expressed at 6h LPS compared to unstimulated BMDCs (**Figure 7C**). Interestingly, JARID1c protein expression was found to undergo a wave-like pattern following LPS stimulation; JARID1c was quickly increased as early as 0.2h post-stimulation, but was quickly decreased by 0.5h, and peaked again at 4h post-stimulation (**Figure 7D**).

Since JARID1c protein increases sharply soon after LPS stimulation, it was hypothesized that JARID1c mRNA may be regulated by miRNAs. TargetScan (327) was used to identify miRNAs that may potentially bind and regulate JARID1c mRNA, and a strong putative binding site for miR-142-3p.2 was identified (**Figure 7E**). miR-142-3p.2 has been shown to regulate phagocytosis and antigen presentation in DCs (328). Overall, JARID1c shows a highly dynamic expression pattern following LPS-stimulation that may be mediated by specific miRNAs. Further, *Jarid1c* and *Jarid1d* are differentially regulated in male- and female-derived BMDCs.

3.4 JARID1c-deficient male- and female-derived BMDCs are less pro-inflammatory compared to wildtype sex-matched BMDCs

A high proportion of immune-related genes are encoded on the X chromosome and escape X-inactivation, including genes involved in DC activation (136, 139). Since *Jarid1c* escapes X-inactivation in female-derived BMDCs (thus allowing for elevated *Jarid1c* expression in female-derived BMDCs), whether JARID1c and JARID1d contribute to sex differences in DC function was determined.

$CD11c^+$ BMDCs were sorted and JARID1c expression in JARID1c-deficient female-derived BMDCs was verified by western blot (**Figure 8A**). JARID1c was reduced by ~30% in $Jarid1c^{fl/wt}$

female-derived BMDCs and ~95% in JARID1c-deficient (*Jarid1c*$^{fl/fl}$) female-derived BMDCs (**Figure 8B**).

The activation phenotype of JARID1c-deficient and wildtype male- and female-derived BMDCs was then examined by analyzing transcript levels of pro-inflammatory genes by RT-qPCR with or without 6h LPS stimulation. LPS-activated JARID1c-deficient female-derived BMDCs were found to express significantly lower levels of pro-inflammatory cytokines (*Il12a*, *Il12b, Il6 and Ifnb*), costimulatory molecules (*Cd40*, *Cd80*, *Cd86*) and MHC II (*H2-Ab1*) compared to wildtype female-derived BMDCs (**Figure 8C**). However, JARID1c-deficient male-derived BMDCs were only found to express significantly lower levels of *Cd80* and *H2-Ab1* in comparison to wildtype male-derived BMDCs, suggesting that JARID1d may partially compensate for loss of JARID1c in most, but not all, aspects of DC activation. Notably, the sex-bias in pro-inflammatory gene regulation that had been previously observed for *Il12a*, *Il12b*, *Cd40* and *Cd80* in wildtype male- and female-derived BMDCs (**Figure 1A**) was no longer present between JARID1c-defcient male- and female-derived BMDCs (**Figure 8C**), suggesting that JARID1c may function as a genetic mediator of sex differences in DC activation.

Surface marker expression was then examined by flow cytometry. A trend for decreased MHC II, CD40, CD86 and CD80 expression was observed among JARID1c-deficient male- and female-derived BMDCs compared to sex-matched wildtype BMDCs (**Figure 9A**). Pro-inflammatory cytokine production (IL-12p40, TNFα and IL-12p70) was next investigated by ELISA and was observed to be significantly reduced in JARID1c-deficient male-and female-derived BMDCs compared to wildtype BMDCs following 6h LPS stimulation (**Figure 9B**). JARID1c-deficient female-derived BMDCs also secreted a significantly reduced amount of IL12-p70 compared to wildtype female-derived BMDCs following 18h LPS stimulation, and JARID1c-deficient male-derived BMDCs secreted significantly reduced amounts of IL-12p40 and IL-12p70 compared to wildtype male-derived BMDCs following 18h LPS stimulation (**Figure 9B**).

3.5 JARID1c-deficient cDC1s and cDC2s from males and females are differentially altered in a sex-dependent and subset-dependent manner

To determine whether JARID1c contributes to DC function *in vivo*, spleens from 10-12-week-old *Jarid1c*$^{fl/fl}$ CD11c-Cre females, *Jarid1c*$^{y/fl}$ CD11c-Cre males and littermate controls were harvested, and cDC1s and cDC2s were analyzed by flow. cDCs were identified as MHCII^{+}Lin^{-} CD26^{+}CD11c^{+} cells. cDC1s were identified as XCR1^{+}, cDC2s as CD172a^{+} (**Figure 2A**).

It was first demonstrated that JARID1c-deficient cDC1s, but not JARID1c-deficient cDC2s, were more abundant than their wildtype counterparts, suggesting that JARID1c may differentially impact cDC subsets (**Figure 10A**). cDC activation at steady-state was then investigated, and it was found that JARID1c-deficient cDCs were differentially activated in a sex-dependent and subset-dependent manner. Splenic female-derived JARID1c-deficient cDC1s, but not male-derived JARID1c-deficient cDC1s, expressed significantly more CD40 and PD-L1 relative to wildtype cDC1s (**Figure 10B**). A trend for increased CD80 expression in both male- and female-derived JARID1c-deficient cDC1s compared to sex-matched wildtype cDC1s was also observed (**Figure 10B**). Splenic female-derived JARID1c-deficient cDC2s expressed significantly lower levels of CD80 and PD-L1, but not CD40 compared to female-derived wildtype cDC2s (**Figure 10B**). Similarly, surface expression of PD-L1, but not CD80 or CD40 was significantly downregulated on male-derived JARID1c-deficient cDC2s (**Figure 10B**). These findings therefore indicate that JARID1c may play different roles in splenic cDC1s and cDC2s in male and female mice.

3.6 JARID1c-deficient pDCs show an altered composition in males and development defects in both males and females

During development in the bone marrow, CDPs commit to either the pre-cDC or pre-pDC lineage, migrate into the periphery and differentiate into cDCs and pDCs (62). Since significant differences in lymph node pDC counts between male and female wildtype mice had been observed (**Figure 4A**), the influence of JARID1c on pDC development was determined. pDCs were identified as MHCII^{+}Lin^{+}CD11b^{-}CD11c^{+}PDCA1^{+}Ly6C^{+} cells (**Figure 2A**). It was observed that splenic male- and female-derived JARID1c-deficient pDCs expressed significantly lower levels of the pDC lineage marker Ly6C at steady-state compared to male- and female-derived wildtype pDCs (**Figure 11A**). Similarly, female, but not male-derived JARID1c-deficient pDCs also expressed

significantly lower levels of the pDC lineage marker PDCA1 at steady-state compared to male- and female-derived wildtype pDCs (**Figure 11A**).

Absolute splenic pDC counts were then examined and it was found that male- and female-derived JARID1c-deficient pDCs were significantly more abundant compared to sex-matched wildtype pDCs (**Figure 11B**). The activation status of JARID1c-deficient pDCs was also investigated and it was found that male- and female-derived JARID1c-deficient pDCs expressed significantly higher levels of surface MHC II compared to sex-matched wildtype pDCs (**Figure 11C**). However, female, but not male-derived JARID1c-deficient pDCs expressed lower levels of CD80 compared to female-derived wildtype pDCs (**Figure 11C**). In addition to potentially regulating the cDC subset, the data presented in this study reveal a putative role for murine JARID1c in pDC activation and development in both sexes.

3.7 Male and female JARID1c-deficient cDCs show a reduced inflammatory phenotype following infection with LCMV-Armstrong

Since JARID1c-deficient BMDCs were found to mount a less potent inflammatory response upon LPS stimulation *in vitro* (**Figures 8 & 9**), whether a similar phenotype was present *in vivo* following 24h infection with LCMV-Armstrong (LCMV-Arm) was determined. Spleen and lymph nodes from *Jarid1c*$^{y/fl}$ CD11c-Cre males and *Jarid1c*$^{fl/fl}$ CD11c-Cre females injected with either $2x10^5$ PFU LCMV-Arm or PBS (Mock) were harvested and digested and cDCs were analyzed by flow cytometry (**Figure 12A**). Similar to the data obtained *in vitro*, JARID1c-deficient cDCs were less activated following LCMV-Arm infection. Male-derived JARID1c-deficient cDC1s in the spleen and lymph nodes infected with LCMV-Arm expressed lower levels of the activation markers CD80 and PD-L1 compared to male-derived wildtype cDC1s infected with LCMV-Arm (**Figure 12B**). Although a significant reduction in CD80 expression on the surface of female-derived JARID1c-deficient cDC1s infected with LCMV-Arm was also observed, there was a trend for increased PD-L1 expression on splenic cDC1s and no significant differences in the lymph nodes compared to female-derived wildtype cDC1s infected with LCMV-Arm (**Figure 12B**).

The effect of viral infection on JARID1c-deficient cDC2s was then examined. Female-derived JARID1c-deficient cDC2s in the spleen and lymph nodes infected with LCMV-Arm expressed significantly lower levels of CD80 and PD-L1 compared to female-derived wildtype cDC2s

infected with LCMV-Arm (**Figure 12C**). Male-derived JARID1c-deficient cDC2s infected with LCMV-Arm also showed reduced surface expression of PD-L1 compared to male-derived wildtype cDC2s infected with LCMV-Arm, but CD80 expression was reduced in the spleen only (**Figure 12C**). T *in vivo* results of this study therefore mirror the *in vitro* results (**Figures 8 & 9**), suggesting a role for JARID1c in cDC activation.

3.8 JARID1c differentially localizes to the promoter regions of *Ciita* or *Il12a* depending on DC activation status

As a H3K4 demethylase, JARID1c acts as a transcriptional repressor. To determine whether JARID1c localizes to the promoter regions of genes involved in DC activation, chromatin immunoprecipitation was performed followed by qPCR (ChIP-qPCR) on precipitated DNA. Since robust and reproducible ChIPs require optimal sonication, nuclear isolation by sonication (NEXSON) was used to generate 300-700bp fragments in a reproducible manner. Crosslinked BMDCs were gently sonicated and stained with DAPI to visualize nuclear isolation efficiency ((324); **Figure 13A**). 120s of sonication was found to efficiently isolate nuclei (**Figure 13B**).

A sonication timecourse was then performed on isolated nuclei to determine the optimal sonication length required to generate 300-700bp fragments. 9 min of sonication was found to produce DNA fragments of the correct size, as measured with an Agilent 2100 Bioanalyzer (**Figure 13C**).

Activated DCs upregulate the expression of pro-inflammatory cytokines including *Il12a*, and lose *Ciita* expression to allow for stable pMHC II complexes on the cell surface. Since JARID1c was found to modulate DC activation, whether JARID1c localizes to the promoter regions of *Ciita* or *Il12a* was determined. Preliminary findings of this study indicate that in the steady-state, JARID1c localizes to the promoter region of *Il12a* (**Figure 13D**). However, following LPS activation, JARID1c disassociates from the *Il12a* promoter and instead localizes to the *Ciita* promoter (**Figure 13D**). Since LPS stimulation is not thought to influence JARID1c localization to the β-actin (*Actb*) promoter, *Actb* was tested as a control. JARID1c binding at the *Actb* promoter was not found to change with LPS (**Figure 13D**). Together, the preliminary ChIP results of this study suggest that JARID1c may preferentially bind to a set of gene loci to either actively restrain DCs in the steady-state or contribute to their activation following exposure to an appropriate stimulus such as LPS.

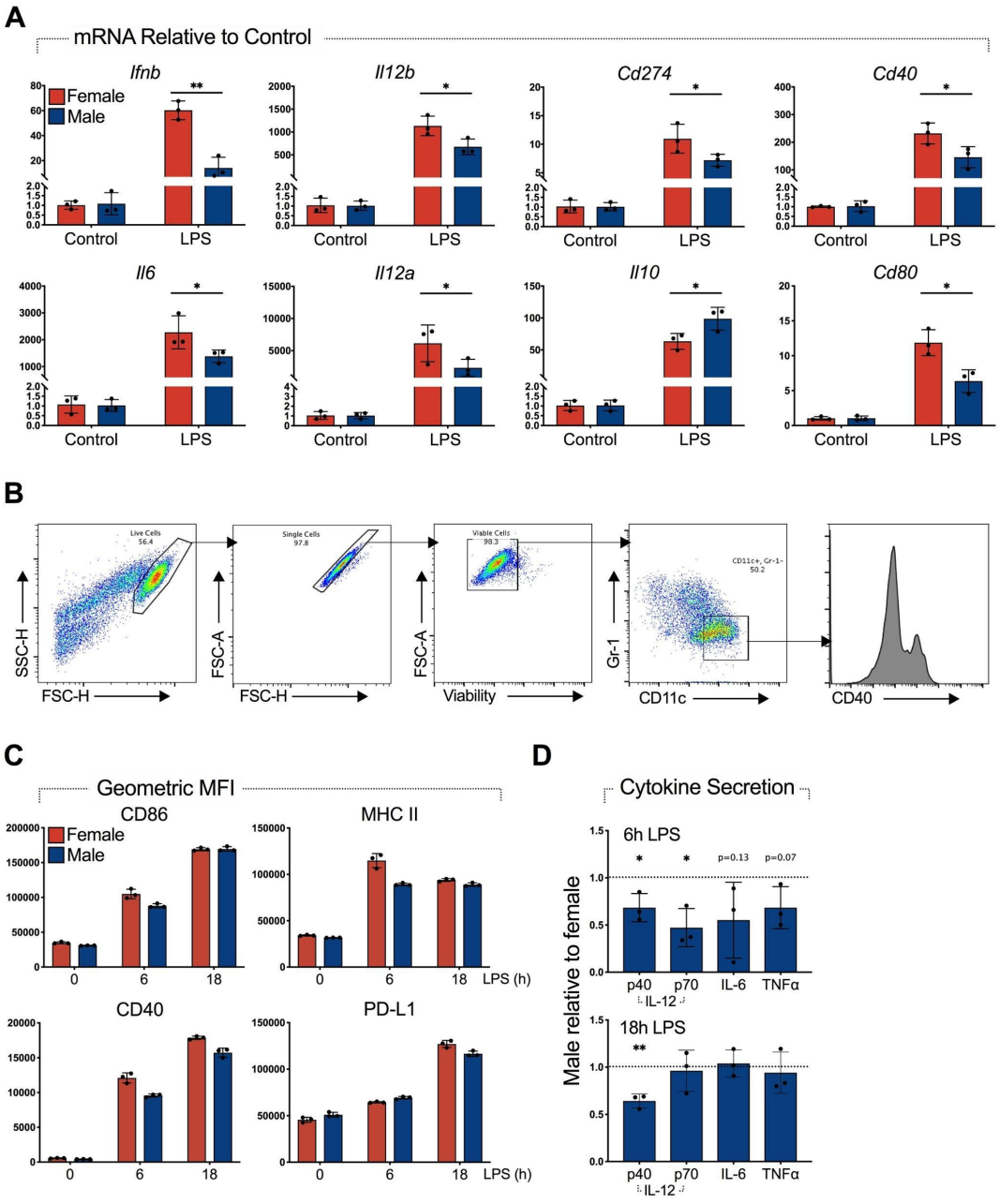

Figure 1. Female-derived BMDCs mount a more robust inflammatory response to LPS compared to male-derived BMDCs. BMDCs from young (6-8 weeks) C57BL/6 mice were stimulated for 6h or 18h with 100ng/mL LPS compared to the unstimulated control. **(A)** RT-qPCR was used to evaluate *Ifnb, Il12b, Cd274, Cd40, Il6, Il12a, Il10* and *Cd80* gene expression normalized to *Hprt* and relative to the mean of control female- and male-derived BMDCs. Data points represent independent experiments (n=3) ± SD. **(B)** Gating strategy for BMDCs analyzed by flow cytometry; viable BMDCs are defined as $CD11c^{+}Gr\text{-}1^{-}$ cells. **(C)** Flow cytometry was used to evaluate the expression of CD86, MHC II, CD40 and PD-L1**.** Data points are from one representative experiment of three independent experiments (n=3). Data are shown as mean ± SD. **(D)** Production of IL-12p40, IL-12p70, IL-6 and TNFα from LPS-stimulated male-derived BMDCs were measured by ELISA relative to the mean cytokine secretion from female-derived BMDCs. Data points represent independent experiments (n=3) and data are shown as ± SD. *p<0.05, **p<0.01, Student t-test.

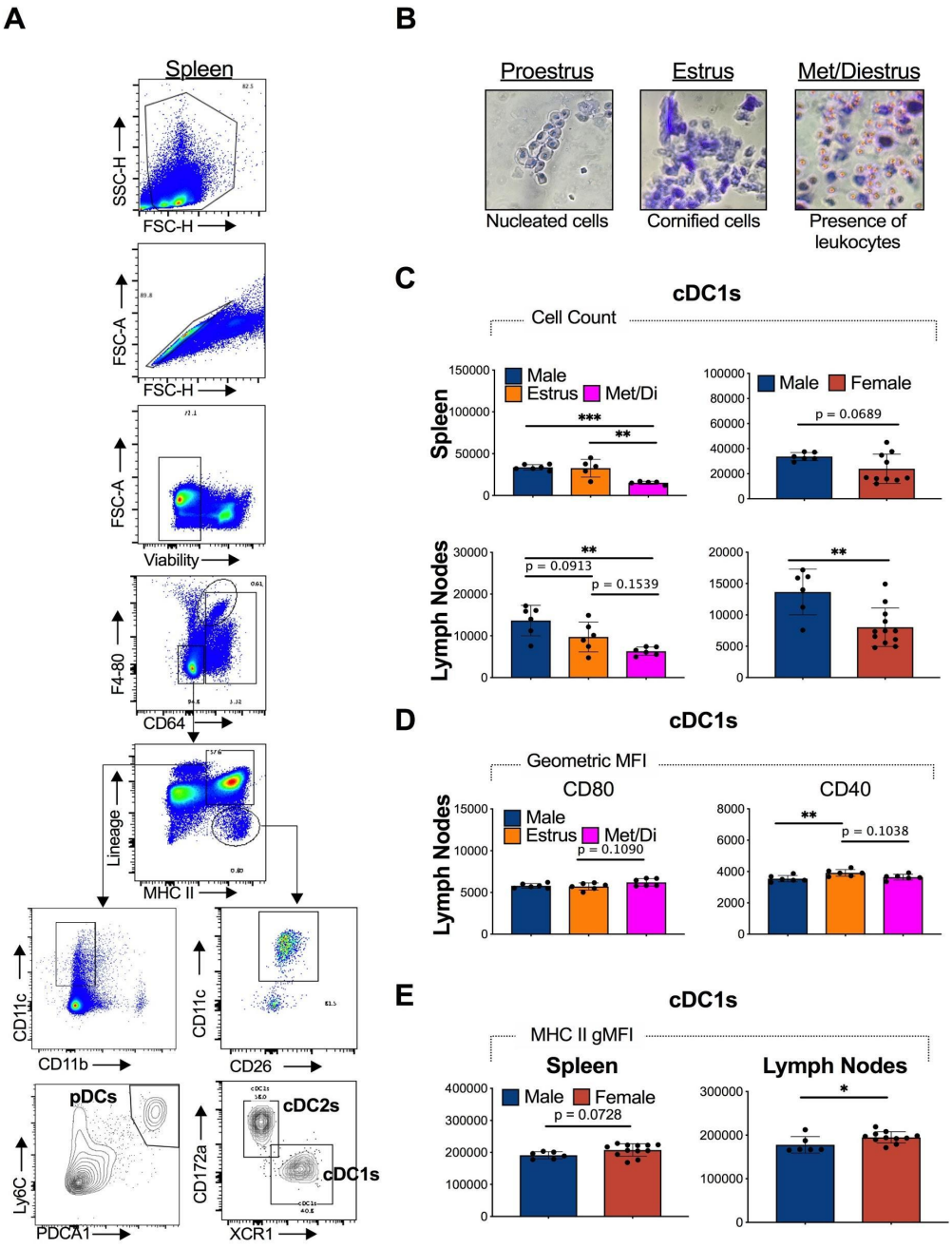

Figure 2. The murine estrus cycle modulates cDC1 composition and activation at steady-state *in vivo*. **(A)** Flow cytometry gating strategy to identify cDC1s, cDC2s and pDCs *in vivo.* **(B)** Vaginal cytology was performed on 8-week old C57BL/6 female mice immediately prior to sacrifice to determine estrus stage. **(C)** Spleens and lymph nodes from males, estrus females and met/diestrus females were digested and stained for flow cytometry. cDC1s from each group were counted. Estrus and met/diestrus females were then pooled and cDC1s from male and female spleens and lymph nodes were counted. **(D)** cDC1 activation in males, estrus females and met/diestrus females was further investigated by evaluating CD80 and CD40 surface expression by flow cytometry. **(E)** Estrus and met/diestrus females were pooled, and cDC1 activation in the spleens and lymph nodes of males and females were evaluating by measuring MHC II surface expression. Data points are from one experiment (n=6 for males and each estrus stage). Data are shown as mean ± SD. *p<0.05, **p<0.01, ***p<0.001, one-way ANOVA (comparing across estrus stage), Student t-test (male-female comparison).

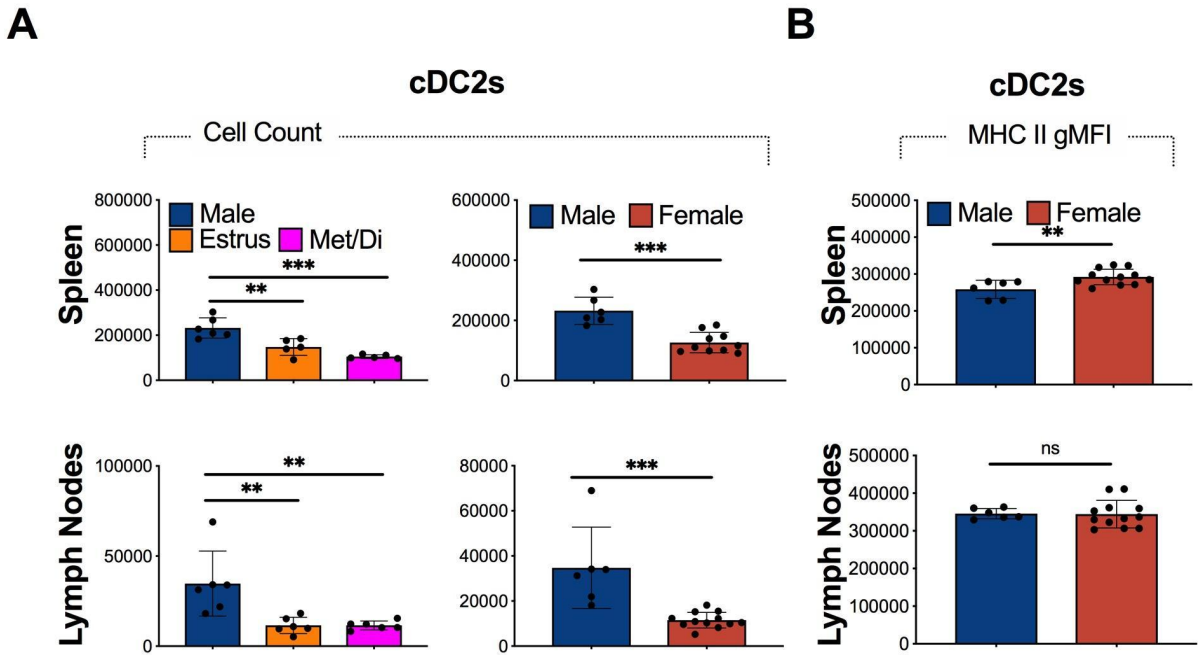

Figure 3. Male-derived cDC2s are more abundant than female-derived cDC2s, but splenic female-derived cDC2s are more activated. Vaginal cytology was performed on 8-week old C57BL/6 female mice immediately prior to sacrifice to determine estrus stage. Spleens and lymph nodes from males, estrus females and met/diestrus females were then digested and stained for flow cytometry. **(A)** cDC2s from each group were counted. Estrus and met/diestrus females were then pooled and cDC2s from male and female spleens and lymph nodes were counted. **(B)** Estrus and met/diestrus females were pooled, and cDC2 activation in the spleens and lymph nodes of males and females were evaluating by measuring MHC II surface expression. Data points are from one experiment (n=6 for males and each estrus stage). Data are shown as mean ± SD. ns = not significant, **p<0.01, ***p<0.001, one-way ANOVA (comparing across estrus stage), Student t-test (male-female comparison).

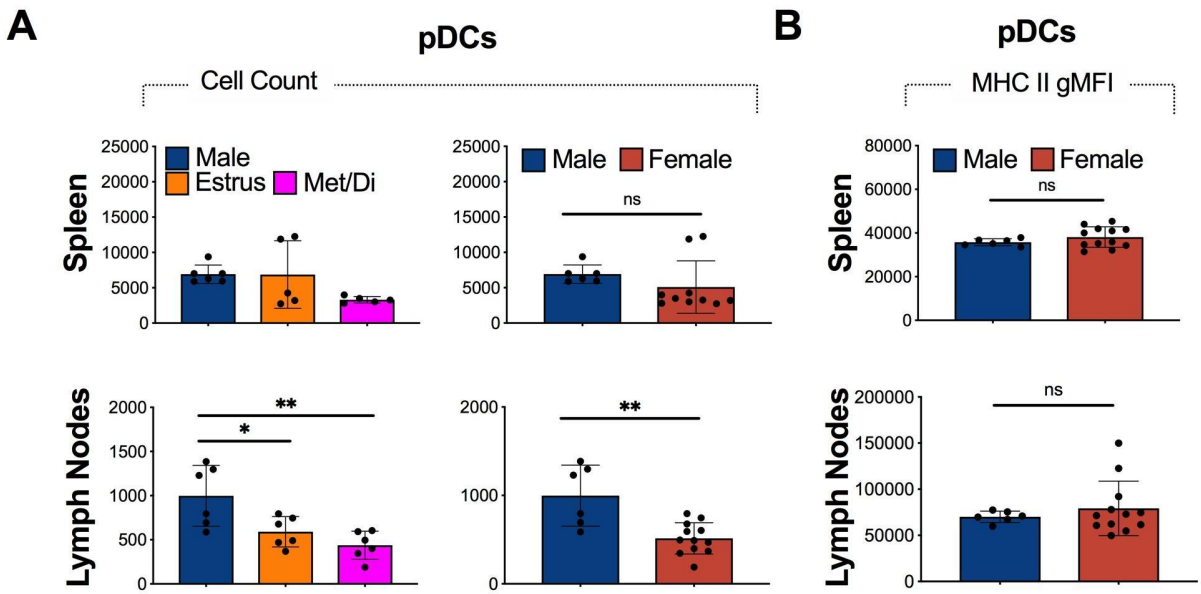

Figure 4. pDCs from males are more abundant than pDCs from females, but pDC composition does not differ across the estrus cycle. Vaginal cytology was performed on 8-week old C57BL/6 female mice immediately prior to sacrifice to determine estrus stage. Spleens and lymph nodes from males, estrus females and met/diestrus females were then digested and stained for flow cytometry. **(A)** pDCs from each group were counted. Estrus and met/diestrus females were then pooled and pDCs from male and female spleens and lymph nodes were counted. **(B)** Estrus and met/diestrus females were pooled, and pDC activation in the spleens and lymph nodes of males and females were evaluated by measuring MHC II surface expression. Data points are from one experiment (n=6 for males and for each estrus stage). Data are shown as mean ± SD. ns = not significant, *p<0.05, **p<0.01, one-way ANOVA (comparing across estrus stage), Student t-test (male-female comparison).

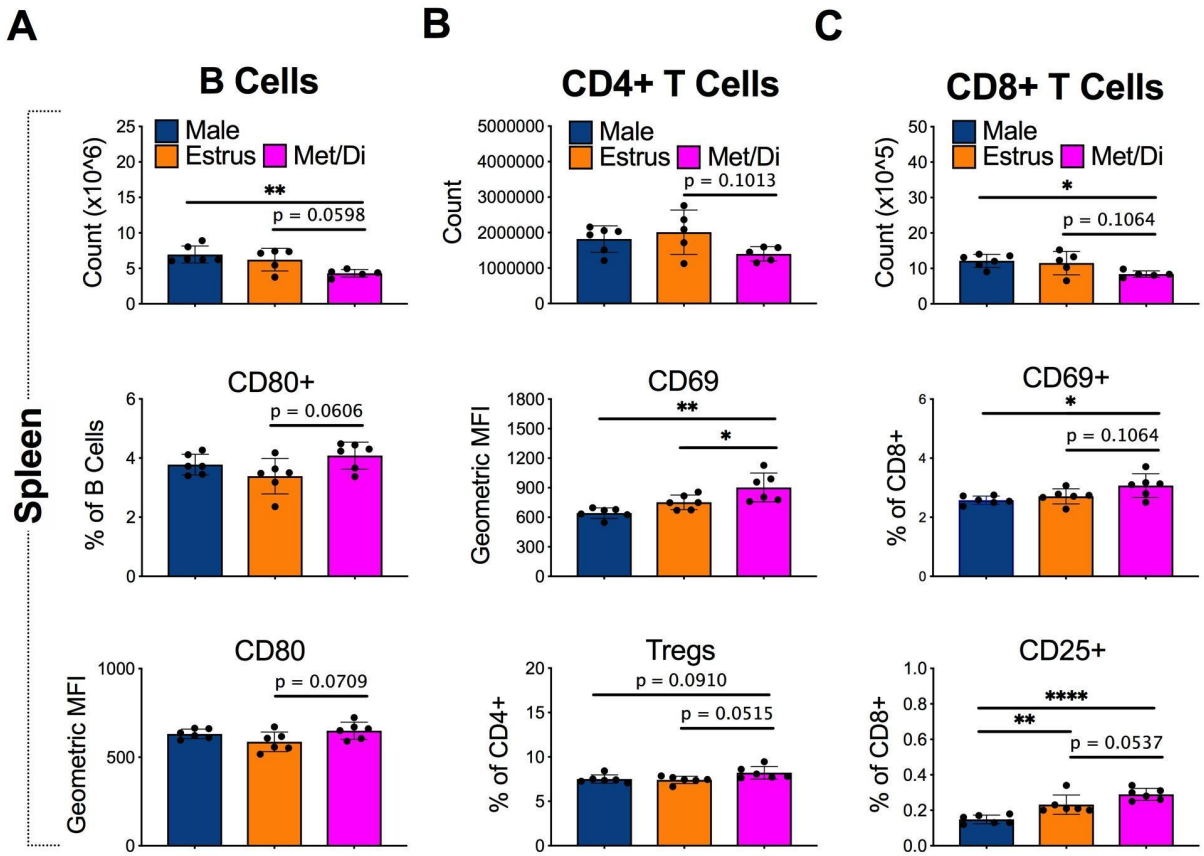

Figure 5. The murine estrus cycle modulates effector cells of the adaptive immune system. Vaginal cytology was performed on 8-week old C57BL/6 mice immediately prior to sacrifice to determine estrus stage. Spleens from males, estrus females and met/diestrus females were then digested and stained for flow cytometry. **(A)** B cells were counted, and B cell activation was evaluated by measuring CD80 surface expression. **(B)** $CD4^+$ T cells were counted, and $CD4^+$ T cell activation was evaluated by measuring CD69 surface expression. The frequency of Tregs was also evaluated. **(C)** $CD8^+$ T cells were counted, and $CD8^+$ T cell activation was investigated by measuring the frequencies of $CD8^+CD69^+$ and $CD8^+CD25^+$ cells. Data points are from one experiment (n=6). Data are shown as mean ± SD. *p<0.05, **p<0.01, ****p<0.0001, one-way ANOVA.

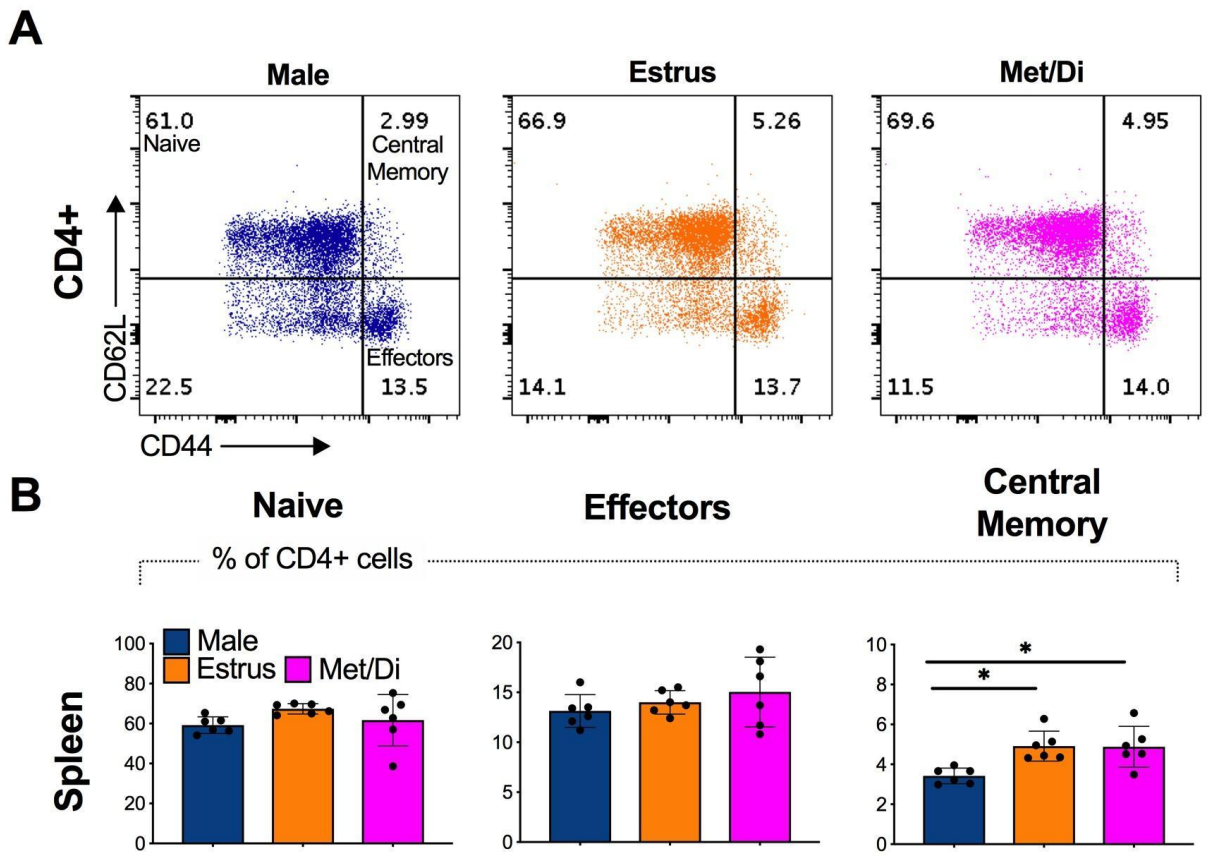

Figure 6. CD4^{+} central memory T cells are more abundant in the spleen of females compared to the spleen of males. Vaginal cytology was performed on 8-week old C57BL/6 mice immediately prior to sacrifice to determine estrus stage. Spleens from males, estrus females and met/diestrus females were then digested and stained for flow cytometry. **(A-B)** Viable CD4^{+} T cells were gated on CD44 and CD62L. Naïve CD4^{+} T cells are CD62L^{low}CD44^{hi}, CD4^{+} effectors are CD62L^{hi}CD44^{low} and CD4^{+} central memory T cells are CD62L^{hi}CD44^{hi}. Data points are from one experiment (n=6). Data are shown as mean ± SD. *p<0.05, one-way ANOVA.

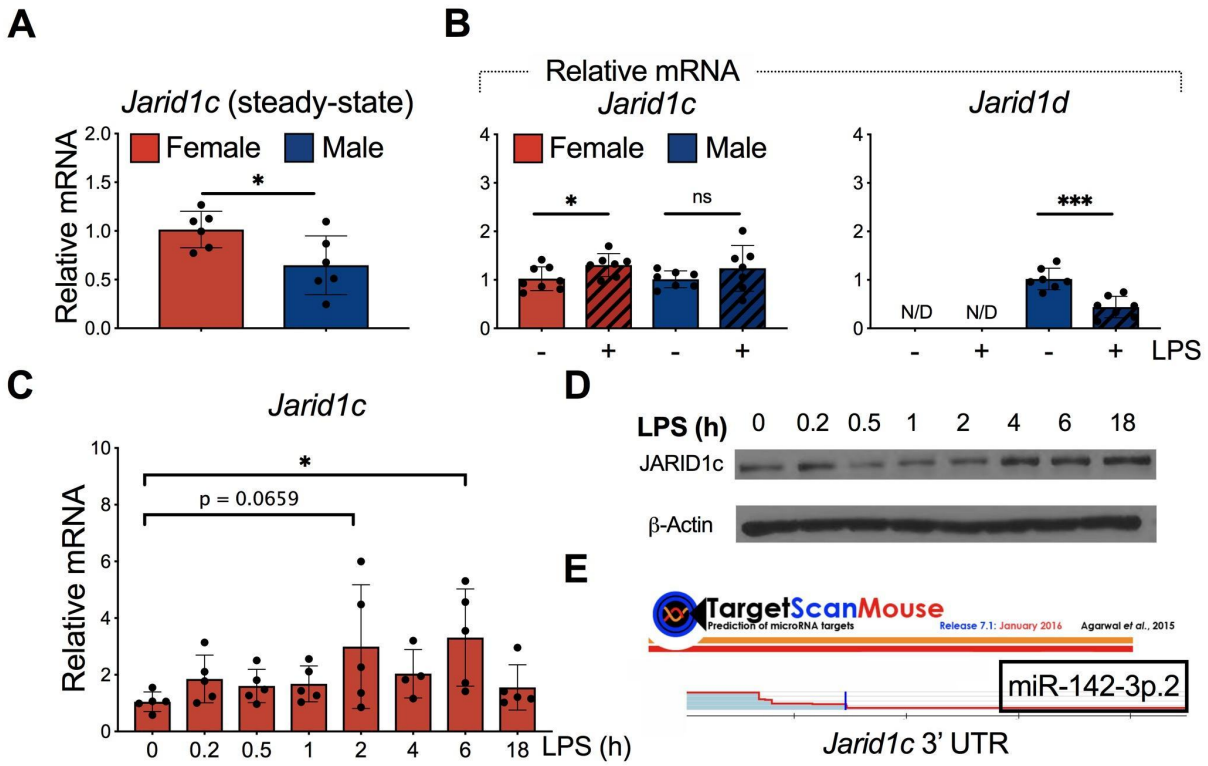

Figure 7. *Jarid1c* and *Jarid1d* are differentially expressed in male- and female-derived BMDCs and JARID1c is dynamically expressed following LPS stimulation *in vitro*. (A) RT-qPCR was used to analyze *Jarid1c* mRNA expression by female- and male-derived BMDCs from young (6-8 weeks) C57BL/6 mice at steady-state. *Jarid1c* expression was normalized to *Hprt* and is relative to the mean of female-derived BMDCs. Data points represent independent experiments (n=6) and data are shown as ± SD. **(B)** RT-qPCR was used to analyze *Jarid1c* and *Jarid1d* mRNA expression by female- and male-derived BMDCs at steady-state and following 18h LPS (100ng/ml) stimulation. *Jarid1c* and *Jarid1d* expression were normalized to *Hprt* and are relative to the mean of unstimulated female- and male-derived BMDCs. Data points represent independent experiments (n=8 for females, n=7 for males) and data are shown as ± SD. **(C)** *Jarid1c* mRNA expression by female-derived BMDCs stimulated with 100ng/mL LPS for 0.2h, 0.5h, 1h, 2h, 4h, 6h and 18h was evaluated by RT-qPCR. *Jarid1c* expression was normalized to *Hprt* and is relative to the mean of unstimulated female-derived BMDCs. Data points represent independent experiments (n=5) and data are shown as ± SD. **(D)** JARID1c protein expression by female-derived BMDCs stimulated with 100ng/mL LPS for 0.2h, 0.5h, 1h, 2h, 4h, 6h and 18h was evaluated by western blot. Results are from one experiment representative of three independent experiments (n=3). Data are shown as mean ± SD. **(E)** TargetScan analysis reveals a putative binding site for miR-142-3p.2 in the *Jarid1c* 3' UTR. ns = not significant, *p<0.05, ***p<0.001, Student's t-test.

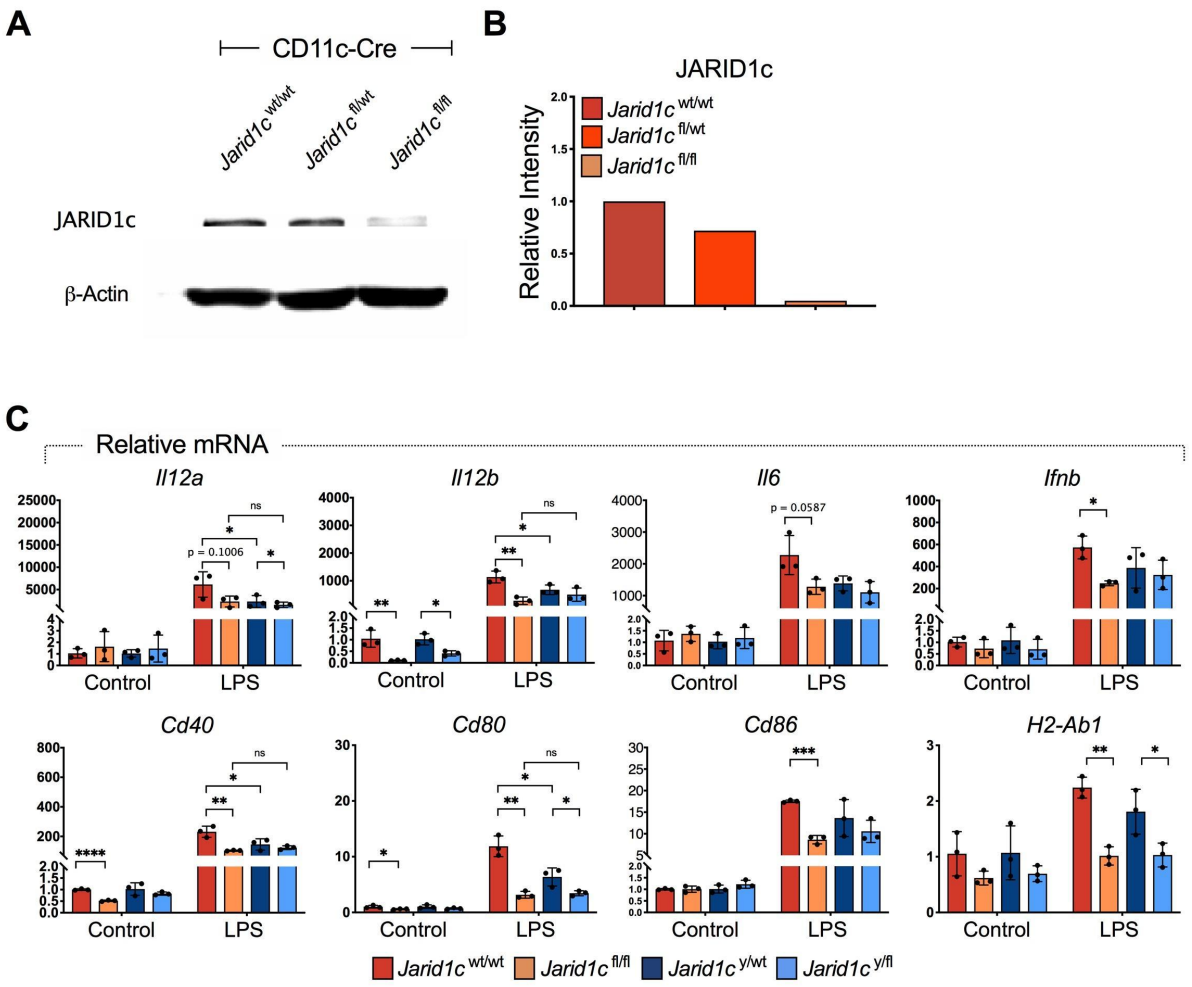

Figure 8. JARID1c-deficient male- and female-derived BMDCs show reduced pro-inflammatory gene expression. (A) BMDCs from *Jarid1c*$^{wt/wt}$, *Jarid1c*$^{y/fl}$ CD11c-Cre males and *Jarid1c*$^{fl/fl}$ CD11c-Cre females were bead-sorted on CD11c^{+} cells and knockout efficiency was assessed by western blot. **(B)** Analysis with Image Lab shows that JARID1c is ~30% reduced in CD11c^{+} cells from *Jarid1c*$^{fl/wt}$ CD11c-Cre females and ~95% reduced in CD11c^{+} cells from *Jarid1c*$^{fl/fl}$ CD11c-Cre females relative to wildtype. **(C)** RT-qPCR was used to evaluate *Il12a, Il12b, Il6, Ifnb, Cd40, Cd80, Cd86* and *H2-Ab1* gene expression by wildtype and JARID1c-deficient male- and female-derived BMDCs. All genes are normalized to *Hprt* and are relative to the mean of control/unstimulated female- and male-derived BMDCs. Data points represent independent experiments (n=3) and data are shown as ± SD. ns = not significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, Student's t-test.

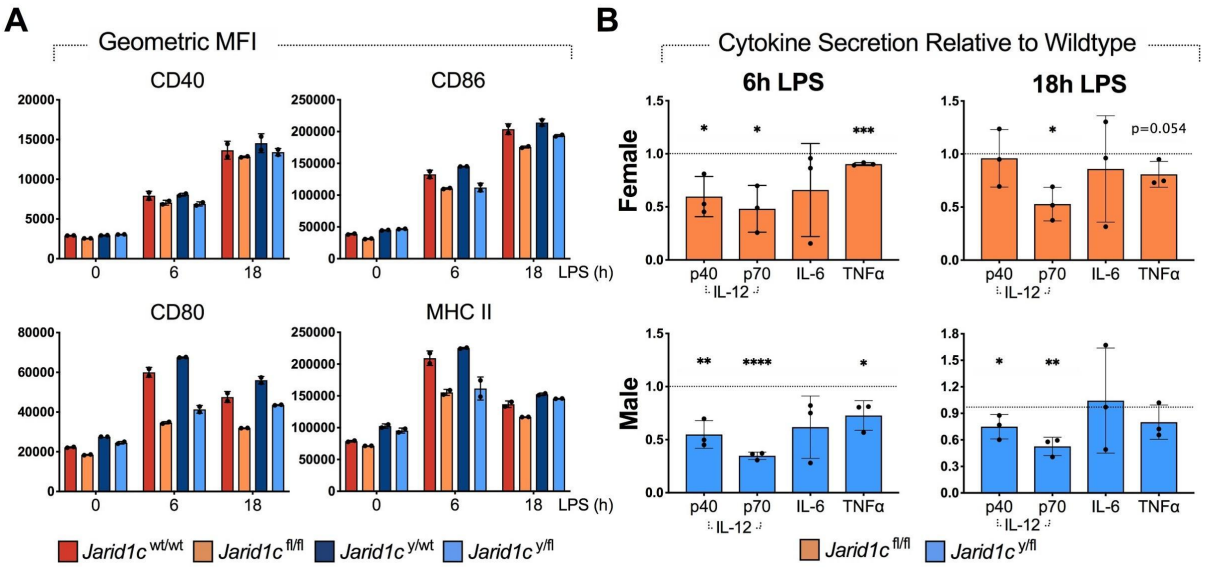

Figure 9. JARID1c-deficient BMDCs express lower levels of surface activation markers and produce lower levels of pro-inflammatory cytokines compared to wildtype BMDCs. JARID1c-deficient BMDCs from young (6-8 weeks) male and female C57BL/6 mice and BMDCs from sex- and age-matched wildtype mice were stimulated for 0h, 6h or 18h with 100ng/mL LPS. **(A)** Flow cytometry was used to evaluate the expression of CD40, CD86, CD80 and MHC II**.** Data points are from one experiment representative of three independent experiments (n=3). Data are shown as mean ± SD. **(D)** Production of IL-12p40, IL-12p70, IL-6 and TNFα from LPS-stimulated male-derived BMDCs were measured by ELISA relative to the mean cytokine secretion from wildtype male- and female-derived BMDCs . Data points represent independent experiments (n=3) and data are shown as ± SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, Student's t-test.

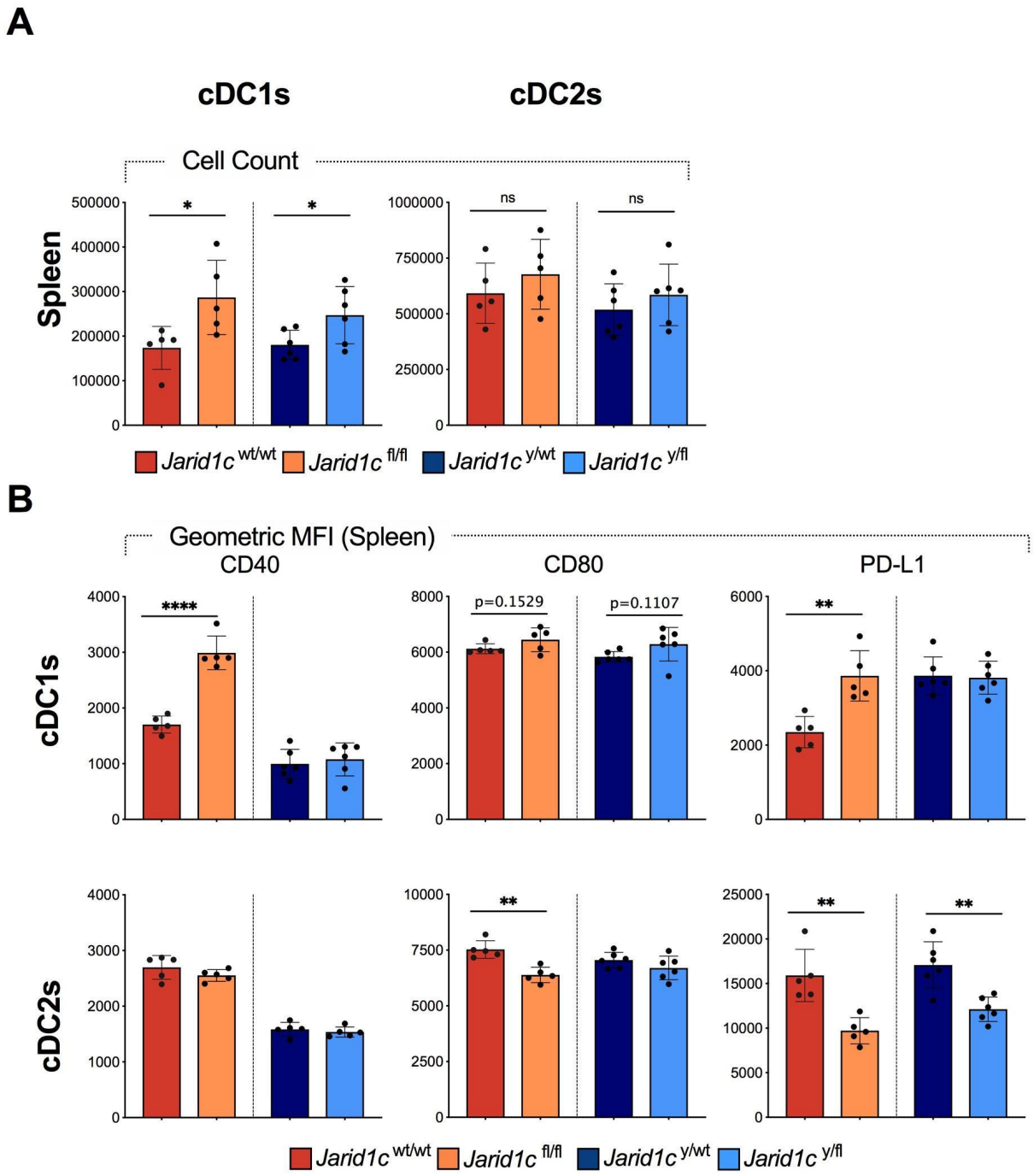

Figure 10. JARID1c-deficient cDCs show sex- and subset-specific activation profiles at steady-state. Spleens from 10-12-week old *Jarid1c*$^{wt/wt}$, *Jarid1c*$^{y/fl}$ CD11c-Cre males and *Jarid1c*$^{fl/fl}$ CD11c-Cre female C57BL/6 mice were digested and stained for flow cytometry. cDC1s and cDC2s were **(A)** counted and **(B)** flow cytometry was used to assess expression of surface activation markers CD40, CD80 and PD-L1. Data points are from one experiment (males; n=5-6) representative of two experiments (females; n=10). Data are shown as mean ± SD. ns = not significant, *p<0.05, **p<0.01, ****p<0.0001, one-way ANOVA.

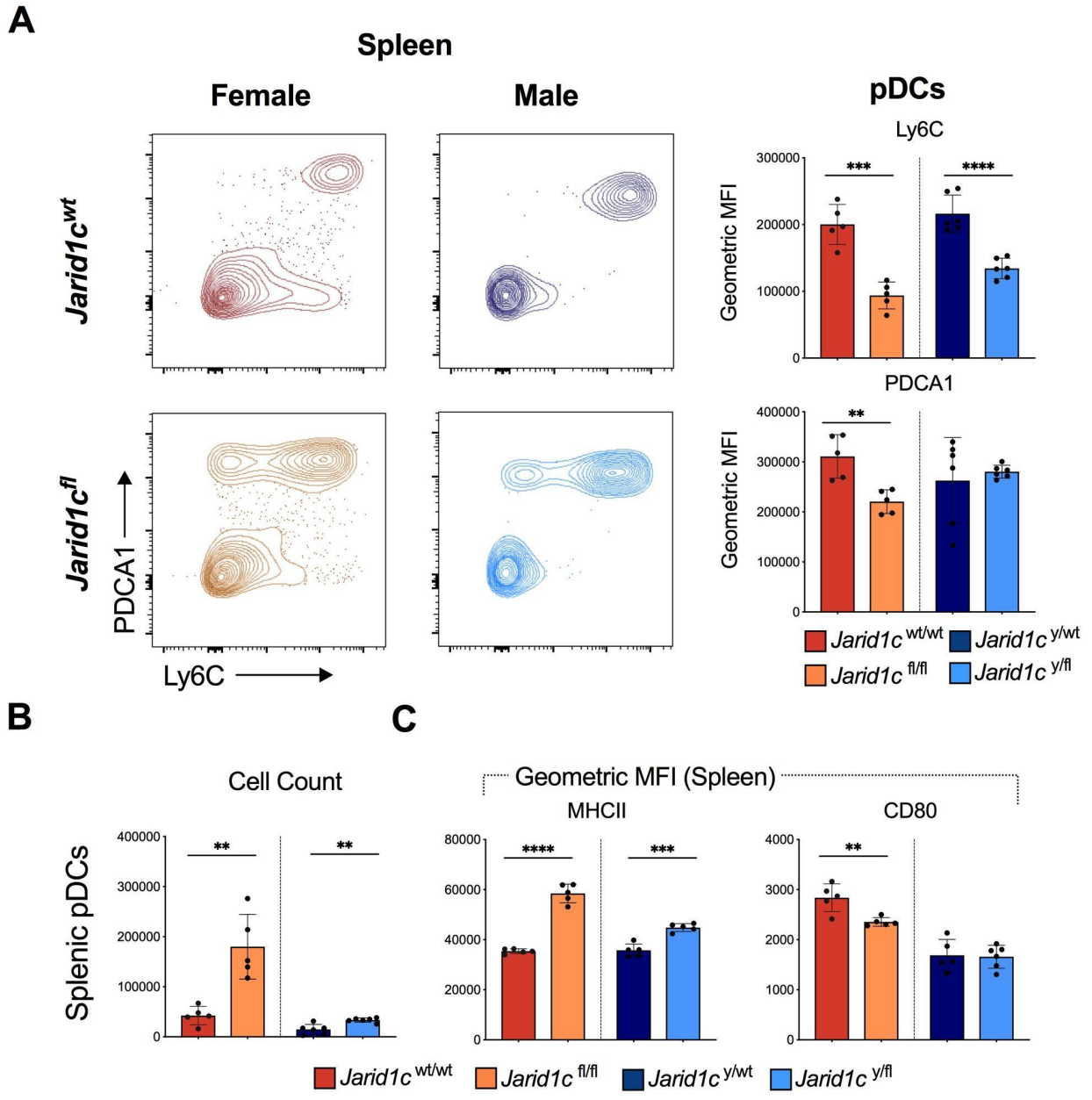

Figure 11. JARID1c-deficient pDCs show developmental defects at steady-state *in vivo*. Spleens from 10-12-week old *Jarid1c*$^{wt/wt}$, *Jarid1c*$^{y/fl}$ CD11c-Cre males and *Jarid1c*$^{fl/fl}$ CD11c-Cre female C57BL/6 mice were digested and stained for flow cytometry. **(A)** Flow cytometry analysis shows that JARID1c-deficient male- and female-derived pDCs decrease Ly6C expression, and females, but not males, decrease PDCA1 expression. Data points are from one experiment representative of three independent experiments (n=3). Data are shown as mean ± SD. Splenic pDCs were also **(B)** counted and **(C)** flow cytometry was used to assess expression of surface activation markers MHC II and CD80. Data points are from one experiment representative of three independent experiments (n=15-18). Data are shown as mean ± SD. **p<0.01, ***p<0.001, ****p<0.0001, Student's t-test.

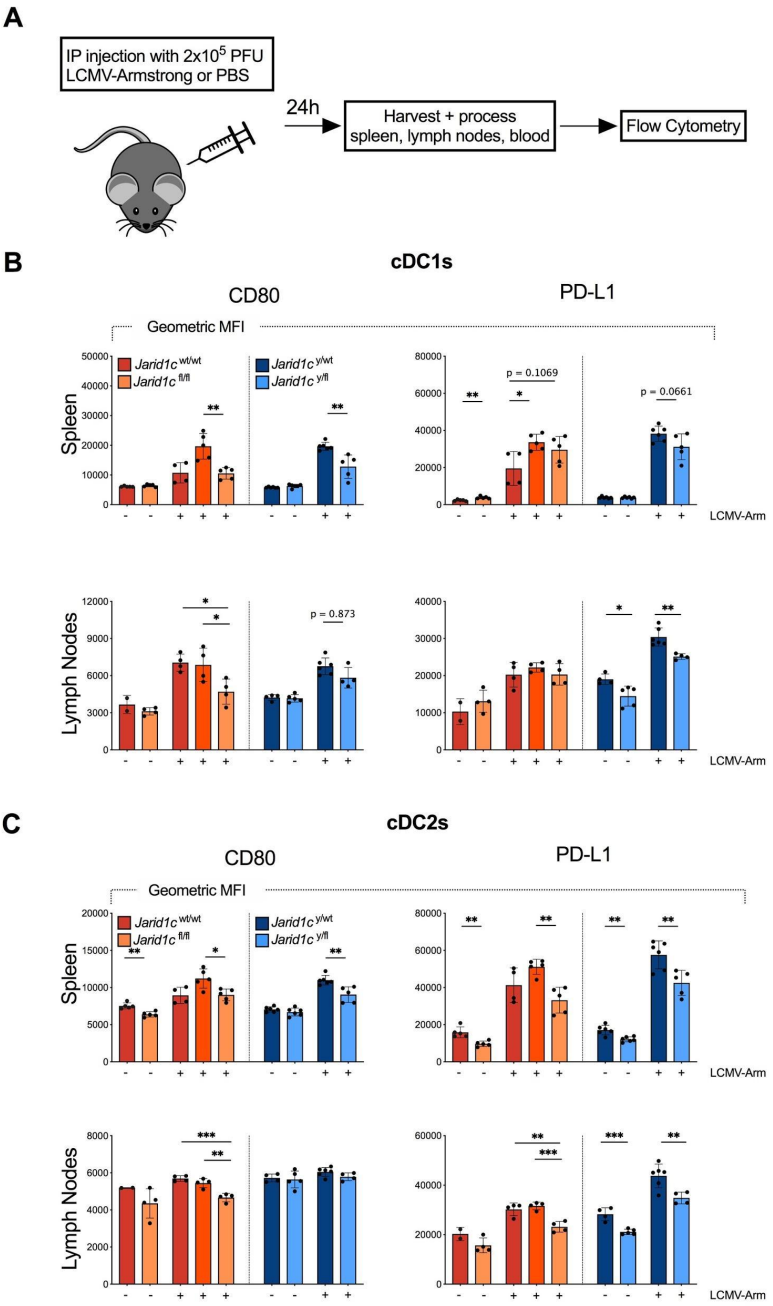

Figure 12. JARID1c-deficient cDCs from males and females mount a reduced inflammatory phenotype compared to wildtype sex-matched cDCs in response to LCMV-Armstrong infection. (A) *Jarid1c*$^{wt/wt}$, *Jarid1c*$^{y/fl}$ CD11c-Cre males, *Jarid1c*$^{fl/wt}$ CD11c-Cre females and *Jarid1c*$^{fl/fl}$ CD11c-Cre female C57BL/6 mice were injected i.p. with 200,000 PFU of LCMV-Armstrong for 24h. Spleens and lymph nodes were then harvested and digested. Flow cytometry was used to evaluate surface expression of CD80 and PD-L1 on **(B)** cDC1s and **(C)** cDC2s. Data points are from one experiment (males; n=4-6) representative of two independent experiments (females; n=6-12). Data are shown as mean ± SD. *p<0.05, **p<0.01, ***p<0.001, one-way ANOVA. Note that the mock mice shown in Figure 12 are the same mice shown in Figure 10.

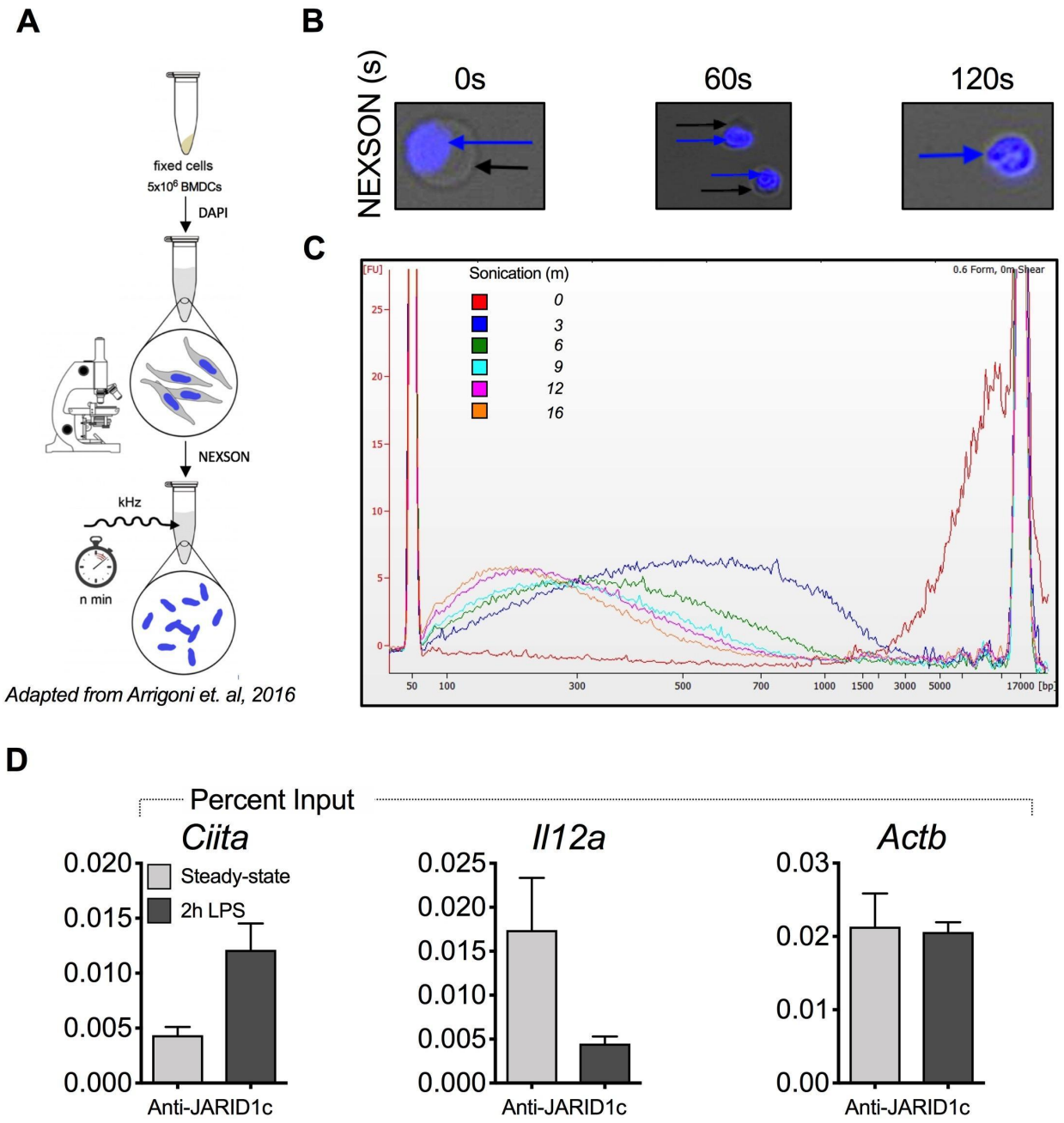

Figure 13. NEXSON ChIP-qPCR reveals that JARID1c differentially localizes to the promoter regions of genes involved in promoting and antagonizing DC activation. (A) For NEXSON, crosslinked BMDCs are briefly sonicated on a low-power setting to promote nuclear isolation. Nuclear isolation efficiency is evaluated by DAPI-staining. **(B)** $5x10^6$ BMDCs underwent 0s, 60s and 120s of NEXSON treatment and stained with DAPI to assess nuclear isolation efficiency. **(C)** NEXSON-isolated nuclei were sonicated for 0m, 3m, 6m, 9m, 12m and 16m. Chromatin fragment size was then assessed with a bioanalyzer. **(D)** ChIP-qPCR was used to determine JARID1c localization in female-derived BMDCs at steady-state and following 2h stimulation with 100ng/mL LPS. Results are from one experiment of two independent experiments (n=2). Data are shown as mean ± SD.

4. DISCUSSION

DCs play a pivotal role in mediating the adaptive immune response to injury and foreign stimuli by presenting antigen and delivering context to adaptive immune cells. For this reason, dysregulation of DC function - particularly at steady-state - can have detrimental effects by promoting a misguided immune response, in the absence of inflammatory threats, to self-tissue. Our lab has previously shown that DC quiescence is epigenetically maintained by PCGF6 and the X-linked histone demethylase JARID1c (269). However, the mechanisms by which JARID1c contribute to DC quiescence remain unclear. Additionally, whether JARID1c also contributes to DC quiescence in both males and females is unknown.

A growing body of evidence suggests marked sexual dimorphism in immune response; females typically mount a more potent response than males to pro-inflammatory stimuli. Although a more potent immune response among females protects against infectious disease and cancer, left unregulated, it can bypass self-tolerance mechanisms and consequently lead to the development of autoimmune pathology or chronic inflammation. Indeed, women account for nearly 80% of autoimmune patients. Most studies have focused on sex hormones to describe sex-biased immunity. It is clear that while sex hormones have an important effect on immune function, they do not completely account for observed differences between the sexes. Further, the influence of varying levels of estrogen and progesterone on immune function outside the female reproductive tract during the estrus cycle has not been documented. Fewer studies have focused on genetic factors; a large proportion of immune genes are encoded on the X-chromosome and at least partially escape X-inactivation in females. This results in the elevated expression of certain X-linked genes in females that may contribute to an enhanced inflammatory response. However, the mechanisms by which these genetic factors drive sex-biased immunity remain unclear and specifically their role in DC activation has not been described.

In this study, sex differences in DC function and activation between males and females were characterized both *in vitro* and *in vivo*, and the influence of the estrus cycle on immune cell function in females was determined. The effect of JARID1c on DC function and activation among males and females both *in vitro* and *in vivo* was also determined. Overall, the results of this study show that DCs from females exhibit elevated pro-inflammatory responses compared to DCs from

males, and that the estrus cycle influences relative compositions and activation of innate and adaptive immune cell subsets. This study also demonstrates that JARID1c-deficient DCs are less inflammatory at steady-state and following activation, and that the observed sex-bias in immune response is no longer present in JARID1c-deficient DCs from males and females. However, the mechanism by which JARID1c contributes to immune function remains unclear and should be addressed in future experiments.

4.1 DCs from females exhibit a more pro-inflammatory phenotype compared to DCs from males

When assessing mRNA expression, surface activation marker expression and cytokine secretion *in vitro*, nearly every assayed pro-inflammatory marker was found to be more highly expressed on female-derived BMDCs compared to male-derived BMDCs following 6h LPS stimulation (**Figure 1**). Exceptions included *Il10* mRNA, which was significantly elevated in male-derived BMDCs 6h post-LPS compared to female-derived BMDCs, and PD-L1, which was unchanged at 6h, but was more highly expressed on female-derived BMDCs 18h post-LPS. Both of these findings are to be expected; IL-10 has known anti-inflammatory properties and may thus contribute to the reduced inflammatory capacity in males, while PD-L1 is known to antagonize T cell activation in the later stages of an immune response. Since an elevated inflammatory phenotype was found among female-derived BMDCs 6h post-LPS, the increased PD-L1 expression 18h LPS may serve as an intrinsic checkpoint to help ensure that the female immune response is kept in check and does not hyperactivate, which can result in serious immunopathologies.

The findings presented here are thus consistent with previous studies indicating more potent inflammation among females. However, a sex-bias was no longer observed following 18h LPS stimulation, particularly in the surface expression of CD86 and MHC II, and the secretion of IL-6, TNFα and IL-12p70 (**Figure 1**). Previous studies have observed that LPS-driven DC activation consists of precise functional stages (326). For example, antigen uptake by DCs peaks 2h post-LPS stimulation, before steadily decreasing, but migratory ability instead peaks 4h post-LPS before declining. By 18h, both functions have decreased considerably. The findings of this study may therefore suggest that DCs from females react more potently to stimuli in the early stages of activation than do DCs from males (and are thus better at antigen uptake and migration than male-

derived DCs), but will exhibit a similar inflammatory phenotype in the later stages of DC activation. A notable exception was IL-12p40 secretion by BMDCs, which was found to be significantly higher in females following 6h and 18h LPS stimulation. This finding may reflect the importance of IL-12p40 in driving T_H1 immunity. Since T cell activation in the draining lymph node occurs 3-4 days post-infection, IL-12p40 secretion by DCs must remain elevated for >18h to promote T_H1 differentiation. Interestingly, T_H1 cells are the main $CD4^+$ T cell effectors that respond to infection with *Mycobacterium tuberculosis* (Mtb) (329). Considering that men are approximately twice as likely to contract and die from Mtb infection than women (330), it is possible that reduced IL-12p40 secretion by male-derived DCs 18h post-activation results in decreased T_H1 differentiation and proliferation, thus putting men at a higher risk of contracting Mtb. Future studies should therefore aim to investigate whether male-derived DCs can differentiate naïve $CD4^+$ T cells into T_H1 effectors as robustly as female-derived DCs, and whether genetically modified male-derived DCs capable of producing female-like quantities of IL-12p40 decreases the incidence of Mtb infection.

The results of this study further demonstrated a marked sex-dependent difference in DC subset composition *in vivo* (**Figures 2C, 3A, 4A**). Males were found to possess a higher number of cDC1s, cDC2s and pDCs compared to females in either the spleen, lymph nodes or both. Although this finding may be due to males having larger spleens, a similar trend was identified when investigating DC subsets as a percentage of viable cells instead of absolute cell counts (data not shown). However, since males typically possess more tissue than age-matched females, the increased number of DCs in male spleens and lymph nodes may simply reflect a requirement for more circulating DCs in males. Alternatively, several studies have shown that stimulated DCs undergo rapid cell death to prevent excessive T cell stimulation in lymphoid organs (331–334). If female-derived DCs are more activated as suggested by the results of this study and others, then it follows that DCs in females would die more quickly than DCs in males, potentially resulting in an increased amount of DCs in males compared to females.

Female-derived cDC1s and cDC2s, but not pDCs, were found to express significantly higher levels of MHC II at steady-state compared to males (**Figures 2E, 3B, 4B**). Since cDC1s are more specialized in activating $CD8^+$ T cells and cDC2s are more specialized in activating $CD4^+$ T cells, the findings of this study suggest that females may possess an intrinsically higher capacity to

stimulate both $CD4^+$ and $CD8^+$ T cell responses. This finding mirrors the observed BMDC data of this study and suggests that BMDCs may be similar to cDCs and not pDCs. Indeed, recent studies have shown that BMDCs are in fact heterogeneous and encompass at least two distinct populations that can be classified as either cDC-like DCs or monocyte-derived macrophages (95). More specifically, cDC-like BMDCs were shown to express the cDC2 marker CD172a, which may indicate that BMDCs most closely resemble cDC2s. Unexpectedly, no sex difference was observed in MHC II expression on pDCs at steady-state (**Figure 4B**). However, pDCs are known to be poor at antigen presentation (87), so MHC II may not be the best measure of pDC activation. Instead, pDCs are specialized in high-level secretion of type 1 interferon (IFN-α/β). Previous studies have indicated that female-derived pDCs produce more IFN following TLR-7 stimulation *in vitro* (137, 138), and that post-translational mechanisms are present in pDCs to ensure rapid IFN secretion following viral challenge. It is therefore possible that sex differences in pDC function are only manifested in an activated state. Future experiments should therefore measure whether IFN-α/β secretion differs between males and females at steady state.

This study and others have confirmed a female-bias in immune response. Yet, it is unknown why evolution has selected for reduced immune capacity among males. Indeed, sex differences in immune response are present throughout the evolutionary tree; innate and adaptive immune response in males from diverse species including insects, lizards, birds and mammals are typically lower than in females of the same species (135). One possible explanation may be that muscular strength was selected for over immune function during the course of evolution. In a pre-agricultural, nomadic world, predators posed a much greater threat to male hunters' survival than infectious disease. Finite metabolic resources were thus funneled into physical ability at the expense of mounting a robust immune response. This manifested itself in higher mammals as testosterone, which bestows upon men physical ability, but also exerts potent anti-inflammatory properties, perhaps to ensure appropriate usage of metabolic resources.

The results of this study have important implications for future research - particularly in immunology. A 2011 study found that fewer than 10% of published immunological studies reported the biological sexes of the animal or human subjects, and 70% failed to report sex at all (219). Since the female and male immune responses differ in key areas, future immunology studies should aim to - if resources allow - consider sex as a biological variable and include both sexes in

research studies. Sex-inclusive studies are especially pertinent in the fields of autoimmunity and immuno-oncology. Considering that 80% of all autoimmune patients are female and that males are twice as likely to develop terminal cancer than females, extra steps should be taken to ensure that biological sex is controlled for in an effort to tailor treatments to the affected sex.

An interesting implication of this study relates to DC-based cancer vaccines, wherein patient-derived DCs are cultured *ex vivo* with cancer antigens, stimulated and re-introduced into the patient. Antigen-loaded DCs activate antigen-specific T cells and help mount an immune response against cancerous cells (335). Since the findings presented here suggest that female-derived DCs exert a more potent immune response than male-derived DCs, clinical trials should analyze data by sex to ensure that DC vaccines are equally effective in male patients. However, at least one recent phase II clinical trial failed to mention the subject's biological sex (336), while another phase I trial indicated subject sex but did not analyze males and females separately (337). Future clinical trial guidelines may consider mandatory analysis by sex.

4.2 The murine estrus cycle as a variable influencing immune cell function

In the mouse, ovulation occurs every 4-5 days. Serum concentrations of E2 and P4 cycle throughout this time; metestrus and diestrus are characterized by low but slowly increasing levels of E2 and a peak in P4, while E2 peaks during the proestrus stage (338). Since immune modulation by estrogen and progesterone have been well characterized, whether the estrus cycle represents an overlooked variable in female immunity was determined. Previous studies have investigated the immune cell composition in the uterus at different estrus stages, however no studies, to our knowledge, have analyzed immune cell composition in SLOs during different estrus stages. Interestingly, it was found that more cDC1s, but not cDC2s or pDCs, are present in the spleen and lymph nodes during the estrus stage relative to the met/diestrus stages (**Figures 2C, 3A, 4A**). cDC1s were found to express higher levels of the co-stimulatory molecule CD40 during estrus and higher levels of CD80 during met/diestrus at steady-state (**Figure 2D**). Although cDC1s were initially expected to exert a decreased pro-inflammatory phenotype during the estrus stage (when both E2 and P4 are low), elevated E2 serum concentrations may not necessarily correlate with higher inflammatory capacity in DCs. A 2005 study showed that E2 has bipotential effects on human monocytes and macrophages; low doses were found to enhance the production of pro-

inflammatory cytokines, but high concentrations reduced cytokine production (177). If also true in murine DCs, this may explain why cDC1s in estrus show a trend for increased expression of CD40. In addition, since changes in gene and surface marker expression may require a few hours to manifest themselves, a possible caveat to the results of this study is that the observed phenotype was induced at the previous stage of the estrus cycle. For example, a trend for increased activation of CD80 among females in met/diestrus compared to females in estrus was found (**Figure 2D**). However, it is possible that the hormone cocktail present during the estrus stage, and not the met/diestrus stage, is what induced CD80 expression. The time required for transcription, translation and CD80 transport to the cell membrane made it so that the mouse had already entered the met/diestrus stage by the time CD80 was expressed on the cell surface. Further studies will have to be conducted to determine the stage of the estrus cycle that is truly responsible for the observed phenotype.

Splenic immune cells of the adaptive system were also investigated at different stages of the estrus cycle and an overall trend for increased activation of B cells, $CD4^+$ T cells and $CD8^+$ T cells during the met/diestrus stage was found (**Figure 5**). Interestingly, there was also a trend for more Tregs during the met/diestrus stage compared to the estrus stage (**Figure 5B**). These findings may have important implications for immune-based therapies and immunization schedules among pre-menopausal females. For example, the findings of this study indicate that cDC1s may possess an elevated baseline activation phenotype during the estrus stage (**Figure 2D**). Considering that cDC1s have the capacity to present antigen to both $CD4^+$ and $CD8^+$ T cells and can thus promote cell-mediated and humoral immunity (339), cDC1s that process antigen during the estrus stage may be better equipped to promote B cell activation later on during the met/diestrus stage. Increased B cell activation may thus correlate with more potent antibody production and increased protection against the pathogen. Future studies should therefore aim to study the effect of estrus stage on immunization efficiency.

The results of this study may have important ramifications for immunological studies that use female mice as animal models. Very few immunology studies have treated estrus cycle stage as a biological variable. However, the results of this study suggest that the estrus cycle modulates both innate and adaptive immunity in females. In addition to implementing sex as a biological variable to control for sex differences in immunity, future studies should consider controlling for estrus

cycle stage to ensure that any differences be the result of the experimental variable and not due to hormonal fluctuations throughout the estrus cycle.

A major limitation of this study is the absence of mice in the proestrus stage. This may be due to the time period at which female mice are likely to enter each estrus stage. When mice are maintained on a standard light-dark cycle (i.e. in the dark between 7PM and 7AM), the estrus phase will usually begin between 12AM and 3AM and continue into the early morning (340). Since proestrus is immediately before estrus and lasts for 6-12 hours, many mice should be in the proestrus phase in the late afternoon and evening. Indeed, our vaginal smears were performed in the morning (roughly 9AM), and a large percentage of mice were found to be in estrus. Considering that E2 peaks in proestrus, future experiments will require the inclusion of females in proestrus to more properly delineate estrus stage as a biological determinant of female immunity. In addition, this study included mice at steady-state; future experiments should assess female immune response to viral/bacterial challenge at different stages of the estrus cycle to determine whether the trends we observed at steady-state are also seen during the course of an immune response.

Females are four-times more likely than males to develop an autoimmune disease during the course of their lives. Mouse studies often use EAE mice to model autoimmune pathology. At least one study has shown that EAE symptoms are attenuated in ovariectomized females with very low serum E2 and P4 concentrations (211), which implicate female sex hormones in autoimmunity. Considering that the findings of this study demonstrate that estrus stage modulates both innate and adaptive immunity, the estrus cycle may play a role in promoting the onset of autoimmune pathologies (i.e. by providing the opportunity to bypass self-tolerance mechanisms) or may exacerbate autoimmune symptoms. Future experiments can test this hypothesis by first ovariectomizing female EAE mice followed by supplementation with hormones in precise concentrations to replicate each stage of the estrus cycle. In this way, female mice would effectively be trapped in one stage of the cycle. Mice in each stage can then be monitored for development of EAE symptoms. In addition, E2 and P4 are common constituents of hormonal contraceptives, which typically work by maintaining high serum concentration of E2 and P4. Several studies have found long-term (>5 years) use of hormonal contraceptives to be associated with an increased risk of several serious autoimmune diseases including Crohn's disease, SLE and interstitial cystitis (341). High E2 and P4 are typical during the proestrus stage. Although this study

did not include females in proestrus, it would be interesting to test whether immune cell subsets are more activated at steady-state in the high E2/P4 proestrus environment. Alternatively, it would be interesting to see whether ovariectomized female mice supplemented with proestrus-like levels of E2/P4 would be at a higher risk of developing autoimmune pathologies.

4.3 JARID1c and JARID1d in male and female response to LPS *in vitro*

Our lab has previously shown that PCGF6 and JARID1c define an epigenetic axis involved in the maintenance of DC quiescence (269). We have previously shown that BMDCs transduced with sh*Jarid1c* exert a more pro-inflammatory phenotype, and therefore characterized the immune response of BMDCs derived from *Jarid1c*$^{fl/fl}$ CD11c-Cre females and *Jarid1c*$^{y/fl}$ CD11c-Cre males. Although previous data indicated that female-derived JARID1c-deficient BMDCs would mount a more robust inflammatory response than female-derived wildtype BMDCs, nearly every pro-inflammatory marker was found to be decreased in female-derived JARID1c-deficient BMDCs (**Figures 8 & 9**) relative to female-derived wildtype BMDCs following LPS stimulation. This was unexpected, although sh*Jarid1c*-mediated knockdown and Cre-mediated knockout may have different biological effects. As a H3K4 demethylase, JARID1c functions as a transcriptional repressor. In this study, preliminary evidence was found to suggest that JARID1c localizes to and represses at least two gene subsets: a subset that is activated to maintain DC quiescence (including *Ciita*) and a subset that is activated to facilitate DC activation (including *Il12a*) (**Figure 13D**).

The findings of this study suggest that in steady-state BMDCs, JARID1c will repress the pro-activation gene subset, but following LPS stimulation, JARID1c disassociates from the pro-activation subset and instead represses the pro-quiescence subset to allow for DC activation. When *Jarid1c* is knocked down by shRNA, the remaining JARID1c protein will preferentially bind to its highest affinity loci. If JARID1c has a higher affinity for the pro-quiescence gene subset than for the pro-activation subset, then the pro-activation subset will be more highly expressed at steady-state, allowing for an increased pro-inflammatory phenotype that we have previously observed. However, if JARID1c is absent, as is the case in BMDCs derived from *Jarid1c*$^{fl/fl}$ CD11c-Cre females and *Jarid1c*$^{y/fl}$ CD11c Cre males, then JARID1c will not be available to repress either gene subset. In this scenario, it is possible that other mechanisms are in place to promote DC quiescence that LPS stimulation can only partially override. Alternatively, if both quiescence and

activation genes are activated, DC quiescence may be favored. Further research will be required to test this hypothesis. Notably, ChIP-Seq should be performed on wildtype BMDCs, BMDCs transduced with sh*Jarid1c*, and JARID1c-knockout BMDCs both at steady-state and following LPS stimulation in order to better understand JARID1c's target loci.

An exciting element of this study is that a sex-bias was not observed in pro-inflammatory gene expression, surface activation markers or cytokine production among LPS-activated JARID1c-deficient BMDCs (**Figure 8**). This finding suggests that JARID1c may represent a novel genetic mediator of sex-biased immunity in DCs. Future research must be conducted to further support this claim. Notably, murine stem cell virus (MSCV)-based strategies can be used to overexpress *Jarid1c* in JARID1c-deficient BMDCs. If a sex difference in DC activation re-emerges, JARID1c may serve as an important mediator of sex-biased DC response. In addition, female-derived JARID1c-deficient BMDCs exerted an attenuated inflammatory phenotype relative to wildtype BMDCs for nearly all markers that were tested (**Figures 8 & 9**). However, male-derived LPS-activated JARID1c-deficient BMDCs showed mixed results; certain markers were significantly less expressed in male-derived JARID1c-deficient BMDCs compared to male-derived wildtype BMDCs, while other markers showed no difference in expression (**Figures 8 & 9**). This may suggest that JARID1c and JARID1d encode redundant functions; JARID1d may be able to at least partially offset the loss of JARID1c. This hypothesis can be tested by overexpressing *Jarid1d* in female-derived JARID1c-deficient BMDCs. If the inflammatory phenotype observed in female-derived wildtype BMDCs is recovered in female-derived JARID1c-deficient *Jarid1d*-expressing BMDCs, then JARID1d and JARID1c may encode redundant functions. Furthermore, a differential regulation of *Jarid1c* and *Jarid1d* was observed in response to LPS activation; whereas *Jarid1c* expression is increased in female-derived BMDCs 18h post-LPS, *Jarid1c* expression does not significantly change in male-derived BMDCs, but *Jarid1d* is significantly downregulated 18h after LPS activation (**Figure 7**). As a result, LPS-activated female-derived BMDCs express more *Jarid1c* than male-derived BMDCs express *Jarid1c/d*, which may have important implications for sex-biased immunity.

The process of LPS-driven DC activation consists of distinct functional stages. For example, antigen uptake is most efficient 2h after LPS stimulation, while migratory capacity peaks at 4h after LPS stimulation. By 18h, both of these processes have returned to slightly above their

baseline levels (326). Considering that each stage requires the regulation of a specific gene subset, massive transcriptional reprogramming must occur at specific timepoints during DC activation (342). Interestingly, JARID1c was found to undergo a wave-like expression pattern following LPS stimulation whereby JARID1c protein level peaks at 0.2h, 4h and 18h after LPS stimulation (**Figure 7D**). Although further investigation is required, it is possible that JARID1c helps to mediate the transcriptional reprogramming that occurs following DC activation. As a transcriptional repressor, JARID1c may localize to and repress a subset of genes at 0.2h, 4h and 18h post-LPS to facilitate DC transition to the next functional stage. Preliminary evidence from this study suggests that at steady-state, JARID1c localizes to the *Il12a* promoter (**Figure 13D**). However, ChIP-qPCR suggested that following DC activation, JARID1c disassociates from *Il12a* and instead localizes to the promoter region of *Ciita* (**Figure 13D**). Considering that *Ciita* is transcriptionally silenced to allow for efficient DC activation (343) and that *Il12a* is expressed by LPS-activated DCs (344), *Ciita* and *Il12a* may be constituents of different gene subsets regulated by JARID1c. Since JARID1c-deficient BMDCs were observed to be less activated following LPS stimulation compared to wildtype BMDCs (**Figures 8 & 9**), it is possible that *Ciita* and other genes repressed during DC activation were not as efficiently silenced in the absence of JARID1c, resulting in an attenuated inflammatory phenotype. To expand on this idea and characterize JARID1c localization genome-wide, ChIP-Seq should be performed on BMDCs at steady-state and at various timepoints following LPS activation.

An interesting observation was that JARID1c protein levels increase as early as 0.2h (or 10 minutes) following LPS stimulation (**Figure 7D**). Including introns, *Jarid1c* is ≅41 kbp in length (UCSC Genome Browser), and JARID1c protein is 1551 amino acids. Average elongation rates by RNA polymerase II (RNAPII) and ribosomes in mammalian cells have been shown to range from 1.3-4.3kb/min for RNAPII (345) and 6-9 amino acids/sec for ribosomes (346). Under these conditions, *Jarid1c* transcription in mammals would require 10-30 minutes, and translation would require an additional 3-5 minutes. Considering that *Jarid1c* mRNA must also be trafficked to the cytosol for translation, 10 minutes is unlikely to be sufficient for the observed increase in JARID1c. Instead, the rapid increase in JARID1c may be due to post-transcriptional regulation by micro-RNAs (miRNAs). miRNAs are small non-coding RNAs that can mediate translational repression or mRNA degradation by binding to target mRNA transcripts (347). TargetScan was used to identify miRNAs that may potentially bind and regulate JARID1c mRNA. A strong

putative binding site for miR-142-3p.2 was determined (**Figure 7E**), which is known to regulate DC function (328, 348, 349). miR-142-3p.2 has been previously shown to interfere with antigen processing and presentation (328). Since it was observed that JARID1c may silence *Ciita* during DC activation, miR-142-3p.2 may interfere with antigen processing by post-transcriptionally silencing JARID1c. If this occurs, JARID1c would no longer silence *Ciita*, thus interfering with antigen presentation by promoting *Ciita* expression and unstable surface MHC II expression. Future biochemical analysis will be necessary to determine whether JARID1c mRNA is targeted by miR-142-3p.2. MSCV-based strategies can be used to transduce a miR-142 sponge into BMDCs. JARID1c protein levels can then be evaluated by western blot to determine whether JARID1c increases in the miR-142-deficient BMDCs. An increase in JARID1c may suggest that miR-142-3p.2 targets JARID1c mRNA.

4.4 JARID1c and JARID1d suppress cDC1 responses and promote cDC2 responses *in vivo*

To study JARID1c *in vivo*, *Jarid1c*$^{fl/fl}$ mice were first bred to CD11c-Cre mice to generate offspring deficient for *Jarid1c* in CD11c-expressing cells. In this model, JARID1c function in CD11c^{+} cells is disrupted through targeted elimination of exons 11 and 12, which encode the enzymatic domain. This disruption strategy is predicted to generate a mutant *Jarid1c* gene encoding an RNA transcript with an in-frame deletion of exons 11 and 12 (311). JARID1c knockout efficiency was assessed by western blot and a 95% reduction in female-derived JARID1c-deficient BMDCs was found (**Figures 8A, 8B**). This finding has also been reported by another group using *Jarid1c*$^{fl/fl}$ mice (311).

To determine whether JARID1c contributes to sex-biased immunity *in vivo*, absolute numbers of JARID1c-deficient cDC1s and cDC2s were first compared at steady-state. JARID1c-deficient cDC1s were found to be more numerous than wildtype cDC1s, but no significant difference was observed for cDC2s (**Figure 10A**). The expression of surface activation markers on splenic JARID1c-deficient and wildtype cDC1s and cDC2s were then compared at steady-state. Interestingly, female-, but not male-derived JARID1c-deficient cDC1s were found to express elevated levels of CD40 and PD-L1 compared to wildtype cDC1s, and a trend for increased activation of CD80 (**Figure 10B**). In contrast, CD80 and PD-L1 expression was lower on female-derived JARID1c-deficient cDC2s compared to female-derived wildtype cDC2s (**Figure 10B**).

Male-derived JARID1c-deficient cDC2s also expressed lower PD-L1 compared to male-derived wildtype cDC2s (**Figure 10B**). These findings suggest that JARID1c regulates cDC1s and cDC2s differently, which may have important implications for cDC1- and cDC2-driven immune responses. cDC1s are potent cross-presenting DCs that are specialized at stimulating $CD8^+$ T cells into CTL effectors responses as well as inducing $CD4^+$ T cell differentiation into T_H1 effectors. In contrast, cDC2s are specialized at inducing $CD4^+$ T cell differentiation into T_H2 and T_H17 effectors. Since T_H1, T_H2 and T_H17 effectors respond to different classes of pathogens, JARID1c in DCs may play an important role in mounting specific modules of the immune response.

Further, JARID1c may also contribute to sex differences in immunopathologies. For example, females typically mount higher T_H2 and antibody responses (350), both of which are driven by cDC2s. The findings presented in this study suggest that JARID1c promotes cDC2, but not cDC1 activation. Considering that female-derived DCs express more JARID1c than male-derived DCs (**Figure 7A**), females may have a genetic predisposition for enhanced T_H2 immunity by expressing higher levels of JARID1c than males. Future experiments should verify whether JARID1c differentially regulates cDC1s and cDC2s. JARID1c can be specifically deleted in cDC1s or cDC2s. Previous studies have identified *Xcr1* as a cDC1-specific marker (351) and *Clec4a4* as a cDC2-specific marker (352). By breeding *Jarid1c*$^{fl/fl}$ mice with either XCR1-Cre or Clec4a4-Cre mice, JARID1c can be specifically targeted in either cDC1s or cDC2s, allowing for the study of JARID1c in cDC1s and cDC2s in isolation. Indeed, another group has recently generated XCR1-Cre mice on the C57BL/6 background (353). These mice can be used to edit and delete genes such as JARID1c in cDC1s.

The cDC response to infection with the murine virus LCMV-Arm was also characterized. Similar to the attenuated inflammatory response observed in JARID1c-deficient BMDCs to LPS, male- and female-derived JARID1c-deficient cDC1s and cDC2s expressed lower levels of CD80 and PD-L1 in the spleen and lymph nodes following infection with LCMV-Arm (**Figure 12**). This finding provides further evidence that JARID1c regulates cDC1s and cDC2s. Interestingly, the decreased surface expression of CD80 and PD-L1 was not sex-specific. Considering that male-derived JARID1c-deficient cDCs were less sensitive than female-derived JARID1c-deficient cDCs at steady-state (**Figure 10B**), this suggests that while JARID1d in male-derived cDCs may partially offset the loss of JARID1c at steady-state, JARID1d is insufficient in the context of

LCMV-Arm infection. Since JARID1c regulates cDC response to LCMV-Arm, cDC-guided adaptive responses may also be impaired. To test adaptive responses, *Jarid1c*$^{fl/fl}$ CD11c-Cre mice can be infected with *Listeria monocytogenes* as a model for T cell activation. By analyzing $CD4^+$ and $CD8^+$ T cell activation 8 days post-infection, we can assess whether the loss of JARID1c in DCs also disrupts T cell activation.

4.5 JARID1c in pDC development and regulation

pDCs are specialized in antiviral immunity. To determine whether JARID1c is involved in pDC development or regulation, JARID1c-deficient pDCs in the spleen were investigated by flow cytometry. pDCs were defined as Ly6C^{hi}PDCA1^{hi} cells (75, 354). Interestingly, male- and female-derived JARID1c-deficient pDCs were found to express significantly lower levels of Ly6C compared to sex-matched wildtype pDCs, while female-derived JARID1c-deficient pDCs also expressed significantly lower PDCA1 (**Figure 11A**). These findings suggest that JARID1c plays a role in pDC development, but future research is required to confirm this finding. A marked increase in the absolute numbers of male- and female-derived JARID1c-deficient pDCs in the spleen compared to sex-matched wildtype pDCs was also found (**Figure 11B**). An exciting observation was the decrease in CD80 expression on the surface of female-, but not male-derived JARID1c-deficient pDCs at steady-state (**Figure 11C**). This suggests that JARID1c in pDCs may regulate CD80 expression and thus promote pDC activation. Considering that females mount an enhanced pDC-mediated antiviral response compared to males (135), females may have a genetic predisposition for enhanced pDC response by expressing higher levels of JARID1c than males.

In an effort to characterize any clinical impact of the work in this study, and to better understand the role of JARID1c in a human context, human subjects with characterized mutations in *JARID1c* would ideally be recruited for immunological studies. Currently, human patients with loss-of-function mutations in *JARID1c* have been shown to be more likely to develop neurological deficiencies, including XLID (298). To the best of our knowledge, the immune response of patients with loss-of-function mutations in *JARID1c* has yet to be tested. Future studies should therefore aim to phenotype the innate and adaptive immune cells of these patients to better understand the role of JARID1c and JARID1d in human immune response.

The results obtained in this study may inform differential functions for JARID1c in different DC

subtypes. For instance, expression of surface activation markers on female-derived JARID1c-deficient cDC1s was found to be enhanced compared to female-derived wildtype cDC1s (**Figure 10B**). However, the opposite trend was observed for female-derived JARID1c-deficient cDC2s (**Figure 10B**), and JARID1c-deficient pDCs showed impaired development (**Figure 11A**). These findings suggest that JARID1c may regulate different gene subsets in cDC1s, cDC2s and pDCs (**Figure 14**). In cDC1s at steady-state, JARID1c may localize to and repress pro-inflammatory genes such as *Il12b* and *Cd40*. Pro-inflammatory genes in JARID1c-deficient cDC1s are thus more likely to become spontaneously activated, resulting in elevated cDC1 activation. In contrast, JARID1c may instead repress pro-quiescence genes such as *Ciita* in cDC2s at steady-state. As a consequence, inflammatory signals may be insufficient to "push" JARID1c-deficient cDC2s towards activation, thus resulting in the reduced cDC2 activation observed in this study. Finally, several transcription factors including *Zeb2* and *Tcf4* have been shown to be required for pDC development (355, 356). JARID1c may repress inhibitors of *Zeb2* and *Tcf4*, such that *Zeb2*, *Tcf4* and other factors involved in pDC development remain at least partially repressed in JARID1c-deficient pDCs, thus impeding pDC development. JARID1c ChIP-seq experiments in cDC1s, cDC2s and pDCs should be conducted to better understand the unique targets of JARID1c in each DC subset.

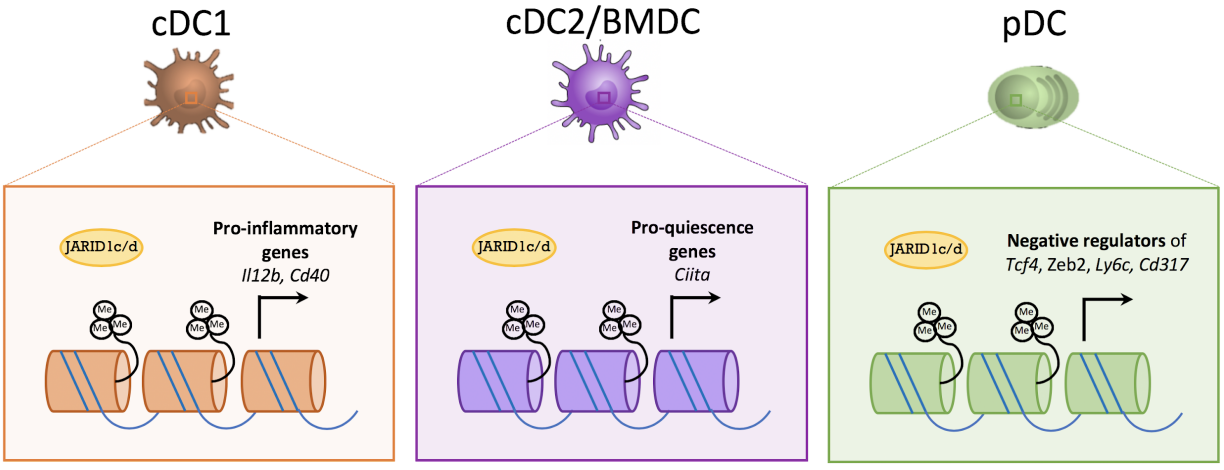

Figure 14. Proposed model for differential JARID1c/d function in cDC1s, cDC2s and pDCs.

Overall, the findings presented in this study implicate JARID1c as a putative genetic mediator of sex-disparate immunity *in vivo*. However, since sex hormones are well-documented mediators of DCs and the immune system, JARID1c and JARID1d must be studied in the absence of sex hormones to fully delineate their contributions to sex-biased DC function *in vivo*. A routine method to study genetic factors in isolation of sex hormones is to surgically remove the gonads from male and female mice to interrupt the production of E2, P4 and testosterone. Future work should therefore aim to characterize JARID1c-deficient cDCs and pDCs from gonadectomized male and female mice. Considering that we also observed significant differences in splenic DCs and adaptive immune cell subsets between females in estrus and females in met/diestrus, splenic immune cell subsets from *Jarid1c*$^{fl/fl}$ CD11c-Cre females should also be characterized to investigate the role of JARID1c in DCs during different stages of the estrus cycle.

4.6 CONCLUSION

Females typically mount more potent immune responses than males, allowing for decreased pathogen loads and increased responses to vaccinations. Enhanced immunity among females is a double-edged sword; the increased capacity for tumor immunosurveillance places females at a lower risk of developing infections and terminal cancers, but a highly active immune response is also more likely to bypass self-tolerance mechanisms and promote autoimmune responses. The influence of sex hormones on immune function, particularly E2 and P4 in females, has been extensively characterized and has been shown to enhance female immune responses. However, few studies have investigated whether the estrus cycle, during which E2 and P4 widely vary, constitutes a variable in immune function. This study determined that immune cell subsets of innate and adaptive immunity are influenced by estrus stage. Considering that few immunological studies separate females by estrus stage, the findings of this study may have important implications for study designs and for female vaccination schedules.

As the antigenic messenger between innate and adaptive immunity, DCs play an important role in guiding immune responses. Previous work from our lab implicated the X-linked transcriptional repressor JARID1c in DC quiescence. This study and others have identified female-derived DCs to exert an enhanced pro-inflammatory phenotype compared to male-derived DCs. This study additionally found that the observed female-bias in DC response is no longer present in the absence of JARID1c. This study therefore implicates JARID1c as a genetic mediator of sex-disparate immunity. In addition, certain germline mutations in human *JARID1c* are known to cause neurological disorders such as XLID. This cohort provides the opportunity to study JARID1c in the context of human immune responses. Finally, This study demonstrates that epigenetic mechanisms are involved in sex-biased immunity, providing a new avenue of study to better understand sex as a biological variable of immune response. Future work will need to be conducted to delineate the roles of JARID1c and its Y-linked paralog JARID1d in various DC subsets in the context of sex-specific inflammatory responses.

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