Molecular Regulation of Dendritic Cell Activation

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TABLE OF CONTENTS

Preface

Abstract

Dendritic cells (DCs) are antigen-presenting cells and are central regulators of the adaptive immune system. DCs are found in a steady state, poised to respond to activating stimuli. Once stimulated, they rapidly undergo dynamic changes in gene expression to adopt an activated phenotype capable of promoting the differentiation of T cell lineages against the particular threat. The exact mechanisms regulating the transition from steady state to activation of DCs are not well understood. In this work, we explored two novel mechanisms of DC regulation.

The first mechanism examined involves microRNA-9 (miR-9). MicroRNAs are emerging as important regulators of immune function due to their fast action and ability to regulate programs of gene expression. We found that miR-9 was upregulated in both bone marrow-derived DCs (BMDCs) and conventional DC1s but not in conventional DC2s following stimulation. miR-9 expression in BMDCs and conventional DC1s promotes enhanced DC activation and function, including the ability to stimulate T cell activation and control tumor growth. We then found that miR-9 reduced the expression of a group of negative regulators, including the transcriptional repressor Polycomb group factor 6.

The second mechanism explored involves the Cystic fibrosis transmembrane conductance regulator (CFTR). Cystic fibrosis is an incurable genetic disease caused by loss of function of the CFTR gene. CFTR loss leads to the creation of an environment suitable for colonization by various pathogenic bacteria in both the lungs and the intestines. Cystic fibrosis patients display an increase in inflammatory signalling that is cell intrinsic and not due to bacterial colonization, while also showing an increased risk for multiple autoimmune and inflammatory conditions. DCs

express CFTR and mice with CFTR knocked out specifically in CD11c expressing cells exhibited increased activation of intestinal DCs which promoted the activation of $Th17^+$ $CD4^+$ T cells. This correlated with a defect in early immune responses towards the intestinal pathogen *C. rodentium*. Finally, we found that loss of CFTR inhibits anti-inflammatory PI3K-Akt signaling in DCs, which may provide a starting point to understand the mechanism linking CFTR and DC regulation.

In summary, this work establishes the novel roles of miR-9 and CFTR in the molecular regulation of DC activation and function.

Résumé

Les cellules dendritiques (CD) sont des cellules présentatrices d'antigène et sont des régulateurs centraux du système immunitaire adaptatif. Les CD se trouvent dans un état stable, prêts à répondre aux stimuli d'activation. Une fois stimulés, ils subissent rapidement des changements dynamiques dans l'expression génique pour adopter un phénotype activé capable de favoriser la différenciation des lignées de cellules T contre la menace particulière. Les mécanismes exacts régulant la transition de l'état d'équilibre à l'activation des CD ne sont pas bien compris. Dans ce travail, nous avons exploré deux nouveaux mécanismes de régulation des CD.

Le premier mécanisme examiné concerne le microARN-9 (miR-9). Les microARN sont en train de devenir d'importants régulateurs de la fonction immunitaire en raison de leur action rapide et de leur capacité à réguler les programmes d'expression génique. Nous avons constaté que miR-9 était régulé à la hausse dans les DC dérivés de la moelle osseuse (MOCD) et dans les DC1 conventionnels, mais pas dans les DC2 conventionnels après stimulation. L'expression de miR-9 dans les MOCD et les DC1 conventionnels favorise l'activation et la fonction des CD, y compris la capacité de stimuler l'activation des cellules T et de contrôler la croissance tumorale. Nous avons ensuite constaté que miR-9 réduisait l'expression d'un groupe de régulateurs négatifs, y compris le répresseur transcriptionnel Polycomb group factor 6.

Le deuxième mécanisme exploré concerne le régulateur de conductance transmembranaire de la mucoviscidose (CFTR). La fibrose kystique est une maladie génétique incurable causée par une perte de fonction du gène CFTR. La perte de CFTR conduit à la création d'un environnement propice à la colonisation par diverses bactéries pathogènes dans les poumons et les intestins.

Les patients atteints de fibrose kystique présentent une augmentation de la signalisation inflammatoire qui est intrinsèque à la cellule et non due à la colonisation bactérienne, tout en présentant également un risque accru de maladies auto-immunes et inflammatoires multiples. Les DC expriment le CFTR et les souris avec le CFTR éliminé spécifiquement dans les DC ont présenté une activation accrue des DC intestinales qui ont favorisé l'activation des cellules T Th17 + CD4 +. Cela était en corrélation avec un défaut des réponses immunitaires précoces envers le pathogène intestinal C. rodentium. Enfin, nous avons constaté que la perte de CFTR inhibe la signalisation anti-inflammatoire PI3K-Akt dans les CD, ce qui peut fournir un point de départ pour comprendre le mécanisme liant la régulation CFTR et DC.

En résumé, ce travail établit les nouveaux rôles de miR-9 et CFTR dans la régulation moléculaire de l'activation et de la fonction des DC.

Acknowledgements

Over the past six years, I have had the pleasure of being trained by three supervisors who have all contributed to my success and influenced me for the better. Firstly, I would like to thank Dr. Connie Krawczyk whom I have trained with since I was an Honours student. Connie's mentorship has laid a strong foundation for my scientific career and has significantly shaped the scientist that I am today. I would also like to thank Dr. Jorg Fritz for his inspiring enthusiasm for science and for teaching me how to think outside of the box when presented with challenging scientific problems. I especially wanted to thank Dr. Samantha Gruenheid, who welcomed me into her lab in the middle of my PhD. Sam's contagious optimism, incredible ability to foster multidisciplinary collaborations, and inspiring leadership are all traits I hope to emulate in my future career.

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Research publications

This is a manuscript-based thesis. This work represents published or soon to be published manuscripts in the following publications:

Chapter 2: **Cordeiro B**, Jeon P, Boukhaled G, Corrado M, Lapohos O, Roy D, Williams K, Jones RJ, Gruenheid S, Sagan S and Krawczyk CM. MicroRNA-9 fine tunes dendritic cell function by suppressing negative regulators. Cell reports 31, 107585, doi:10.1016/j.celrep.2020.107585 (2020).

Chapter 3: **Cordeiro B**, Ackroyd T, Yousefi M, Ji D, Mindt B, Zhu L, Krawczyk CM, Hanrahan J, Gruenheid S, Fritz J. Loss of CFTR function promotes susceptibility to *Citrobacter rodentium* infection and immune dysregulation in the gastrointestinal tract. (*Manuscript in preparation*).

Other research contributions not appearing in this thesis

I have also contributed the following manuscripts that are not included in this thesis:

Pardy RD, Valbon SF, **Cordeiro B**, Krawczyk CM and MJ Richer. 2020. An Epidemic Zika Virus Isolate Suppresses Antiviral Immunity by Disrupting Antigen Presentation Pathways. Nature Communications. *(In press).*

Huang F, Gonçalves C, Bartish M, Rémy-Sarrazin J, Issa ME, **Cordeiro B**, Guo Q, Emond A, Attias M, Yang W, Plourde D, Su J, Gimeno MG, Zhan Y, Galán A, Rzymski T, Mazan M, Masiejczyk M, Faber J, Khoury E, Benoit A, Gagnon N, Dankort D, Journe F, Ghanem GE, Krawczyk CM, Saragovi

HU, Piccirillo CA, Sonenberg N, Topisirovic I, Rudd CE, Miller WH Jr, Del Rincón SV. Inhibiting the MNK1/2-eIF4E axis impairs melanoma phenotype switching and potentiates antitumor immune responses. J Clin Invest. 2021 Apr 15;131(8):140752. doi: 10.1172/JCI140752. PMID: 33690225.

Boukhaled GM, **Cordeiro B**, Deblois G, Dimitrov V, Bailey SD, Holowka T, Domi A, Guak H, Chiu HH, Everts B, Pearce EJ, Lupien M, White JH, Krawczyk CM. The Transcriptional Repressor Polycomb Group Factor 6, PCGF6, Negatively Regulates Dendritic Cell Activation and Promotes Quiescence. Cell Rep. 2016 Aug 16;16(7):1829-37. doi: 10.1016/j.celrep.2016.07.026

Contributions of authors

I wrote this thesis and designed all figures and tables presented. This thesis was thoughtfully edited by Rebecca Rabinovitch, Dr. Samantha Gruenheid, Dr. Jorg Fritz and Dr. Connie Krawczyk. The specific contributions to the work presented in this thesis are as follows:

Chapter 2

I constructed the miR-9 expression vector and miR-9 sponge expressing vectors. I prepared all BMDC cultures, T cell co-culture and antigen sensitivity assays. I performed all western blot and flow cytometry experiments. Peter Jeon and I performed RNA extractions and RT-qPCR experiments. Orsolya Lapophos, Peter Jeon and I performed ELISA experiments. Giselle Boukhaled, Mario Corrado, Dominic Roy and I performed the B16 melanoma experiments and monitored the mice for tumour growth. Kelsey Williams assisted with designing figures.

Experiments were designed by Connie Krawczyk, Samantha Gruenheid and I, with input from Selena Sagan. The manuscript was written by Connie Krawczyk and I.

Chapter 3

I performed all experiments involving CD11cCre and MeoxCre mice including; *C. rodentium* infections, assessing bacterial burdens and all immunophenotyping. I grew BMDC cultures, performed all T cell co-culture and antigen sensitivity assays. I performed all ELISA, western blot, and flow cytometry experiments on BMDCs (Figure 3-7). Travis Ackroyd, Mitra Yousefi, Barbara Mindt, Lei Zhu and Daisy Ji performed experiments and analysis involving the CFTR KO, ΔF508, VillinCre, gut corrected and LysMCre mice including; infection with *C. rodentium*, assessing bacterial burdens and immunophenotyping (Figure 1-3). Travis Ackroyd also performed immunohistochemistry staining and prepared histology slides for histopathological analysis. Travis Ackroyd also performed RNA extractions and RT-qPCR experiments for Figure 2. Electrophysiological analysis was performed in the Hanrahan lab. Experiments were designed by Samantha Gruenheid, Jorg Fritz and I. I wrote the manuscript and it was edited by Samantha Gruenheid, Connie Krawczyk and Jorg Fritz.

Contributions to original knowledge

The work presented in this thesis contributed several original findings to our understanding of the regulation of DC activation:

- We are the first to show that microRNA-9 plays a role in regulating DC activation and function.

- This study contributes strong evidence that miR-9 regulates the expression of multiple negative regulators of DC activation, simultaneously. These results add to the growing evidence that single miRNA can govern programs of expression in immune cells.
- We found that miR-9 regulates the activation and function of cDC1s but has no appreciable affect in cDC2s. These results contribute to evidence that cDC1s and cDC2s are regulated differently.
- We are the first to show that Cystic fibrosis mice are more susceptible to infection with *C. rodentium*.
- This study confirms that loss of CFTR induces an inflammatory state in the gastrointestinal tract in mice that is independent of intestinal obstruction and accumulation of mucus. Instead, we provide strong evidence that loss of CFTR induces dysregulated immune activation that contributes to the enhanced inflammatory state.
- We are the first to show that CFTR is a negative regulator of DC activation, potentially through inhibiting anti-inflammatory Akt signaling.

List of figures

Chapter 2:

Chapter 3:

List of tables

Chapter 2:

Chapter 3:

List of abbreviations

A/E- attaching and effacing
Ago argonaut
AIRE- autoimmune regulators
ALS- amyotrophic lateral sclerosis
AP-1- activator protein 1
APCs- antigen presenting cells
ARID5a- Adenine-thymine rich interactive domain 5a
BATF3- Basic leucine zipper ARF-like transcription factor 3
BCR-B cell receptor
BMDCs- bone marrow-derived DCs
BMDM- bone marrow-derived macrophages
CADM1 - cell adhesion molecule 1
CARD9- caspase recruitment domain family member 9
CCR2- C-C Chemokine receptor 2
CCR7- chemokine receptor 7
CD- Crohn's disease
cDCs- classical/conventional dendritic cells
CF- Cystic Fibrosis
CFTR- cystic fibrosis transmembrane conductance regulator
CFTR F508- CFTR Δ508 mutation mice
CFTR KO- CFTR knockout mice
CIITA- Class 2 trans-activator complex
CLEC9A- C-type lectin domain 9A
CLP- common lymphoid precursor
CLRs- C-type lectin receptors
cMoPs- common monocyte progenitor
CMP- common myeloid precursor
coREST- co-repressor Repressor-element-1 silencing transcription factor

CTLA-4- cytotoxic T-lymphocyte-associated protein 4
DAMPs- danger-associated molecular patterns
DCs- Dendritic cells
DGCR8- DiGeorge syndrome critical region
DP T cells – double positive T cells
EHEC- enterohemorrhagic *Escherichia coli*
EPEC- enteropathogenc *Escherichia coli*
ERK- extracellular signal-regulated kinase
FLT3- Fms-like tyrosine kinase 3
FLT3L- Fms-like tyrosine kinase 3 Ligand
FOXP3- forkhead protein 3
GAPs- goblet cell associated passages
GATA-3 - GATA binding protein 3
GM-CSF- Granulocyte-monocyte colony stimulating factor
GMP- granulocyte-monocyte progenitor
H2Aub- mono-ubiquitination of H2A
H3K27ac- acetylation of lysine 27
H3K27me3- Tri-methylation of lysine 27
H3K4me3- tri-methylation of lysine 4
H3K9me3- Tri-methylation of lysine 9
HDAC- histone de-acetylases
HDM- house dust mite
HSCs - Hematopoietic stem cells
IBD- inflammatory bowel disease
ICAMs- intercellular adhesion molecules
IDO- Indoleamine 2,3- dioxygenase
IECs- intestinal epithelial cells
IFN-β- interferon-beta
IFN-γ- Interferon-gamma
IKK- Ikappaβ kinase
IL- interleukin
IL-10R- IL-10 receptor
ILCs- innate lymphoid cells
inf-cDC2- inflammatory cDC2
IRF- interferon regulatory factor
iTreg- induced Treg
JMJD3 Jumonji domain-containing protein D3
JNK- Jun nuclear kinase
KDM4D Lysine demethylase 4d
KDM5c Lysine demethylase 5c
LFA-1- lymphocyte function-associated antigen 1

lin28a- lin-28 homolog A
LPS- lipopolysaccharide
M cells - microfold cells
MAPK- mitogen-activated protein kinase
MHC- major histocompatibility complex
miR-142- microRNA-142
miR-146- microRNA-146
miR-155 – microRNA-155
mir-181 – microRNA 181
miR-223- microRNA-223
miR-224- microRNA-224
miR-9- microRNA-9
miRNAs microRNAs
MLN- mesenteric lymph nodes
moDCs- monocyte-derived dendritic cells
MS- multiple sclerosis
mTECs- medullary thymic epithelial cells
MyD88- myeloid differentiation primary-response protein 88
NCOR1- Nuclear co-repressor 1
NF-κβ - nuclear factor-kappa Beta
NOD2- Nucleotide-binding oligomerization domain containing-2
nTreg- natural Treg
PAMPs- pathogen-associated molecular patterns
PBRM1 Polybromo1
PCGF6 Polycomb group factor 6
PD-1- programmed cell death-1
pDCs- plasmacytoid dendritic cells
PDLIM2- PDZ and LIM domain 2
PRC- Polycomb repressive complexes
PRC1.6- non-canonical PRC1 6
pre-miRNA- precursor-miRNA
pri-miRNA- primary miRNA
PRRs- pattern recognition receptors
RA- Rheumatoid arthritis
RALDH2- retinaldehyde dehydrogenase 2
RBPs- RNA binding proteins
RegIIIγ- regenerating islet-derived protein 3-gamma
REST- Repressor-element-1 silencing transcription factor
RIP- receptor interacting protein-1
RISC RNA-induced silencing complex
RNF125-ring finger protein 125

RORyt- RAR-related orphan receptor gamma
RSV- respiratory syncytial virus
RUNX3- Runt-related transcription factor 3
SHIP1- SH2 domain containing inositol phosphatase 1
SIBO- small intestinal bacterial overgrowth
SIN3α- SIN3 Transcription Regulator Family Member A
SIRT1- Sirtuin 1
SMAD- Sma and Mad proteins from *Caenorhabditis elegans* and Drosophila
SOCS1- Suppressor of cytokine signaling 1
STAT-3 signal transducer and activator of transcription 3
Syk- spleen tyrosine kinase
T-bet- T-box transcription factor
TCR- T cell receptor
TEDs- trans-epithelial dendrites
TGF-β- transforming-growth factor beta
TGF-βR- transforming growth factor-beta receptor
Th1 - Type 1 helper T cells
Th17 -Type 17 helper T cells
Th2- Type 2 helper T cells
TIR- Toll/IL-1R
TLRs- toll-like receptors
TME- tumour microenvironment
TNF- tumour necrosis factor
TNFAIP3 TNF-α-induced protein 3
tolDCs – tolerogenic dendritic cells
TRADD- tumor necrosis factor receptor type 1-associated DEATH domain protein
TRAF6 TNF receptor associated factor 6
Treg- regulatory CD4+ T cells
TRIF- TIR-domain containing adaptor protein inducing IFNB
TSLP- thymic stromal lymphopoietin
TTP Zinc-Finger protein tristetraprolin
UC- ulcerative colitis
USP38- ubiquitin specific peptidase 38
UTR untranslated regions
ZBTB46- Zinc finger and BTB domain containing 46
ZymD- Zymosan-depleted
Δ508- deletion of a phenylalanine residue at position 508

Chapter 1: Literature Review

1.1 The mammalian immune system

The immune system is a complex network of cells that defend the host organism from pathogens. Bacteria have a very simple immune system consisting of numerous enzymes to protect from infection by bacteriophages, but large multicellular organisms require a more complex immune system to defend against more diverse threats [1, 2]. The mammalian immune system utilizes both molecular- and cellular-based mechanisms to combat infection and maintain homeostasis [3]. The primary lymphoid organs, such as the thymus and bone marrow, are critical for production of immune cells. The spleen, lymph nodes, and mucosa-associated lymphoid tissue are secondary lymphoid organs and are where the immune cells will primarily communicate and engage responses. These immune sites are connected with the circulatory system, and each other, through the lymphatic system. Tissues are bathed in interstitial fluid, which accumulates waste products, cellular debris, proteins, and bacteria. This lymph fluid is drained into the lymphatic system and is filtered through the immune organs, exposing the immune cells to antigens [4].

The mammalian immune system is comprised of two arms, the innate immune system and the adaptive immune system. The innate immune system is an ancient defensive strategy and is the first line of defense against pathogens. This response is characterized by a rapid non-specific response involving both chemicals and recruitment of a variety of immune cells. The adaptive immune response is unique to vertebrates and is composed of more specific responses to infection [2].

1.1.1 The innate immune system

The innate immune system is comprised of many different cell types which all serve a functional and temporally specific purpose in combating threats to homeostasis. The innate immune system is non-specific and does not require prior exposure to a pathogen to engage a response, unlike the adaptive immune system. Innate immune cells are germline-encoded to respond to a variety of signals from both pathogens and host cells. These signals, usually in the form of danger-associated molecular patterns (DAMPs) or pathogen-associated molecular patterns (PAMPs), will dictate the type of response initiated against the particular threat [2]. Innate immune cells can be programmed to alter their response to secondary exposures to a previously encountered stimuli, either increasing or decreasing the strength of the response. This mechanism, termed trained immunity, enables innate immune cells to confer context-adjusted responses to stimuli [5].

Pathogens first come into contact with various barrier surfaces, such as the skin and mucosa, where various chemical and biological barriers render these surfaces impermeable to most pathogens [6]. If these barriers are breached, epithelial cells and resident innate immune cells like macrophages and dendritic cells (DCs) will detect the pathogens through their PAMPs. These innate cells will then respond through secretion of various chemical factors, such as cytokines and chemokines, to recruit other immune cells including neutrophils [7]. Immune cells recruited to the area of infection will attempt to quickly control pathogen burden through direct cell killing, phagocytosis, and secretion of pro-inflammatory cytokines. Different pathogens will elicit different innate immune responses through the detection of their specific

PAMPs by innate immune cells. For example, if a parasite breaches the barrier surface, the resident innate immune cells would react to parasite-specific molecular patterns and specifically eosinophils and basophils would be recruited to expel the parasite [8]. If innate immunity fails to clear the invading pathogen, then the adaptive immune system would need to be engaged to produce a more specific immune response [9].

1.1.2 Antigen presenting cells

The innate immune system is often sufficient to clear small doses of certain pathogens [10]. However, many pathogens have evolved strategies to evade the innate immune system and instead can only be completely cleared by the adaptive immune system [11, 12]. Antigen presenting cells (APCs) act as the bridge between the innate and adaptive immune systems. During an immune response, APCs residing in the infected tissue or in the draining lymph node will become exposed to antigens from the invading pathogen. APCs have the unique ability to phagocytize, process, and present these antigens to cells of the adaptive immune system, T lymphocytes (T cells) and B lymphocytes (B cells). This will lead to the creation of an immune response specifically targeted towards the antigen presented.

Major histocompatibility complex (MHC) proteins are used to present antigens to T cells. MHC class I proteins are present on all nucleated cells and are used to present endogenous peptide sequences on the surface of these cells for immune recognition. MHC class I will typically present self-peptides during homeostasis and antigens from any invading intracellular pathogens to cells of the adaptive immune system [13]. MHC class II proteins are present only

on APCs and allow these cells to directly present exogenous antigens to adaptive immune cells [14].

Macrophages, DCs and, B cells are all professional APCs but differ in their ability to activate adaptive immunity [15, 16]. DCs have a unique capacity to present extracellular antigens on MHC class 1, termed cross-presentation, to induce adaptive immune responses against intracellular pathogens without needing to first be infected. Macrophages are less efficient than DCs at presenting antigens to T cells [17]. DCs express the highest amount of MHC class 2 and thus can present more antigens to T cells then other APCs [17]. The lysosome of macrophages is highly acidic and thus destroys many potential peptides that can be presented to T cells. The lysosome in DCs is less acidic and thus more peptides can be processed from the same antigen, increasing the likelihood of encountering antigen-specific T cells in the lymph nodes [18].

1.1.3 Dendritic cells

In 1973, Ralph Steinman and Zanvil Cohn observed cells in the spleen with large neuron-like processes projecting from their cell bodies. These cells were morphologically distinct from macrophages and were soon discovered to be able to induce antibody and T cell responses [19]. These cells were formally named dendritic cells and were further classified as classical/conventional DCs (cDCs), which were later subdivided into two groups: cDC1s and cDC2s [20, 21]. Years after the discovery of cDCs another subset of DCs were discovered and named plasmacytoid DCs (pDCs). The ability of plasmacytoid DCs to present antigen like cDCs is

controversial but it is well established that their primary function is to secrete large amounts of Type 1 interferon (IFN) following viral infection [22]. DCs are now understood to be the most proficient antigen presenting cells, patrolling the local environment to sample antigens and inducing potent adaptive immune responses against varied threats [23].

1.1.4 Murine DC ontogeny

Hematopoietic stem cells (HSCs) are multipotent progenitors in the bone marrow that give rise to all cells of the blood, including immune cells [24]. HSCs give rise to the common myeloid precursor (CMP) and the common lymphoid precursor (CLP) based on expression of the transcription factor PU.1 , where CLPs express high amounts of PU.1 [24, 25]. Originally, it was thought that the CMP gave rise to all cells of the innate immune system and that the CLP gave rise to all cells of the adaptive immune system. It is now understood that innate lymphoid cells (ILCs) and most pDCs derive from the CLP [26, 27].

The CMP gives rise to the granulocyte-monocyte progenitor (GMP), which give rise to the common DC progenitor (CDP) and the common monocyte progenitor (cMoPs) [28]. The CDP will give rise to all cDCs, but not to any macrophages or other myeloid cells. The development of cDCs from the CDP is dependent on Fms-like tyrosine kinase 3 (FLT3) and its ligand FLT3L (aka CD135) [29, 30]. FLT3 is expressed highly in CLP, CDP, pre-cDCs, but is not expressed in other myeloid cell lineages or precursors [31, 32]. PU.1 drives expression of FLT3L in DC precursors and maintains FLT3L expression in mature DCs [33]. cDCs also express the transcription factor Zinc finger and BTB domain containing 46 (Zbtb46), which is not absolutely required for their

development but has been shown to reinforce commitment to the cDC lineage [34]. Granulocyte-monocyte colony stimulating factor (GM-CSF) is a critical growth factor for macrophage and monocyte development but has been shown to be dispensable for lymphoid-resident cDC development [35, 36]. GM-CSF has been shown to be important for maintaining non-lymphoid tissue resident cDCs, particularly cDC2s in the intestine [36, 37].

Pre-cDC precursors arise from the CDP and migrate out of the bone marrow and into the tissues [23]. Once in the tissues, pre-cDC precursors will differentiate into cDC1s and cDC2s. Originally it was hypothesized that once in the tissues, pre-cDCs would differentiate into cDC1s and cDC2s based on the tissue microenvironment [38]. With the advent of single-cell sequencing and mass cytometry, it is now appreciated that the CDP is comprised of a heterogeneous pool of fate determined pre-pDC, pre-cDC1, and pre-cDC2 precursors [39-42]. This fate determination is initiated by the expression of specific transcription factors at the CDP stage. Commitment to cDC1 and cDC2 lineages is distinguished by the mutually exclusive expression of either interferon regulatory factor (IRF) 8 or 4. Specification of the CDPs to pre-cDC1 has been found to be IRF8 dependent [40, 43]. In contrast, development of cDC2s is dependent on IRF4 expression [44]. Commitment to the pDC lineage is dependent on IRF8 as in cDC1s, but pDCs also express E2-2, which inhibits IRF8 [45]. cDC1s express Id2, which inhibits the function of E2-2 and thus potentiates IRF8 expression [45]. Basic leucine zipper ARF-like transcription factor 3 (Batf3) expression is also required for the development of $IRF8^{+}$ cDC1s, where it is responsible for potentiating $IRF8^{+}$ expression in mature cDC1s [46]. Recently, the cDC2 lineage was found to be comprised of two lineages; cDC2A and cDC2B [47]. cDC2A and cDC2B perform divergent functions through the expression of distinct sets of transcription

factors and unique repertoires of chemokine receptors. cDC2A express T-bet and are primarily associated with anti-inflammatory signaling whereas cDC2B express RORγt and are associated primarily with pro-inflammatory signaling [47].

Aside from the CDP, DCs can also arise from monocytes during inflammatory conditions [48, 49]. Monocytes are mononuclear innate cells which are derived from cMoPs in the bone marrow [50]. There are two major subsets of monocytes denoted by their expression of Ly6c: classical Ly6c^{Hi} and non-classical Ly6c^{Lo} monocytes [51, 52]. Non-classical monocytes patrol the circulation, interact with endothelial cells and aid in tissue repair [52, 53]. Classical monocytes are found in both the blood and in the tissues, where under inflammatory conditions they have historically been thought to differentiate into monocyte-derived dendritic cells (moDCs) and monocyte-derived macrophages [54]. moDCs, also known as inflammatory DCs, have been shown to accumulate at the sites of infection and present antigens to T cells to induce adaptive immune responses [55]. They have also been implicated in allergy: mediating T cell responses to the allergen house dust mite (HDM) [56]. moDCs have also been implicated in adaptive immune activation by components in vaccine preparations [57, 58]. Dissecting which functions are due to moDCs or cDCs in inflammatory conditions has proven difficult. moDCs and cDC2s express similar surface markers, which can lead to contamination during flow cytometry analysis [55, 59, 60]. It was recently shown that what was thought to be a population of moDCs in the lung was actually a mixture of monocyte-derived macrophages, with little ability to stimulate T cell responses, and a newly described bona fide cDC-derived inflammatory cDC2 (inf-cDC2) [61]. These inf-cDC2s express CD64, which is typically used to remove macrophages from cDC gating strategies, and express markers that were associated with moDCs [61]. It is

currently unclear whether inf-cDC2s are present in other tissues, but their existence in the lung makes it essential to revisit the roles of moDCs in other tissues.

1.1.5 Murine Classical/conventional dendritic cells

Multiparameter flow cytometry has facilitated the characterization of multiple DC subsets using the expression of surface receptors. Two major subsets of conventional DCs were then described in mice lymphoid tissues: $XCR1^+$ $CD8^+$ cDC1s and $CD172\alpha^+CD11b^+$ $CD4^+$ cDC2s [37, 62, 63]. However, analyzing these populations in non-lymphoid tissues led to difficulties in distinguishing the two subsets [64, 65]. For example, in inflammatory conditions, it became unclear whether differences in surface expression between groups of DCs were due to different subsets, modified versions of the same cell, or due to infiltration of moDCs [66, 67].

It was later revealed that many of the markers used to identify DCs were shared with other cell types, most notably macrophages [68, 69]. For example, both macrophages and DCs express CD11c and MHC class II, the prototypical markers used for DCs *in vitro* [68]. F4/80 had been used extensively to remove macrophages from analysis of cDCs, but cDC2s were found to also express F4/80 in inflammatory conditions [70]. It was hypothesized that cDCs were a specialized subpopulation of macrophages due to sharing many of the characteristic surface markers and performing similar functions [64, 71]. However, the discovery of DCs arising from a precursor that was distinct from the CMP solidified DCs as a subset of cell distinct from macrophages [38, 72]. Furthermore, distinct ontological progenitors for pDCs, cDC1s, and cDC2s solidified them as distinct subsets. To end the confusion, a new ontology-based nomenclature

for all myeloid cells was proposed: IRF8$^+$ cDC1s, IRF4$^+$ cDC2s, IRF8$^+$ E2-2$^+$ pDCs, macrophages, and moDCs [65].

The use of lineage markers to identify cDC1s and cDC2s *in vivo* has proven to be difficult. Macrophages and moDCs could not be completely removed from the analysis of DCs in tissues. An unsupervised high-dimensional analysis of surface marker expression of DCs, macrophages and monocytes across multiple tissues has since revealed that cDC1s and cDC2s can be identified through expression of unique surface markers while also excluding macrophages and other contaminating cells [70]. IRF8$^+$ cDC1s express high levels of chemokine XC receptor 1 (XCR1), whereas IRF4$^+$ cDC2s express high levels of CD172α. This same analysis has led to the creation of gating strategies that can analyze cDCs in a variety of mouse and human tissues using the same markers [70].

Functionally, cDC1s and cDC2s perform complementary but non-redundant functions in regulating adaptive immunity. cDC1s are particularly adept at cross-presenting antigen on MHC class I [73]. They are also an early source of IL-12, which is an important cytokine for skewing the immune response against intracellular pathogens [74, 75]. cDC1s are also essential for anti-tumour responses, where they have been shown to be essential in inducing T cell infiltration of tumours [73, 76]. cDC2s on the other hand, are critical for immune responses to extracellular pathogens using MHC class II [77]. cDC2s have also been implicated in allergic airway responses and in B cell responses [56, 78].

1.1.6 Human cDCs

Distinguishing macrophages from cDCs in human tissues has been as challenging as it has been in mice [79, 80]. Human DC subsets have historically been defined through the expression of CD141 (cDC1), CD1c (cDC2), and CD123 (pDC) [81-83]. These markers have limitations, however, as macrophages and monocytes also express CD141 and can upregulate the expression of CD1c in inflammatory conditions [70, 84, 85]. The pDC CD123 marker was also found to be expressed in cDC precursors [84, 86]. As seen in mouse DCs, new ontogeny-based nomenclatures and advances in flow cytometry gating strategies have changed how human cDCs are described [65, 70, 86]. These new approaches also allow for alignment of mouse, human, and primate DCs across multiple tissues [70]. Human cDC1s are defined as $XCR1^{+}$ but , unlike murine cDC1s, express intermediate to low amounts of CD11c [70, 87]. It is thus important to use CD26 along with CD11c to gate on $CD26^{+}CD11c^{low\text{-}int}$ cDC1s [70]. Human cDC1s also express cell adhesion molecule 1 (CADM1), which can be used in conjunction with XCR1 for increased accuracy [70, 88]. Human cDC2s express high amounts of CD172α and CD1c, but CD1c is also expressed on moDCs and cDC1s [85, 89]. Human cDC2s also express CD64, which in mice is used to remove macrophages and monocytes from DC analysis. For this reason, CD14 and CD16 are used to remove macrophages instead of CD64/F480 in mice [70].

Overall, cDC1s and cDC2s in humans have similar functions to mice cDCs. $XCR1^{+}$ cDC1s mediate efficient anti-viral T cell responses [90, 91]. $XCR1^{+}$ cDC1s have been shown to also be excellent cross presenters to T cells, but cross presentation is not as restricted to cDC1s in humans as it is in mice [90, 92, 93]. Human cDC2s have been shown to be able to cross present

antigens to T cells [94-96]. Human cDC1s secrete low amounts of IL-12 compared to their mouse counterparts, while human cDC2s secrete large amounts of IL-12 [85, 95]. Human cDC2s are proficient at engaging a host of immune responses and are not restricted to primarily allergic and anti-parasitic immune responses [97, 98].

Recently, high resolution single cell sequencing has defined a possible new lineage of human cDCs called DC3s [99, 100]. DC3s share phenotypic markers of both human cDC2s and monocytes, which has made it challenging to distinguish them [100]. DC3s do not arise from the CDP or the cMoPs, but instead develop from a subset of the GMP that expresses low CD123 [101, 102]. The cytokines required for the development of DC3s are not well understood. FLT3L treatment has been found to expand DC3s in the blood of human volunteers, which is consistent with its effects on other cDC subsets in humans [100]. In contrast, another group found that GM-CSF and not FLT3L was required for the differentiation of DC3s [102]. Similarly, the function of DC3s has been controversial. This is thought to be due to the difficulty in separating DC3s from cDC2s and moDCs in inflammatory conditions [100-102]. Currently, no mouse equivalent of DC3s has been identified.

1.1.7 Tissue distribution of cDCs

cDCs are found throughout lymphoid tissues but they are also located in nearly every non-lymphoid tissues such as the intestines, lungs, and skin. cDCs in these tissues are critical for capturing antigens from pathogens, migrating to the local lymph node and engaging the adaptive immune response. However, analysis of cDC subsets in different tissues have been

evolving separately, resulting in a number of different tissue specific markers and subsets being characterized. This has led to confusion within the field as to whether a tissue specific subset is a bona fide new subset or analogous to other tissue subsets. With the advent of ontogeny-based nomenclature, and new more accurate flow markers to remove contaminating cells, non-lymphoid cDCs have been more uniformly classified [65, 70].

In the intestine, cDCs play a critical role in engaging protective immune responses against diverse intestinal microbes [103-105]. They also induce oral tolerance to innocuous food antigens and the commensal microbial communities in the gut [106, 107]. Intestinal cDCs are found in the lamina propria (LP) of the small intestine, the Peyers patches, the mesenteric lymph nodes (MLN) and the colon [108, 109]. Aside from the typical cDC1 and cDC2 markers, intestinal cDCs are also defined by the expression of CD103 and CD11b. In mice, cDC1 intestinal DCs are also $CD103^+$ and $CD11b^-$ [110]. cDC2s are divided into two subsets, $CD103^-CD11b^+$ cDC2s and $CD103^+$ $CD11b^+$ cDC2s [69, 111] . These populations of intestinal cDCs are maintained in human intestines [70, 112]. $CD103^+$ $CD11b^+$ cDC2s were originally thought to only be present in the gut but have recently been described in the nasal cavity as well [113]. $CD103^-$ $CD11b^+$ cDC2s will become $CD103^+$ $CD11b^+$ in response to the cytokine transforming-growth factor beta (TGF-β). $CD103^+$ $CD11b^+$ cDC2s are transcriptionally closely related to $CD103^-$ $CD11b^+$ cDC2s but differ in their ability to regulate immune responses in the intestinal tract [114]. $CD103^+$ $CD11b^-$ cDC1s are the major DC subset in the colon but $CD103^+$ $CD11b^+$ cDC2s dominate in the small intestine [69, 110]. The reason for this difference is not well understood but is thought to be due to differences in microbial communities between the small and large intestine [115].

In the lungs, cDCs contribute to defense against inhaled pathogens through engaging adaptive immune responses to presented antigen. cDCs are also capable of engaging in maladaptive immune responses that are detrimental to the host. There are two major cDC subsets in murine lungs: $CD103^+$ $CD11b^-$ cDC1s and $CD103^-$ $CD11b^+$ cDC2s [56, 70, 116, 117]. Interestingly, $CD103^+$ cDC1s also express langerin (aka CD207), which is predominantly expressed on DCs in the skin [118, 119]. $CD103^+$ $CD207^+$ cDC1s are found in the epithelial layers of the lung whereas $CD103^-$ cDC2s are found in the underlying lamina propria [120, 121]. Both are important for the direct recognition of inhaled antigens, with cDC1s primarily responding to viral pathogens and cDC2s responding to allergens [57, 122, 123]. cDCs are also indispensable for suppressing immune responses towards innocuous inhaled antigens, though the exact cDC subset responsible is not well understood [120]. In the human lung, cDC1s and cDC2s are differentiated based on different markers: CD141 and CD1c respectively [85, 124].

In both murine and human skin, cDCs are primarily found in the dermis and can be subdivided into $XCR1^+$ cDC1s and $CD172a^+$ cDC2s as in other tissues. A critical difference is that skin cDC1s express CD207 and can be further subdivided by the expression of CD103: $CD103^-$ cDC1s and $CD103^+$ cDCs [125, 126]. $CD103^+$ cDCs are proficient cross-presenters of skin tumour antigen and have shown to be potent inducers of $CD8^+$ T cell responses against fungal pathogens [127-129]. $CD103^-$ cDC1s express high amounts of C-type lectin domain 9A (CLEC9A) which allows them to capture and clear dead cell debris [130, 131]. $CD172\alpha^+$ cDC2s can be subdivide based on the expression of CD11b: $Cd11b^+$ cDC2s and "double negative" $CD11b^{lo}$ $Cd11c^{lo}$ cDC2s [129]. Past-research on the function of $CD11b^+$ cDC2s is hard to interpret due to contamination with monocytes, which also express CD11b and other typical DC markers [125].

Nonetheless, it is clear that CD11b$^+$ cDC2s are potent producers of retinoic acid, the rate limiting metabolite in vitamin A synthesis [126]. Retinoic acid production from CD11b$^+$ cDC2s promotes suppression of immune responses to the beneficial skin microbiome as well as tempering the immune response against continual exposure to antigens on the skin [126, 132]. CD11b$^+$ cDC2s have also been shown to be potent inducers of antibody and allergy responses to skin allergens [133]. The function of double negative cDC2s is not well understood [125]. Both subsets of cDC2s express high amounts of C-C chemokine receptor 2 (CCR2), which is associated with migration to the local skin lymph nodes, whereas cDC1s do not express CCR2 [48]. A double negative cDC2 equivalent has yet to be found in human skin [134].

1.1.8 Bone-marrow derived DC culture systems

Due to the rarity of DCs in tissues and their short lifespans in *ex vivo* cultures, studying DCs *in vitro* is challenging. Many immortalized DC cell lines only recapitulate some of the features of wild type DCs [135]. Instead, much of the biology of DCs has been discerned through the use of *in vitro* cultures of hematopoietic precursors differentiated into DCs through the use of growth factors [136, 137]. The most popular protocol involves culturing mouse bone marrow with GM-CSF to generate CD11c$^+$ MHC class II$^+$ cells that resemble DCs [138, 139]. These bone marrow-derived DCs (BMDCs) have been used extensively to elucidate the molecular mechanisms of various DC functions including cross presentation of soluble antigen and anti-tumour responses [140].

It was known that BMDC cultures generated with the use of GM-CSF are a heterogenous culture of DCs and bone marrow-derived macrophages (BMDM) [138]. GM-CSF BMDC cultures include both adherent and loosely adherent cells. The BMDMs were thought to be contained in the adherent cell populations and thus could be removed from downstream analysis by examining only the loosely adherent populations which contained BMDCs [141, 142]. It was recently revealed that the non-adherent population includes both DCs and also macrophages [143]. Both populations express CD11c, and both cell populations were found to respond to stimulation but displayed different functional properties, which impacts the interpretation of the results obtained from these cultures [143]. For example, upon stimulation BMDMs in culture will preferentially express interferon-stimulated genes, which led to the belief that BMDCs expressed these genes [143, 144]. To decrease the amount of BMDMs generated, some protocols include the addition of IL-4 to the GM-CSF cultures but these cultures remain heterogenous [143]. These findings are not absolute for all protocols however, as frequencies of BMDMs in the BMDC cultures vary between research groups, and so it is possible that some protocols lead to cultures lacking BMDMs [143]. A greater understanding of the role of FLT3L in generating cDCs *in vivo* has led to the generation of BMDC cultures using FLT3L instead of GM-CSF [38, 145, 146]. BMDCs generated from these cultures give rise to CD172α^{hi}, CD172α^{lo}, and pDCs which have shown to resemble comparable DC populations in the spleen. These cultures generate significantly fewer cDCs but may be more phenotypically and functionally comparable to cDCs then other BMDC culture protocols [38].

1.1.9 The adaptive immune system

Once DCs acquire antigens from the periphery, they will migrate to the local lymph nodes and engage the adaptive immune response by interacting with lymphocytes. Although initially slower to respond then the innate immune system, the adaptive immune system is capable of generating a specific response against invading pathogens and is often required for a sterilizing resolution of infection [147].

The adaptive immune system is comprised of two groups of lymphocytes, which are distinguished by their antigen specific receptor. T cells recognize antigen through the T cell receptor (TCR) and are subdivided into two major classes based on the co-receptor associated with their TCR: $CD4^+$ T cells and $CD8^+$ T cells. $CD8^+$ T cells respond to antigens presented on MHC class I molecules and are typically involved in cytotoxic responses against intracellularly infected host cells and anti-tumour immunity. $CD4^+$ T cells (also known as helper T cells) respond to antigens presented on MHC class II molecules and communicate to other immune cells to enhance their responses against particular threats [148]. B cells on the other hand recognize antigen through their B cell receptor (BCR), which is a cell surface-bound immunoglobulin. B cells are involved in antibody production, and direct responses against whole antigens. Antibodies perform many functions including: inactivating toxins, opsonizing pathogens to aid in clearance through innate cells, and direct cytotoxic effects on pathogens. B cells can also present whole antigens to helper T cells which activates both the T cell and the B cell [149].

Adaptive immunity is only possible due to a lymphocyte specific process known as VDJ recombination that only occurs in T cells and B cells during their development. The antigen binding domains of the TCR and BCR contain variable (V), diversity (D) and joining (J) genes which will be randomly assembled. This generates unique antigen binding domains on each TCR or BCR of each lymphocyte. This produces an enormous repertoire of antigen binding domains which can bind over one hundred billion different antigens, allowing lymphocytes to recognize and respond to most pathogens [150]. Innate immune cells are limited to what they can respond to by their germline encoded Pattern recognition receptors (PRRs) but adaptive immune cells can respond to novel pathogens not encountered by ancestors, and pathogens that mutate frequently [4].

VDJ recombination is a costly process as all recombinations must end in-frame for the final protein product to be produced [150]. The randomness intrinsic to VDJ recombination will lead to some lymphocytes with antigen binding domains that recognize proteins on host cells and can lead to immune responses against self [147]. Two strategies have evolved to prevent immune reactions to self-proteins: central and peripheral tolerance. Central tolerance is the process of removing self-reacting T cells and B cells during development. In brief, specialized cells expressing autoimmune regulator (AIRE) will present self-antigens to lymphocytes, resulting in self-reacting lymphocytes to die by apoptosis. Peripheral tolerance occurs when lymphocytes encounter self-antigens after development is complete and functions to inhibit the activation of the self-reacting lymphocytes. Anergy is one mechanism of peripheral tolerance, which occurs when lymphocytes encounter self-antigen without receiving proper activation signals from APCs. This causes the T cell to become anergic, preventing any

responses to that self-antigen. Central tolerance is limited by what self-antigens can be produced by the AIRE, so most tolerance is established through peripheral tolerance [147].

Another critical aspect of adaptive immunity is immunological memory. Once exposed to an antigen, some of the antigen-specific lymphocytes will differentiate into long-lived memory lymphocytes, which can respond more quickly to another exposure to that pathogen. This process is exploited in vaccination, where antigens from a pathogen are artificially delivered to the adaptive immune system in the hopes of creating a population of memory lymphocytes to respond during an actual infection with that pathogen [149].

1.1.10 T cell development

T cells are first generated from the CLP in the bone marrow as early T cell precursors, which then migrate to the thymus to develop. The thymus is the primary location for T cell development and is where T cells undergo central tolerance. Early T cell precursors entering the thymus do not express either $CD4^+$ or $CD8^+$ co-receptors and will undergo TCR rearrangement to generate double positive $CD4^+$ $CD8^+$ T cells (DP T cells). DP T cells will then come into contact with cortical epithelial cells, where DP T cells must bind to either MHC class I or MHC class II molecules to survive positive selection and continue developing. Positive selection ensures that T cells have properly functioning TCRs which can bind to self-MHC class I or self-MHC class II molecules with high enough affinity. These DP T cells will then undergo negative selection by interacting with thymic DCs presenting self-antigens on MHC class I or MHC class II [151-153]. Any DP T cells that bind to self-antigens too strongly will undergo apoptosis to prevent self-

reacting T cells from leaving the thymus. Central tolerance ensures that any T cells that interact too strongly with self-antigen are deleted but also ensures that T cells can interact with self-MHC molecules and signal appropriately through their TCRs [153, 154] . The remaining DP T cells will become single positive T cells expressing either $CD4^+$ or $CD8^+$ co-receptors [152]. These T cells will then migrate out of the thymus and can mostly be found in the secondary lymphoid organs [155].

1.1.11 T cell activation

Once a T cell completes development, it leaves the thymus as a naïve T cell and is considered immature until it encounters the antigen its TCR can bind. Once an APC presents the T cell with its cognate antigen, it will rapidly undergo clonal expansion, where the antigen-specific T cell will rapidly proliferate to give rise to identical antigen-responding T cells. At this stage, the T cell is no longer naïve and is considered an effector T cell [156].

A naïve $CD8^+$ T cell that encounters its cognate antigen will differentiate into a cytotoxic T lymphocyte (CTL). These effector $CD8^+$ T cells will migrate out of the local lymph node and will patrol the tissue in search of its cognate antigen. Since $CD8^+$ T cells respond to intracellular antigens, these antigens will be presented on MHC class I molecules in virally infected or cancerous cells to allow the T cell to detect the antigen. Once the CTL detects its antigen in the context of MHC class I, it will directly kill the cell using potent effector molecules such as perforin and granzyme B [157].

Effector $CD4^+$ T cells are subdivided into different classes depending on the type of immune response they shape. These subtypes are identified through the expression of different master transcription factors that control the immune program they elicit. Type 1 helper T cells (Th1) express the transcription factor T-box transcription factor (T-bet) and primarily promote control of intracellular pathogens. Type 2 helper T cells (Th2) express GATA binding protein 3 (GATA-3) and shape responses against large parasites and help B cells to produce antibodies. Type 17 helper T cells (Th17) express RAR-related orphan receptor gamma (RORyt) and shape responses against extracellular bacteria and fungi. There are also regulatory $CD4^+$ T cells (Treg) that express forkhead protein 3 (FOXP3), which suppress immune responses as part of peripheral tolerance and are critical for resolution of adaptive responses after the threat is removed. A number of other effector $CD4^+$ T cell subtypes have been identified and are still an active area of research [156].

Naïve T cells which differentiate into effector T cells will also upregulate the expression of various surface activation markers essential for effector function. CD44 expression is significantly upregulated upon T cell activation and is widely accepted as a marker of antigen experience in mice and humans. CD44 is an integrin which mediates T cell extravagation into inflamed tissues through interactions on endothelial cells [158]. CD69 is a surface molecule that is used as a T cell activation marker *in vitro* but is not reliable *in vivo*. CD69 is upregulated at the early phase of T cell activation, where it temporarily inhibits T cell egress from the lymph node. At the peak of activation, CD69 becomes downregulated and the reduced expression enables T cells to leave the lymph node [159]. The expression of the alpha chain of the IL-2 receptor (aka CD25) is upregulated upon T cell activation and is critical for the clonal expansion of effector T

cells [160]. CD25 increases the affinity of the IL-2 receptor for IL-2, a cytokine critical for engaging T cell proliferation and survival [160]. Effector T cells will also decrease the surface expression of proteins that prevent effector function. Naive T cells express high amounts of CD62L which is a homing receptor from the thymus to the lymph node [161, 162]. CD62L expression is rapidly downregulated in effector T cells to allow them to leave the lymph node [163].

1.2 DC activation and function

As naïve T cells enter the lymph node, they migrate to the T cell zone and come into contact with resident DCs or DCs migrating in from the periphery. Naïve T cells will frequently make close contacts with many DCs in the lymph node, scanning MHC complexes through the TCR, in search of its cognate antigen [164]. Once a naïve T cell binds to a DC presenting its cognate antigen, the T cell will differentiate to an effector T cell and begin the adaptive immune response [165]. In the process of differentiating, naïve T cells must receive three signals from DCs: antigen presentation, co-stimulation, and cytokine production to fully transition into effector T cells [165]. DCs cannot confer the three signals to T cells without receiving proper stimulation from the environment. In the absence of inflammatory signals from the environment, DCs are found in a “resting” or steady state and are poor activators of T cell responses [166, 167]. Only through differentiating into activated or mature DCs can they confer the three signals to T cells and properly engage the adaptive immune response. This three-

signal requirement is thought to protect the host from spontaneous T cell activation, which could lead to auto-immune disease [160, 161].

1.2.1 Sensing the environment

DC activation is strongly induced by pathogens or stimulatory compounds in the environment. DCs sense the environment through specialized germline-encoded PRRs [168, 169]. PRRs consist of both surface-bound and intracellular receptors that initiate signaling cascades upon exposure to stimulatory signals [169]. PRRs detect conserved molecular motifs associated with pathogens that are not expressed by host cells [168]. These PAMPs will typically be components of the bacterial cell wall, such as lipopolysaccharide (LPS) which is found on gram negative bacteria [170]. PRRs can also detect microbes through binding bacterial-specific features found on their nucleic acids, such as unmethylated CpG-islands [171, 172]. It is now appreciated that PRRs can also detect molecular motifs associated with host-cell death or damage, indicative of infection or sterile-injury. DAMPs are intracellular host molecules that normally wouldn't be found outside the cell, such as heat shock proteins or mitochondrial DNA [173]. Both DAMPs and PAMPs will activate PRR receptors and lead to DC activation.

The best characterized surface bound PRRs are the toll-like receptors (TLRs), which are evolutionarily conserved receptors present in a variety of different species [174, 175]. TLRs were originally discovered in *Drosophila* but are present in most vertebrates and invertebrates and are considered one of the most ancient components of the immune system [174, 175]. Different species express different members of the TLR family. For example, there are ten TLRs

in humans (TLR1-10) whereas mice do not express TLR10 but do express three TLRs not found in humans (TLR 11-13) [176]. This difference in expression of TLRs across species is thought to be related to differential exposure to certain pathogens. Each TLR member is specialized in detecting specific PAMPs and can influence the initiation of specific immune responses against that type of pathogen [169, 174]. For example, TLR5 can detect bacterial flagellin and initiate anti-bacterial immune responses [177]. TLR-6 can detect the fungal cell wall component zymosan and engage anti-fungal immune responses [178]. Intracellular TLRs, such as TLR7-9, can engage anti-viral immunity through recognition of viral genomic material [179, 180]. Some PRRs can detect PAMPs and DAMPs simultaneously, such as TLR4 which can detect LPS from bacteria and also various host cell heat shock proteins [181].

Many different cell types express differing members of the TLR family in order to sense danger and engage immune responses [9, 169]. DCs express an array of TLRs and thus can surveil for many different pathogens and initiate adaptive immune responses specific for the detected threat [23]. Interestingly, different DC subsets express distinct TLRs which contribute to that subsets' specialized function. Plasmacytoid DCs, specialized for anti-viral responses, express high levels of viral RNA/DNA sensing TLRs, such as TLR7 and TLR9 [182, 183]. Murine cDC1s express high levels of TLRs associated with detection of intracellular pathogens, such as viruses and bacteria while cDC2s express high levels of TLR4/TLR5 and specialize in the detection of extracellular bacteria [183, 184].

C-type lectin receptors (CLRs) are another family of surface-bound PRRs that are particularly known for detecting fungal pathogens but can also bind mycobacterial, viral, and helminth PAMPs [185]. Dectin-1, for example, is a CLR that binds to B-glucan carbohydrates

found on the cell wall of fungal pathogens [186]. TLRs and CLRs can overlap in the microbes they can detect by binding to different PAMPs expressed by the same microbe [187]. For example, the allergen HDM is composed of PAMPs that can be detected by both TLR4 and the CLR Dectin-2 [188]. TLR4 and Dectin-1 can also both detect different PAMPs composing the fungal cell wall component Zymosan [187]. Zymosan that has been treated with hot alkali, Zymosan-depleted (ZymD), is detected by Dectin-1 but destroys the PAMP that is detected by TLR4 [189].

1.2.2 TLR signaling

Most TLR signaling leads to the production of various immunoregulatory factors, mainly pro-inflammatory cytokines, by engaging the activity of two major signaling pathways: nuclear factor-kappa beta (NF-κβ) pathway and activator protein 1 (AP-1) pathway [190]. Some TLRs will also induce the production of interferons, such as Interferon-gamma (IFN-γ) or interferon-beta (IFN-β), which are cytokines that enhance responses against intracellular bacteria and viruses. This induction occurs through translocation of Interferon regulatory factors (IRFs) into the nucleus [191]. All three of these major pathways lead to increased transcription of pro-inflammatory genes necessary for innate immune responses against pathogens [185, 192, 193].

TLRs bind their ligands through leucine-rich repeat motifs on their extracellular domains, which form a concave surface for PAMP/DAMP binding [194]. TLRs differ in their LRR motifs but all TLRs share a conserved intracellular signaling domain known as the Toll/IL-1R (TIR) domain [195]. Upon binding of PAMPS/DAMPs, TLRs will dimerize and TIR domains will initiate

intracellular signaling through the recruitment of adaptor proteins [174]. For most TLRs, this will involve recruitment of myeloid differentiation primary-response protein 88 (MyD88) which will link TLRs with downstream signaling molecules [174]. Typically, MyD88 recruitment will lead to the activation mitogen-activated protein kinase (MAPK) family members such as extracellular signal-regulated kinase (ERK) 1/2, p38 and Jun nuclear kinase (JNK) [196, 197]. These proteins mediate phosphorylation of AP-1, which allows it to traverse into the nucleus and activate the transcription of target genes [174, 196]. MyD88 recruitment can also activate NF-κβ signaling, allowing the translocation of NF-κβ into the nucleus and activation of the transcription of pro-inflammatory genes [190]. Expression of pro-inflammatory cytokines, such as IL-6 or tumour necrosis factor (TNF), require AP-1 and NF-κβ activity in the nucleus [174, 198].

Some TLRs can also signal using MyD88-independent pathways, usually involving members of the TIR-domain containing adaptor protein inducing IFN-β (TRIF) family [191, 199]. This pathway is predominantly associated with the activation of IRFs and the production of interferons [199]. MyD88-independant pathways can also lead to activation of MAPK and NF-κβ signaling [174, 198]. TLR4 utilizes both MyD88-dependant and independent signaling pathways when detecting LPS, with each pathway inducing the expression of different pro-inflammatory genes [200].

C-type lectins like Dectin-1 will also initiate pro-inflammatory gene expression through NF-κβ signaling but use different adaptor molecules. When Dectin-1 detects PAMPs, spleen tyrosine kinase (Syk) is recruited and induces the formation of a molecular complex which includes caspase recruitment domain family member 9 (CARD9) [185]. This complex will

mediate the activation of NF-κβ by degrading its inhibitor Ikappaβ kinase (IKK). NF-κβ is then free to translocate to the nucleus and induce expression of target genes [186, 201].

PRR signaling induces the differentiation of DCs to the activated state and leads to many phenotypic consequences, especially concerning antigen presentation. Steady-state DCs express high amounts of MHC class II, but PRR signaling will induce further expression of MHC class II to increase the amount of peptide MHC-antigen complexes on the surface [202]. PRR signaling will also increase the ability of DCs to phagocytize antigen and process it for presentation, but only for a brief time as PRR signaling will eventually shut down phagocytosis and antigen processing [202]. This is thought to ensure that DCs present specifically the antigens in the environment that activated them, to induce an adaptive immune response tuned to the particular stimuli in the inflamed tissue. PRR signaling will also stabilize peptide MHC-antigen complexes on the surface of the activated DCs in order to give DCs more time to find and prime antigen-specific T cells. DCs will also upregulate the expression of the chemokine receptor 7 (CCR7), which will allow them to migrate to the local lymph node and interact with T cells [203, 204].

1.2.3 Co-stimulation and Co-inhibition

Recognition of the cognate-peptide MHC complex by the TCR will lead to TCR signaling but co-stimulation is required to fully induce the differentiation of naïve T cells into effector T cells. PRR signaling will induce the expression of multiple co-stimulatory molecules on the surface of DCs, which will bind ligands on the interacting T cell [205, 206].

CD80 (B7.1) and CD86 (B7.2) are the most important and most widely studied co-stimulatory molecules on DCs [207]. CD80/CD86 interact with CD28 on T cells, which promotes the expansion and differentiation of effector T cells [208]. CD28 signaling stabilizes the production of IL-2, which is consumed by proliferating T cells to support their growth [209]. CD28 signaling can also reduce Treg differentiation by inhibiting signaling by IL-10, an immunosuppressive cytokine [210].

As T cells are co-stimulated by DCs, the expression of other co-stimulatory receptors will increase and allow for other signaling events. CD40 is a co-stimulatory molecule expressed on activated DCs that has been shown to play an important role in cellular and humoral immunity [192, 211, 212]. CD40 binds CD40L on T cells, which is only expressed when the T cell has already been activated [192, 212]. Instead of the DC stimulating the T cell, the CD40:CD40L interaction will further promote the activation of the DC. This interaction can take place with either the DC that initially activated the T cell or a new DC presenting the same antigen [192]. This allows the activated T cell to promote the immune response through a feed-forward mechanism [213].

Interestingly, CD80/86 can also suppress T cell responses by binding cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) instead of CD28 on T cells [214, 215]. CTLA-4 is expressed by activated T cells and can compete with CD28 for CD80/86 binding. CTLA-4 has a higher binding affinity for CD80/86 than CD28 and increases in expression as the T cells remain activated, providing a negative feedback loop to eventually shut down T cell responses [214]. The difference in the amount of CD80/CD86 on DCs correlates with the extent of activation induced in T cells [216]. Immature DCs are not efficient at inducing T cell responses, as the low

expression of CD80/86 on steady-state DCs will preferentially bind with CTLA-4 and prevent T cells from becoming activated [214].

T cells can also be suppressed through the action of programmed cell death-1 (PD-1) [217]. PD-1 is expressed on T cells and binds two ligands, PD-L1 (B7-H1) and PD-L2 (B7-H2), expressed on DCs [217]. PD-1 signaling decreases TCR signaling, reduces T cell activation and cytokine secretion [218]. However, strong co-stimulation through CD80/CD86 can overcome the suppressive signaling through PD-1 [219].

1.2.4 Cytokine production

The final signal conveyed from the DC to the T cell is delivered via cytokine secretion, which will give the T cell the cue to differentiate into a particular effector subset [220-222]. PRR signaling will generally lead to the production and secretion of cytokines by DCs [223]. Different PAMPs will elicit the secretion of particular cytokines related to the immune response needed to clear the specific threat [223]. For example, the detection of LPS by TLR4 will induce DCs to produce Th1-skewing cytokines such as IL-12 and TNF-α. Detection of fungal cell wall components by Dectin-1 will instead lead to the secretion of Th17-schewing cytokines such as IL-23 and IL-6 [224, 225].

Cytokines will bind to extracellular cytokine receptors on T cells and initiate signaling cascades that will drive the differentiation of $CD4^+$ T cells into particular effector subsets [221, 226]. These signals will also promote further production of cytokines from the T cells themselves, which can feed forward the polarization signals or activate other immune cells

[227]. The differentiation of Th1 effector T cells is primarily accomplished through IL-12 and IFN-γ, which will activate T-bet signaling and induce a transcriptional program to enhance the elimination of intracellular pathogens [228, 229]. Th1 derived IFN-γ can enhance the function of macrophages and other phagocytes by increasing phagocytic activities, and can also induce the differentiation of more Th1 T cells [230]. Th1 $CD4^+$ T cells can also promote $CD8^+$ T cell responses through "licensing" DCs via CD40 co-stimulatory signaling to further promote Th1 immune responses [231, 232]. CD40:CD40L signaling will promote IL-12 cytokine secretion in previously activated DCs, which can then make contacts with $CD8^+$ T cells and induce their differentiation into CTLs [233]. These same DCs will secrete IL-2, a critical proliferative and survival signal for $CD8^+$ T cells [234].

IL-4 signaling on $CD4^+$ T cells will activate GATA-3 signaling and induce differentiation into the Th2 subset, enhancing anti-parasite immunity [235]. Th2 T cells secrete IL-4, IL-5 and, IL-13 which will promote the clearance of parasites through effects on both innate immune cells and epithelial cells [236]. Th2 derived IL-4 can enhance the function of mucus secreting goblet cells and enhance the contractibility of smooth muscles cells in the intestinal tract to aid in the expulsion of gastrointestinal parasites [235, 237].

Th17 differentiation is elicited through multiple key cytokines including IL-6, IL-23 and, TGF-β [238, 239]. These cytokines signal through RORyt, which will allow Th17 T cells to engage immune responses against extracellular bacteria and fungi [238]. Th17 T cells secrete cytokines that increase recruitment of neutrophils to infection sites and enhance their cytotoxic function against fungal structures [240].

DCs can also induce the differentiation of Tregs through cytokine secretion [241]. There are two major subtypes of $CD4^+$ Treg cell: induced Treg (iTreg) and natural Treg (nTreg). Natural Tregs are produced in the thymus during selection and naturally express high levels of FOXP3. The role of DCs in promoting nTreg differentiation is controversial but it has been shown that thymic DC-derived cytokines can drive their differentiation in the thymus [242-244]. Naïve $CD4^+$ T cells can be induced to express FOXP3 via IL-10 and TGF-β secretion by DCs, becoming $FOXP3^+CD25^+$ iTregs [245]. Both subsets of Treg secrete high amounts of IL-10 and potently reduce the immune response. They are critical for limiting damage from active immune responses and for preventing autoimmunity [245, 246].

Although this discussion outlines the basic principles of $CD4^+$ T cell differentiation, this process can be far more complex, and may involve the integration of numerous signaling events [220, 221, 247]. Within a given lymph node there is a diversity of secreted factors making up the cytokine milieu, which signal to T cells and other immune cells [221]. As more activated DCs migrate into the lymph node, the cytokine milieu will shift towards driving the appropriate immune response and outpace the other signaling pathways. Further complicating matters, some cytokines can contribute to different immune responses depending on the amounts of other cytokines present [248, 249]. DC-secreted IL-6 can skew T cells into Th1 and Th17 profiles, depending on other cytokines signaling to the T cell [31, 220]. It is now appreciated that effector $CD4^+$ T cells are a highly heterogenous cell population that undergo a continuum of polarization levels when activated by mature DCs [221, 247]. Even under controlled *in vitro* conditions, stimulation of effector $CD4^+$ T cells generates heterogenous populations with hybrid cytokine expression profiles [250-252]. This has recently been shown *in vivo*, where single cell

sequencing showed that effector $CD4^+$ T cells in the gastrointestinal tract exist as a continuum of effector phenotypes and do not segregate into the various Th subsets [253]. These results indicate that effector $CD4^+$ T cells existing in a more plastic state than previously appreciated. Further work is needed to dissect this complex regulatory network responsible for T cell activation [226].

1.2.5 Direct DC: T cell interactions via the immune synapse

T cells constantly patrol the secondary lymph organs, scanning DCs in the lymph node in search of their cognate antigen [254, 255]. Scanning T cells will pause on each DC in the lymph node, forming short lived interactions as their TCR samples peptide-MHC complexes [256, 257]. Once a T cell encounters its cognate antigen in the context of MHC, it establishes a prolonged contact with the DC involving extensive cell surface reorganization [258-260]. This interaction forms the immune synapse, which was named after similarities were found with the neuronal synapse [261]. There are profound differences in immune synapse formation between the different APCs [262]. B-cells form a very rigid synapse with T cells, consisting of concentric rings of segregated clusters of proteins, whereas the DC:T cell synapse is very dynamic and involves multiple contacts between the cells [262, 263]. This is due to DCs possessing cell membranes with multiple veils and ruffles, which increase in density during maturation due to cytoskeletal reorganization [264, 265]. These structures allow for the formation of multiple CD28/co-stimulation signaling complexes instead of a singular macrocomplex observed in B cell: T cell

interactions [263, 266]. The immune synapse is required for DCs to proficiently convey the three signals to T cells to induce their specific differentiation into effector T cells[261].

At the onset of synapse formation, the cell membranes of both the DC and T cell undergo significant structural changes [267, 268]. This is initialized through binding of DC intercellular adhesion molecules (ICAMs) to lymphocyte function-associated antigen 1 (LFA-1) on T cells [268, 269]. This interaction retains both cells in close proximity and allows for further reorganization events. Binding of LFA-1 to ICAM also initiates pro-inflammatory signaling events which reinforce TCR signaling, increasing both T cell activation and proliferation [270-272]. ICAM:LFA-1 binding will cluster TCRs and MHC molecules together through actin-cytoskeleton remodeling, creating multiple TCR:MHC microdomains [267]. ICAM will also mediate recruitment of co-stimulatory molecules to the various TCR:MHC microdomains, allowing for co-stimulation and antigen presentation at multiple foci [263, 266]. This realignment of the actin cytoskeleton to these microclusters is critical for T cell activation, as TCRs are normally spaced out from each other by large cell surface glycoproteins and must be moved closer together to form proper signaling complexes [273]. Also, the tensile strength of ICAM:LFA-1 interactions are needed to physically maintain long term interactions between the relatively short MHC and TCR proteins [274, 275]. Finally, DC vesicular transport becomes polarized to the DC:T cell interface allowing direct secretion of pro-inflammatory cytokines to induce local cytokine signaling on the interacting T cell [276].

A naïve T cell only needs to recognize a single peptide-MHC complex on the surface of an activated DC to commence formation of the immune synapse, which it must identify amongst hundreds of peptide-MHC complexes [277, 278]. T cells are also able to respond to

low levels of antigen in the tissues to engage immune responses. This is only possible due to naïve T cells being exquisitely sensitive to their cognate antigen [279-281]. T cell antigen sensitivity is a measure of the threshold of antigen required to elicit an effector response. The higher the sensitivity, the lower the amount of antigen needed to trigger full T cell activation [280]. Antigen sensitivity is not only affected by the affinity of the receptor for peptide ligands but also surface receptor interactions during APC interactions and efficiency of the signal transduction through the TCR during activation [282]. Sensitivity to antigen is initially hardwired during thymic selection, where only highly sensitive T cells are able to signal when presented with low levels of antigen in the thymus [283, 284]. After egress from the thymus, environmental factors during inflammation have shown to dynamically regulate sensitivity to antigen [285-287]. IL-12 and IFN-β have shown to increase the antigen sensitivity of $CD8^+$ T cells through enhancement of TCR signaling [287]. IL-10 signaling has shown to have the opposite effect, reducing antigen sensitivity through decreasing TCR clustering on the cell surface [286]. While scanning DCs in the lymph node, short lived interactions with non-cognate antigen bearing DCs induces tonic low-level TCR signaling that is important for maintaining T cell responsiveness to foreign antigen [288]. DCs dynamically regulate the activation and antigen sensitivity of T cells through multiple means and further understanding of the mechanisms governing these interactions could lend important insights into T cell dysfunction.

1.3 Molecular regulation of DC activation and function

DCs are at the center of both innate and adaptive immune responses and thus their function must be tightly regulated to maintain proper immune responses [166, 289]. DCs are regulated throughout their life cycle by both pro-inflammatory and suppressive signaling, which can affect the immune responses they elicit. DCs are actively maintained in the steady state by multiple regulatory pathways, which are only inhibited once the DC is appropriately stimulated through PRR signaling. Failure to maintain the resting state in DCs can lead to improper activation and the induction of inappropriate or autoreactive immune responses [290]. Once in the active state, DC function is regulated through other pathways to fine tune the elicited immune response. From migration to the regional lymph node to immune synapse formation, extrinsic and intrinsic mechanisms are used to terminate, dampen, or heighten the immune response [291].

1.3.1 Regulation of PRR signaling

DC activation can be extrinsically modulated indirectly through soluble factors or directly through interactions with other cells [292, 293]. One of the most prominent soluble factors known to suppress DCs is the cytokine IL-10 [294]. IL-10 is produced by many cell types, including DCs, and is known to suppress a wide variety of immune cells [295]. IL-10 binds to the IL-10 receptor (IL-10R) which is composed of a constitutively expressed IL-10R2 and the activation-induced IL-10R1 [296, 297]. Signaling through the IL-10R leads to the phosphorylation of signal transducer and activator of transcription 3 (STAT-3), which

translocates to the nucleus and promotes transcription of a variety of immunosuppressive genes [298, 299]. IL-10 can directly inhibit PRR signaling in DCs and completely suppress DC function [300]. In already-activated DCs, IL-10 can suppress pro-inflammatory cytokine secretion and promote DC-secreted IL-10 [294]. IL-10 can also inhibit actin cytoskeletal rearrangements that are critical for immune synapse formation [293].

Another potent soluble suppressor of DC activation is TGF-β [292, 301, 302]. TGF-β functions in numerous physiological processes including wound healing, angiogenesis, and immune cell regulation [303, 304]. TGF-β signals in both innate and adaptive immune cells, affecting different cell functions depending on cell context and can also affect signaling by other cytokines [305]. TGF-β signals through binding to TGF-β receptor (TGF- βR), which induces formation of the small worm phenotype in Drosophila (SMAD) signaling complex. The SMAD complex will translocate to the nucleus and initiate cell context specific transcription of target genes [306, 307]. In DCs, the SMAD complex prevents upregulation of co-stimulatory markers CD80 and CD86 upon PRR induction by inhibiting NF-κβ and MAPK [308].

In order to limit PAMP-mediated signaling, PRR receptors are often downregulated following DC maturation [309]. TLR4-mediated detection of LPS leads to the rapid endocytosis of the TLR4 signaling complex and fusion with the lysosome for degradation [310, 311]. This process terminates LPS-induced production of pro-inflammatory cytokines, preventing overstimulation of other immune cells. TLR9 signaling induces its own proteasomal degradation through activation of the ring finger protein 125 (RNF125), preventing overproduction of IFN-β [311]. However, many RNA viruses can enhance the expression of RNF125 to prevent anti-viral immunity [312].

PRR signaling induced transcription factors can be post-translationally modified to enhance or disrupt their function to modulate DC activation [313]. PDZ and LIM domain 2 (PDLIM2) is a nuclear ubiquitin ligase that has been shown to terminate NF-κβ function through proteasomal mediated degradation. PDLIM2 is expressed following LPS stimulation, migrates to the nucleus, and poly-ubiquitinates the p65 subunit of NF-κβ [314]. Loss of PDLIM2 led to increased autoimmunity and differentiation of effector T cells, showcasing its importance in preventing overactivation of NF-κβ [315]. TNF-α-induced protein 3 (TNFAIP3) has been shown to induce the ubiquitin mediated degradation of several key NF-κβ signalling molecules including receptor interacting protein-1 (RIP) and TNF receptor associated factor 6 (TRAF6) [316, 317]. DC-specific loss of TNFAIP3 led to DC autoactivation and the induction of auto-reactive B cell responses [290].

SH2 domain containing inositol phosphatase 1 (SHIP1) will post-translationally modify a variety of PRR signaling components to inhibit signaling in the steady-state [318]. SHIP1 is basally expressed in steady-state BMDCs, where it maintains low MAPK and NF-κβ signaling by de-phosphorylating critical signaling components [319]. SHIP1 expression is reduced upon proper activation, removing its suppressive effects on PRR signaling [319, 320]. However, in response to IL-10 signaling SHIP1 expression is increased in activated DCs and can suppress their ability to induce immune responses [318]. DC-specific deletion of SHIP1 in naïve mice causes splenomegaly, with large increases in the number of activated T cells and B cells, due to stimulant-independent activation of DCs in the periphery [321, 322].

Suppressor of cytokine signaling 1 (SOCS1) is another SH2-domain containing protein that inhibits NF-κβ signaling. TLR signaling induces the expression of SOCS1, which acts as a

negative feedback loop to limit activation to appropriate levels. Loss of SOCS1 function induces DCs to secrete more pro-inflammatory cytokines and greater T cell responses [323, 324]. However, DC-specific loss of SOCS1 can cause lethal autoimmunity in mice, caused by over-induction of $CD8^+$ T cell responses [325]. Mutation in SOCS1 is also associated with multiple autoimmune conditions, including multiple sclerosis (MS) and asthma [326, 327].

1.3.2 Epigenetic regulation of DC activation

The main consequence of PRR signaling is the translocation of key activation-induced transcription factors into the nucleus, where they will bind to the promoter regions of target genes to initiate transcription [328]. The availability of the binding sites of these transcription factors represents another layer of regulation of DC activation and is primarily dependent on epigenetic regulation [329-331].

Epigenetic regulation affects the chromatin landscape, which is composed of compact areas of heterochromatin and open areas of euchromatin [332]. Heterochromatin is inaccessible to most transcription factors and prevents the expression of the contained genes. Through histone modifications and other mechanisms, heterochromatin can be unwound into euchromatin, allowing access to transcription factors [332]. Epigenetic factors can regulate chromatin architecture through a combination of mechanisms, including physically unwinding DNA from histones and covalent modification of histones [332].

Epigenetic regulation of gene expression is crucial for regulating cell development but also plays a role in regulating cell function, mostly through governing the binding dynamics of

the large network of transcription factors present in the nucleus [329, 332, 333]. A variety of immune cells utilize epigenetic regulation for rapid integration of environmental cues, which can be modified to regulate transcription factor binding and initiation of immune programs [329, 334, 335] A large body of evidence has shown that even after development DCs require alterations to the chromatin landscape for function [329, 330, 335]. PRR stimulation correlates with increases in chromatin accessibility in areas containing genes involved in DC activation, and the initiation of transcription of these genes [336-338]. Epigenetic changes in DCs are also notably stable and maintain activation-induced transcription. For example, single cell sequencing of migrating activated DCs has shown that transcriptional programs engaged by specific PAMP detection are maintained from the site of stimulation to the local lymph node [339].

Changes in the chromatin landscape are primarily mediated through chromatin modifying complexes which will typically contain enzymes that chemically modify histone tails to either increase or decrease chromatin accessibility [340]. The most studied histone tail modifications are methylation, acetylation, and ubiquitination, which all dynamically regulate chromatin accessibility [341]. A single histone tail can have different modifications that function together to determine the accessibility of nearby genes, forming a histone code that is deciphered by a variety of different proteins [341]. Histone acetylation is typically associated with active transcription whereas histone methylation can denote active or repressed transcription depending on the specific modification. Tri-methylation of lysine 27 (H3K27me3) and lysine 9 (H3K9me3) denote repressed promoters, whereas acetylation of lysine 27 (H3K27ac) and tri-methylation of lysine 4 (H3K4me3) denote transcriptionally active promoters

[341, 342]. Histone subunits H2A and H2B are frequently ubiquitinated and promote other histone modifications such as methylation and acetylation [343]. For example, mono-ubiquitination of H2A (H2Aub) allows for the binding of transcription repressor complexes that will remove H3K4me3 marks from the same histone [344, 345]. Mono-ubiquitination of H2B (H2Bub), on the other hand, will promote the addition of H3K4me3 marks [346, 347]. Upon stimulation with LPS, BMDCs were found to have rapid increases in histone marks associated with active transcription, including H3K27ac and H3K4me3, and removal of inhibitory markers, such as H3K27me3 and H3K9me3 [348-350]. These modifications reflected the initiation of transcription of stimulus-dependent transcription factor associated genes, including NF-κβ and AP-1 [350].

During the steady-state, the promoters of many genes necessary for activation are silenced by multiple histone modifying complexes through either removal of histone marks associated with transcription or by the addition of marks associated with gene silencing [328, 351]. Multiple histone de-acetylases (HDAC) have been shown to be critical for regulating DC activation in the steady-state by actively removing acetylation marks on histones [352]. HDAC4 represses the transcriptional activity of AP-1 in steady-state DCs [353]. Upon stimulation with LPS, HDAC4 expression was significantly downregulated which relieved its repression of AP-1 binding [353]. Sirtuin 1 (SIRT1) is a histone deacetylase that de-acetylates histones associated with the NF-κβ binding site on the IL-12 promoter, preventing the promotion of IL-12 transcription [354]. SIN3 Transcription Regulator Family Member A (SIN3α) forms a complex with HDAC1/2 to deacetylate the MHC class 2 promoter, preventing expression of MHC class II in most cell types [355]. The Class 2 trans-activator complex (CIITA) recruits multiple histone

acetyltransferases to acetylate the MHC class II promoter to counteract SIN3α in activated DCs [355].

Polycomb repressive complexes (PRC) are a family of chromatin modifying complexes associated primarily with gene silencing, which have been shown to be critical regulators of DC steady-state and activation [356-358]. There are two major PRC families: PRC2, which is primarily responsible for silencing transcription through catalyzing H3K27me3 marks, and PRC1, which has more varied roles depending on the constituents of the complex. PRC2 is recruited to the promoter of viral DNA sensing PRRs through the action of Polybromo1 (PBRM1), inhibiting anti-viral immune responses through catalyzing H3K27me3 histone marks [359]. PBRM1 expression is increased in response to IFN-γ secretion, serving as a negative feedback loop to control IFN-γ secretion by innate cells through PRC2 methylation activity. A variety of histone demethylases that counteract H3K27me3 inhibitory marks have been shown to promote DC activation. The histone demethylase Jumonji domain-containing protein D3 (JMJD3) removes H3K27me3 marks from the promoters of pro-inflammatory cytokines and co-stimulatory receptors upon LPS stimulation [358]. JMJD3 expression is upregulated in response to PRR signaling in macrophages and BMDCs [360]. DCs pulsed with an inhibitor of JMJD3 showed a reduction in IL-6 production, mediated by an increase in H3K27me3 marks at the IL-6 promoter [361]. Adoptive transfer of BMDCs treated with an inhibitor of JMJD3 into a mouse model of gut inflammation led to a reduction in effector Th1 T cells and an increase in the differentiation of iTregs in mouse colons [362].

There are multiple functionally distinct PRC1 complexes that mediate different chromatin modifications depending on the composition of the complex [356]. The canonical

role of PRC1 is to recruit PRC2 to genomic areas for silencing through catalyzing H2Aub marks but several non-canonical PRC1 complexes perform different functions [356]. Polycomb group factor 6 (PCGF6) is a component of the non-canonical PRC 6 (PRC1.6), which is typically associated with gene silencing through mediating the addition of H3K9me3 to histone tails [356]. The PRC1.6 complexes mediate the addition of H3K9me3 inhibitory marks on the promoters of pro-inflammatory cytokines IL-12 and IL-23 in steady-state DCs, which are removed by Lysine demethylase 4d (KDM4D) following PRR stimulation [349]. PCGF6 is also known to interact with Lysine demethylase 5c (KDM5c) which has been shown to remove H3K4me3 marks to suppress transcription [363-365]. Our group has shown that PCGF6 regulates the levels of H3K4me3 marks on genes critical for DC activation, such as IL-12 and MHC class 2, through its interaction with KDM5c [366]. PCGF6 expression is rapidly reduced upon DC stimulation, followed by an increase in H3K4me3 marks and the transcription of activation-induced genes [366]. H3K4me3 marks can also be removed by another Lysine demethylase, KDM5B, to inhibit transcription initiation at the IL-6 promoter. LPS stimulation upregulates the expression of histone de-ubiquinitase ubiquitin specific peptidase 38 (USP38), which removes H2Bub marks that are needed for the recruitment of KDM5B and thus inhibits its silencing activity on the IL-6 promoter [367].

Dysregulation of epigenetic silencing has profound effects on the maintenance of the DC steady-state, promoting aberrant stimulant-independent responses which can drive autoimmunity or inflammatory disease [340, 368, 369]. Knockdown of PCGF6 in steady state BMDCs promotes the stimulus-independent activation of BMDCs and increases the activation of co-cultured T cells *in vitro* [366]. Promoting the expression of SIRT1 in lung DCs led to

exacerbated lung inflammation and dysregulated anti-viral immunity [370]. HDAC4 deficient BMDCs were more activated and were skewed towards promoting a Th17 immune response which promoted lung pathology in a mouse model of emphysema [353]. Pathogens can also co-opt factors regulating gene silencing to disrupt immune responses. For example, respiratory syncytial virus (RSV) induces the expression of KDM5B, which inhibited the expression of pro-inflammatory cytokines necessary for DC-mediated clearance of RSV [371].

1.3.3 microRNA overview

Regulation of DC activation does not stop at the initiation of transcription following PRR stimulation. A plethora of transcripts produced post-activation are further regulated post-transcriptionally to control the length and strength of generated responses [372-374]. MicroRNAs (miRNAs) are a large group of RNA molecules that post-transcriptionally regulate mRNA and have been shown to rapidly fine tune immune responses due to their fast action [375, 376]. miRNAs are short non-coding RNA molecules which bind to specific target sequences in the 3' untranslated region (3' UTR) of target mRNA to suppress its expression [377]. Originally it was believed that miRNAs primarily reduced mRNA translation directly without affecting mRNA levels but now it is appreciated that mammalian miRNAs predominantly directly reduce mRNA levels [378, 379]. miRNAs are evolutionary conserved across species and contribute to tissue specific and cell type specific protein expression [380-382]. In DCs, miRNAs are crucial for fine tuning PRR signalling and for promoting or inhibiting activation [375, 376, 383].

miRNA sequences are normally found within introns of protein encoding genes but can also be identified in the exons and introns of non-coding RNA [384]. Typically, miRNAs are transcribed via RNA polymerase 2 to form the primary miRNA (pri-miRNA) transcript. Pri-miRNA are large single stranded-RNA molecules maintained in the nucleus, which consist of a stem-loop structure surrounded by unpaired flanking sequences. [385, 386]. Pri-miRNA are then processed by the microprocessor complex, which consists of nuclear endonuclease Drosha and its co-factor DiGeorge syndrome critical region (DGCR8), which removes the flanking sequences and creates a much smaller precursor-miRNA (pre-miRNA) [387]. The stem-loop structure of the pre-miRNA is recognized by the nuclear exporter exportin-5, which mediates export of the pre-miRNA out of the nucleus [388]. The hairpin structure is then cleaved by the dicer complex in the cytoplasm, forming a mature miRNA duplex [389]. One strand of the miRNA duplex is loaded into the RNA-induced silencing complex (RISC), which consists of argonaut (Ago) proteins and the dicer complex [390]. The miRNA strand then will interact with the target mRNA 3'UTR, guiding the RISC to the target to mediate degradation [390]. Binding of the miRNA to its target mRNA is mediated through interactions with the miRNA "seed" sequence, which is centered on nucleotides 2-7 of the 5' region of the miRNA, to the mRNA. This binding isn't completely complementary as wobble base pairing is tolerated, and thus determination of putative miRNA is based on algorithm based approaches [391, 392]. Direct miRNA targeting of a specific sequence can then be validated through the use of a variety of assays, such as ectopic expression and luciferase assays [393].

1.3.4 microRNA regulate programs of expression in DCs

miRNA represent only a small percentage of the genes transcribed in mammalian cells and yet they have tremendous influence on expression of the genome [394, 395]. Most mammalian mRNAs (60%) have been shown computationally to be targeted by miRNA, including by multiple miRNA simultaneously [396, 397]. On average, a single miRNA can target around 200 different mRNA transcripts, with some ancient miRNA targeting over 1000 different genes [391, 392]. Genome-wide analysis that compared miRNA targets against functional annotations found that coordinated repression of multiple genes in a process by a single miRNA is prevalent in mammalian genomes [398]. Single miRNAs have since been proven to govern various programs of expression in mammalian cells, including developmental and metabolic pathways [399-401]. In the immune system single miRNA are implicated in the regulation of many immune response programs. For example, microRNA-181 (miR-181) which targets multiple phosphatases that regulate T cell activation [284].

microRNA-155 (miR-155) is the most studied miRNA in both murine and human DCs [402-404]. miR-155 is required for optimal activation of both murine and human DCs, modulating a variety of activation pathways by targeting multiple genes [405, 406]. For example, miR-155 deficient BMDCs were defective in their ability to present antigen and activate T cells whereas BMDCs ectopically expressing miR-155 expressed more co-stimulatory CD80 and were able to further promote the activation of T cells [402, 407]. miR-155 promotes the activation of DCs by targeting multiple negative regulators of activation, including SHIP1 and SOCS-1 [320, 408]. miR-155 expression is regulated by multiple factors that regulate DC

activation. For example, miR-155 expression is downregulated by suppressive factors, such as TGF-β and IL-10, while also promoting the expression of its targets, leading to feed-forward suppression of DC activation [409-411]. DCs isolated from the tumour microenvironment in breast cancer show reduced miR-155 expression. Ectopic expression of miR-155 in these DCs promoted greater migratory potential and function in breast cancer tumours [412].

The microRNA-146 (miR-146) family is composed of two genes, miR-146a and miR-146b, which have the same seed region but are differentially expressed in different immune subsets [413, 414]. As with miR-155, miR-146a expression is also upregulated in response to TLR stimulation, but instead promotes inhibition of DC activation [415]. IL-10 signalling also induces the expression of miR-146b, cooperatively reducing DC function [416]. Ectopic miR-146a expression reduces the production of pro-inflammatory IL-12, IL-6 and TNF-α in both murine and human DCs [417]. miR-146b knockout mice have enlarged spleens filled with activated DCs and other myeloid cells along with spontaneous intestinal inflammation [418]. miR-146a directly targets TRAF6 and IRAK1, which are critical components of the TLR4 signalling cascade which induces NF-κβ translocation to the nucleus [419]. miR-146 functions as an NF-κβ negative feedback loop, where PRR signalling induced NF-κβ signalling increases miR-146a expression which then decreases NF-κβ function to prevent over activation [419]. As with other negative regulators of immune activation, various pathogens co-opt miR-146 to suppress immunity and promote pathogenesis [420, 421]. For example, Epstein-Barr virus encodes various factors that significantly upregulate miR-146a expression to reduce NF-κβ signalling and reduce anti-viral immunity [422].

miRNA profiling of human DCs has revealed that the expression of many other miRNA are modulated by TLR stimulation or by suppressive cytokines, though the exact function of these miRNA in DC activation are just beginning to be understood [375, 376, 423]. miR-142 is highly expressed in steady-state BMDCs but is rapidly downregulated following TLR stimulation. Ectopic expression of miR-142 in BMDCs severely reduces the ability of BMDCs to activate CD4+ T cells through miR-142 specifically targeting IL-6 mRNA [424]. miR-223 is upregulated in human DCs stimulated with LPS and is one of the most highly upregulated miRNA in myeloid cells from multiple sclerosis (MS) patients. DC-specific miR-223 knockout mice show reduced infiltrating myeloid cells and Th17+ T cells in the brain. miR-223 knockout BMDCs express significantly less th17 skewing cytokines such as IL-23 and IL-6 while also expressing more inhibitory receptors such as PD-L1 [425]. The exact mRNA target of miR-223 in DCs remains to be elucidated. MicroRNA-9 (miR-9) was also found to be upregulated in LPS stimulated human DCs, but its exact function in DCs remains to be elucidated.

1.3.5 microRNA-9

miR-9 is an ancient miRNA that has been evolutionary conserved since the transition to triploblasty [426, 427]. Mammals express three copies of miR-9 (miR-9-1,2,3) which are encoded in different areas of the genome but encode the same mature miRNA so are often collectively referred to as miR-9 [428]. The distribution of miR-9 family members in the genome allows for differential regulation and even cell context-specific expression [428]. miR-9 is predominantly studied in neurodevelopment, as it is highly enriched in developing and mature nervous systems of vertebrates [429, 430]. In mammals, miR-9 is highly expressed specifically in

neural progenitors but absent in mature neurons [431, 432]. miR-9 inhibits the proliferation of neural progenitors in order to increase their differentiation [433]. miR-9 directly targets multiple negative regulators of neural differentiation, many of which are epigenetic regulators that silence genes critical for neural differentiation [434-436]. Repressor-element-1 silencing transcription factor (REST) and its co-repressor (coREST) form a transcriptional silencing complex that epigenetically silences neuronal genes in neural stem cells [436]. miR-9 mediated inhibition of this complex allows for the expression of these neural genes and promotes the differentiation of neural progenitors [433]. miR-9 also directly targets HDAC4 and SIRT1, which also act in chromatin silencing complexes that inhibit neural differentiation [434, 435]. miR-9 expression is repressed in proliferating neuronal progenitors by lin-28 homolog A (lin28a) which directly binds to pre-miR-9 and promotes its degradation [437].

miR-9 was originally thought to be expressed only in the developing brain but it is now understood that miR-9 regulates cellular function in many other systems. Aberrant expression of miR-9 is associated with many cancers, including various malignancies not associated with the brain [438, 439]. miR-9 plays a diverse role in cancer biogenesis, where it is seen as a potent oncogene in some cancers but also a tumour suppressor in others [439, 440]. Tissue expression of miR-9 has also been shown to be upregulated in various inflammatory diseases, including inflammatory bowel disease (IBD), amyotrophic lateral sclerosis (ALS) and MS [441-443]. miR-9 is expressed by multiple immune cells in both the innate and adaptive immune systems and is known to play a role in modulating the function of these cells [444-446]. LPS stimulation increases the expression of miR-9 in neutrophils and human monocytes, where it was shown to target NF-κβ [444, 447]. The promoter of the primary miR-9-1 transcript (pri-miR-9-1) contains

a putative NF-κβ site therefore miR-9 may be involved in regulating its own expression similarly to what was observed with miR-155. Inhibiting miR-9 in macrophages led to reduced secretion of pro-inflammatory cytokines and reduced inflammation in a model of sepsis [448]. Interestingly, many of the validated targets of miR-9 are previously mentioned negative regulators of DC function including SIRT1, HDAC4 and TGF-βR1 [434, 435, 449]. An algorithm-based approach to determine putative targets of miR-9 shows that miR-9 putatively targets many other negative regulators of DC activation such as PCGF6, PBRM1 and PDLIM2 [391]. Since human DCs rapidly upregulate miR-9 expression, it is possible that activation-induced expression of miR-9 aids in the promotion of DC activation by inhibiting the expression of these negative regulators.

1.4 DC function in homeostasis and disease

DCs play an important role in maintaining immune homeostasis as they are involved in both promoting immune responses to pathogens and preventing autoimmunity [450-452]. The microenvironment provides DCs with the context necessary to either induce activation or promote suppression of immunity. At the same time, DCs can influence the microenvironment through secretion of pro-inflammatory or anti-inflammatory cytokines. Disruption of DC activation and function can induce profound changes in the microenvironment, which can alter the balance between maintaining homeostasis and promoting inflammation [450]. This can have particularly negative consequences in tissues where the immune system is constantly exposed to both pathogens and commensals, such as the intestinal tract and skin, where dysregulated DC function can cause chronic inflammatory disease or autoimmunity [205, 450].

1.4.1 DCs in immune tolerance

Immune tolerance is a state of unresponsiveness of the immune system against self and innocuous antigens. Multiple mechanisms are present to regulate immune tolerance to prevent autoimmune pathology, while ensuring proper immune activation to pathogens [450]. DCs actively suppress immune responses in certain contexts, but it was originally thought that they played no major role in the induction of tolerance [450]. However, mice lacking DCs displayed strong autoimmune pathology, with uncontrolled migration of pro-inflammatory innate cells and aberrant T cell effector responses to self antigens [453, 454]. Alternatively, expanding DC numbers in mice predisposed to autoimmune diabetes showed reduced onset of disease compared to controls [455]. These experiments show that DCs play a critical role in the maintenance of immune tolerance.

As discussed previously (see section 1.10), T cells undergo central tolerance in the thymus to remove strongly self-antigen reacting T cells during development. Previously, it was thought that medullary thymic epithelial cells (mTECs) were predominantly responsible for central tolerance but now it is understood thymic DCs play an important role as well [456-458]. Thymic DCs present tissue specific and blood-derived self-antigens produced by AIRE expressing mTECs to DP T cells and induce negative selection [459]. Half of mTEC dependant negative selection is dependant on antigen presentation by thymic DCs [458]. Thymic DCs also aid in the migration of developing T cells to the medulla of the thymus by producing CCR4 ligand, which attracts post-positive selection T cells to undergo negative selection [460]. Non-thymic DCs also aid central tolerance, as cDC2s from the periphery can migrate into the thymus to present

peripheral self-antigens to lymphocytes to induce negative selection of self-reacting T cells [461].

Central tolerance is insufficient to eliminate all autoreactive T cell clones and so peripheral tolerance is required to fully prevent T cell related autoimmunity [462]. As previously described, DCs can suppress immune responses in inflammatory contexts through secretion of anti-inflammatory cytokines and induce the differentiation of naïve T cells into Tregs [450]. It is now understood that some DCs can also induce specific peripheral tolerance to self-antigens instead of only dampening activation in inflammatory conditions. DCs involved in promoting peripheral immune tolerance are categorized as tolerogenic DCs (tolDCs), which are thought to be produced by an alternative differentiation pathway of steady-state DCs induced by the encounter of antigen in the absence of activating stimuli or in the presence of inhibitory factors [463, 464]. For example, steady-state DCs which encounter self-antigens from cells undergoing apoptosis will differentiate into tolDCs [465-467]. Unlike activated DCs, tolDCs express low levels of co-stimulatory markers, secrete high amounts of anti-inflammatory cytokines, and function to induce peripheral tolerance to antigens in the periphery through a variety of mechanisms [468, 469]. When a tolDC presents antigen to T cells in the lymph node they will not fully deliver the required activation signals to naïve T cells and instead induce anergy, preventing T cell responses against that antigen. TolDCs are also specialized for suppressing self-antigen specific effector T cell responses through inducing the differentiation of iTregs by secreting large amounts of anti-inflammatory IL-10 and TGF-β [470]. TolDCs also express high amounts of Indoleamine 2,3- dioxygenase (IDO), which metabolizes tryptophan, that is essential

to T cell activation [471]. In the absence of high levels of tryptophan, T cells become tolerized and anergic [471].

It is not yet clear if cDC1s and cDC2s play differing roles in the induction of peripheral tolerance, as studies on tolDC differentiation in tissue-resident cDCs are marred by contamination of moDCs and macrophages [450]. However, delivering self-antigen to cDC subsets *in vivo* through chimeric antibody delivery has shown that a population of splenic cDC1s and cDC2s can induce T cell anergy [472]. With the advent of new gating strategies to remove contaminating cells from analysis of tissue cDCs, tolDC differentiation in cDCs can be studied more accurately.

1.4.2 Regulation of tolerogenic DC function

As previously discussed, activation of steady-state DCs is dependent on the integration of signals from the environment followed by large gene expression changes governed by tight regulation. TolDCs follow an alternative differentiation pathway that is now understood to be similarly induced and regulated [292, 450, 473]. PRR signalling can induce differentiation into an activated DC or into tolDCs, depending in which microenvironment the PRR signalling takes place [474]. For example, ligation of TLR-2 by pro-inflammatory Zymosan will typically induce DCs to promote a pro-inflammatory immune response. However, if the zymosan-TLR2 interaction takes place within a microenvironment containing high amounts of IL-10, the induction of tolDC differentiation will be favoured [475]. Differentiation of tolDCs from steady-state DCs involves expression changes as large as those observed for activated DCs, including many of the same genes and regulators [476] One such regulator is TNFAIP3, previously mentioned for its role in maintaining the DC steady-state, which is highly expressed in tolDCs

and promotes tolDC differentiation by dampening TLR signalling [290]. Epigenetic mechanisms are also critical for regulating tolDC function. Nuclear co-repressor 1 (NCOR1) is expressed in activated DCs and recruits histone deacetylases to promote silencing of genes required for tolDC function, such as IL-10 and IDO [473].

In some cases the tissue microenvironment can induce dysregulated tolDC differentiation and skew the balance of promoting tolerance or inflammation [477]. DCs play a critical role in the elimination of many cancers by presenting tumour antigens to cytotoxic T cells and promoting anti-tumour immune responses [478]. In immunogenic tumours, steady-state DCs that migrate into the tumour microenvironment become activated by pro-inflammatory cytokines and DAMPS released from dying cells [479]. However, in some cases, steady-state DCs entering the tumour microenvironment acquire an aberrant tolerogenic phenotype which promotes tumour persistence [480]. DCs entering the non-immunogenic tumour microenvironments are exposed to multiple tumour-secreted suppressive factors, including IL-10 and TGF-β, which promote tolDC function [481, 482]. TolDCs can induce tolerance to tumour-derived antigens and further promote the suppressive microenvironment by inducing iTregs in the tumour [482]. Many anti-tumour therapies can skew the tumour microenvironment towards promoting DC activation and reducing the number of tolDCs [478]. Addition of TLR agonists, such as poly-I:C, to the tumour microenvironment can skew DCs towards activation and away from tolerance [483]. Checkpoint blockade therapy can also inhibit the suppressive functions of tolDCs in the tumour microenvironment and increase recruitment of anti-tumour CD8 T cells [484].

Chronic inflammation induced by autoimmune disease can also lead to a tissue microenvironment that dysregulates tolDC function. Tissues undergoing continuous immune activation due to autoimmunity create a microenvironment with high levels of pro-inflammatory cytokines that alter the regulatory activity of tolDCs, promoting activation-like phenotypes that potentiates inflammation [477, 485]. Rheumatoid arthritis (RA) is caused by an autoimmune response against synovial fluid which is promoted through dysregulated DC function [486]. Steady-state DCs which migrate into inflamed joints of RA patients encounter self-antigens but become aberrantly activated due to the high levels of pro-inflammatory IL-6 [487, 488]. These activated DCs will then potentiate the autoimmune response by inducing further expansion of autoreactive T cells. Altering the microenvironment of the inflamed joint through the addition of anti-inflammatory mediators or of patient-derived tolDCs can skew the microenvironment towards promoting tolerance, which will inhibit T cell responses [488-490].

1.4.3 DCs promote intestinal tolerance

The immune system residing in barrier tissues maintains a fine balance between immune activation to pathogens and maintenance of tolerance to not only self-antigens but also antigens from commensal organisms that are critical to health. This is an especially daunting task in the gastrointestinal tract, where the intestinal immune system must be tolerant towards antigens from trillions of commensal microorganisms and non-harmful food antigens while also promoting immune responses against intestinal pathogens [491].

In a healthy state, the intestinal microbiota in the lumen are kept separate from the immune system by a single layer of intestinal epithelial cells (IECs). Tight junctions between

epithelial cells restrict trans-epithelial permeability and goblet cells in the epithelial layer maintain a layer of mucus, which keep the microbiota away from the mucosal surface [492]. It was originally believed that tolerance to the microbiota was not an active process, but was achieved because the microbiota were compartmentalized away from the immune system [68]. However, it is now understood that microbiotal antigens are actively presented to cells of the adaptive immune system. A large amount of microbiota-specific IgA is secreted by plasma cells located in the intestinal lamina propria [493, 494]. It has also been shown that microbiota-specific effector T cells are found in the lamina propria but are kept in check by Treg cells to inhibit effector responses [495, 496]. Tolerance to the microbiota is also restricted to the intestinal tract, unlike most mechanisms of peripheral tolerance which are systemic [497]. This is critical for systemic immune responses against opportunistic pathogens within the microbiota, which can cross into the circulation under inflammatory conditions [498]. All these results indicate that microbiota-derived antigens are acquired by intestinal APCs, which are presented to adaptive immune cells located specifically in the intestinal tract and that compartmentalized peripheral tolerance is induced to protect the microbiota from intestinal effector T cells [492].

Although physically separated by the epithelial layer, antigens from the intestinal lumen are actively sampled by intestinal cDCs in the lamina propria and are presented to naïve T cells in intestinal lymph nodes to induce tolerance to the microbiota or induce effector responses against pathogens [499]. As discussed previously (see Section 1.17), there are three subsets of intestinal cDCs: $CD103^+CD11b^-$ cDC1s, $CD103^-CD11b^+$ cDC2s and $CD103^+CD11b^+$ cDC2s. In steady-state conditions, $CD103^+CD11b^-$ cDC1s and $CD103^+CD11b^+$ cDC2s are commonly believed

to be primarily responsible for the induction of tolerance in the intestine. However, it is worth noting that these previous studies did not take into account contaminating $CD103^{-}CD11b^{+}$ cDC2s [500-502]. It is now appreciated that no singular cDC subset is responsible for the induction of peripheral tolerance to the microbiome and that all cDC subsets in the intestine can develop a tolDC phenotype in the steady-state [500]. Intestinal cDCs induce a state of tolerance towards the microbiota by inducing anergy in microbiota-specific naïve T cells and through generation of iTreg responses in the intestinal mucosa [497, 503] Steady-state intestinal cDCs also express IDO, secrete suppressive cytokines, and co-inhibitory markers to reduce inflammation [492, 501, 504].

It was previously thought that steady-state intestinal cDCs primarily obtained microbiota-derived antigens by extending trans-epithelial dendrites (TEDs) directly between epithelial cells or through specialized microfold cells (M cells) at small intestine Peyers patches [505-508]. However, this process would expose intestinal cDCs to PAMPs in the lumen, which has been shown to induce cDC activation [509]. Also, the presence of TEDs is rare in steady-state intestines but significantly induced in pro-inflammatory conditions [510]. This is thought to be due to the inflammatory microenvironment affecting the integrity of the epithelial tight junctions [511]. M cells are also rare in the steady-state but are induced upon pro-inflammatory signalling by innate immune cells in the Peyers patches [512]. In contrast, goblet cells are commonly filled with luminal antigens in the steady-state and deliver them to intestinal cDCs through goblet cell associated passages (GAPs) [513]. GAP-mediated delivery of antigen is now thought to be the principal mechanism for the induction of tolerance by intestinal cDCs and associated with increased tolDC function [514, 515].

In the steady-state intestine, intestinal cDCs induce tolerance to microbiota-derived antigens because the intestinal microenvironment maintains cDCs in a tolerogenic state [492, 516]. Various cells contribute to the tolerogenic microenvironment in the intestinal tract, including IECs, other immune cells and the microbiota itself. IECs produce copious amounts of thymic stromal lymphopoietin (TSLP) and TGF-β, which induce tolDC differentiation and inhibits pro-inflammatory cytokine secretion [517, 518] . Intestinal cDCs uniquely express retinaldehyde dehydrogenase 2 (RALDH2), which generates retinol from dietary vitamin A obtained from IECs and stromal cells. Retinol promotes tolDC differentiation by enhancing the induction of Treg differentiation [519, 520]. Dietary tryptophan can also directly influence anti-inflammatory functions of tolDCs by enhancing IDO expression [521].The microbiota itself directly influences the function of immune cells in order to promote tolerance. *Bacteroides* and *Bifidobacteria* members of the microbiota produce soluble factors that directly induce human tolDC differentiation *ex vivo* [522]. Components of the *Bacterioides fragilis* cell wall drive IL-10 production in DCs in the colonic lamina propria [523]. Members of the microbiota can also interact with IECs to increase their expression of TSLP and TGF-β to further enhance DC tolerance [524]. The microenvironment of the MLN is also intrinsically tolDC-promoting [525].

1.4.4 DCs promote intestinal inflammation

During inflammation, the tolDC phenotype of many intestinal cDCs is inhibited and instead a pro-inflammatory activation state is promoted [500]. Inflammation changes the composition of the intestinal microenvironment, which promotes DC activation and inhibits tolDC function [526]. Inflammation inhibits the expression of TSLP and TGF-β in IECs, skewing

the cytokine milieu towards a pro-inflammatory environment [527]. IECs that come into contact with intracellular pathogens secrete chemokines that promote the migration of DCs to the site of infection [528]. Virally infected IECs secrete large amounts of chemokines to attract $CD103^+$ $CD11b^-$ cDC1s, which can then obtain viral antigens presented on MHC class I [529, 530]. Not all cDCs transition to an activated state during immune responses to pathogens, as microbiota-specific immune responses are still inhibited by homeostatic Treg generation [500].

In inflammatory conditions, intestinal cDC1s are potent secretors of pro-inflammatory IL-12 which drives Th1 immune responses [75]. Intestinal cDC1s can also acquire antigens from intestinal epithelial cells infected with intracellular pathogens to induce $CD8^+$ T cell responses in the MLN [530]. $CD103^-$ $CD11b^+$ intestinal cDC2s promote clearance of intestinal parasites by inducing Th2 mediated CD4+ T cell responses [104]. The unique intestinal population of $CD103^+$ $CD11b^+$ intestinal cDC2s promote Th17 mediated immune responses and secrete anti-microbial peptides such as regenerating islet-derived protein 3-gamma (RegIIIγ) [531, 532].

Intestinal DCs are required for the initiation of pro-inflammatory immune responses to many intestinal pathogens, including attaching and effacing (A/E) enteropathogens such as *Citrobacter rodentium* [533, 534]. *C. rodentium* is a gram negative mouse-specific pathogen, which intimately adheres to intestinal epithelial cells and causes rearrangement of the underlying cytoskeleton to form pedestal-like structures [535, 536]. The A/E lesions caused by *C. rodentium* are indistinguishable from those caused by the human diarrheal pathogens enteropathogenic *Escherichia coli* (EPEC) and enterohemorrhagic *E.coli* (EHEC) [536, 537]. *C. rodentium* shares many of the key pathogenic mechanisms of EHEC and EPEC, including expression of many of the same effector proteins necessary to cause A/E lesions [538]. Since

mice are naturally resistant to EPEC/EHEC, *C. rodentium* is used as a small-animal model to study A/E enteropathogens.

C. rodentium primarily infects the large intestine of mice, causing colitis, diarrhea, and colonic hyperplasia. Disease severity is dependant on the genetic background of the host, with susceptible mouse strains succumbing to lethal diarrhea and inflammation a week after infection, while resistant mice clear the infection 3-4 weeks post-infection [539]. *C. rodentium* infection induces an initial Th1 response but ultimately requires a potent Th17 response for full clearance [540-542]. Intestinal cDCs are required for the induction of Th17 effector T cell-mediated clearance of *C. rodentium* infection [542, 543]. Inhibition of MyD88 in intestinal cDCs increases susceptibility to *C. rodentium* infection and inhibition of protective adaptive immune responses [534]. $CD103^{+}CD11b^{+}$ cDC2s secrete large amounts of IL-23 in response to *C. rodentium* infection, which promotes the differentiation of Th17 $CD4^{+}$ T cells and induces critical ILC responses. Mice lacking $CD11b^{+}$ cDC2s succumbed to infection due to impaired induction of protective immune responses [543].

1.4.5 Dysregulated DC function promotes inflammatory diseases of the intestine

Dysregulated DC function due to intrinsic genetic factors or environmental alterations is associated with the induction of multiple autoimmune diseases, including systemic lupus erythematosus, multiple sclerosis, and RA [544, 545]. Dysregulated cDC function is particularly pathogenic in the intestinal tract, where breaks in tolerance towards the microbiota are associated with multiple inflammatory diseases of the intestinal tract, including IBD [492].

IBD is a group of chronic relapsing inflammatory diseases in the intestinal tract caused by an inappropriate immune response to the microbiota, chronic inflammation due to continuous stimulation of the immune response, or failure to re-establish homeostasis after a pro-inflammatory response [546, 547]. Crohn's disease (CD) and ulcerative colitis (UC) are two major forms of IBD which share common features but develop in different areas of the gastrointestinal tract. CD involves uncontrolled inflammation which can present throughout the gastrointestinal tract, whereas UC is mainly limited to the distal colon and rectum [548]. Pathogenesis of both CD and UC involves dysregulated immunity in the intestinal tract, including increased recruitment of pro-inflammatory Th1 and Th17 $CD4^+$ T cells and a dramatic reduction in the numbers of Tregs [549]. IBD also drastically affects the composition of the microbiome, with decreased diversity and increased prevalence of species associated with inflammation [550]. The exact causes of IBD are unknown but the disease is known to be dependent on both environmental and genetic factors [548].

Genome-wide association studies have identified multiple genes associated with susceptibility to IBD, many of which are related to immune regulation [551]. For example, defective IL-10 signalling was quickly identified as a potent driver of IBD pathogenesis [552, 553]. Monogenic defects in either *IL10* or *IL10R* genes are associated with development of IBD in human patients and are associated with severe early-onset illness [553]. Follow-up experiments in mice showed that IL-10 knockout mice developed severe colitis caused by a large influx of activated cDCs and effector $CD4^+$ T cells in the small intestine and colon [554]. In human cDCs, loss of IL-10 signalling promoted aberrant expression of TNF-α and uncontrolled Th1 and Th17 effector T cell responses *ex vivo* [516, 555].

Dysregulated intestinal cDC function is heavily associated with IBD susceptibility and severe disease [556, 557]. Aberrant tolDC function can contribute to hyper activation of the immune response, through impaired secretion of IL-10, full activation in the presence of self-peptides, or defective antigen processing [553, 558, 559]. Single cell RNA sequencing experiments on intestinal mucosa of patients with CD and UC showed a shift towards pro-inflammatory activation in all cDC populations in both diseases [549]. Colonic cDCs from UC patients express increased amounts of TLR2 and TLR4, are more reactive to bacterial PAMPs, and show increased expression of co-stimulatory markers [560]. In CD patients, production of pro-inflammatory IL-6 and TNF-α by colonic cDCs correlates with disease severity [561].

Many of the immune-related genes associated with susceptibility to IBD can be mapped to intestinal cDC function, including negative regulators of DC function and PRR signalling components [548, 551]. Nucleotide-binding oligomerization domain containing-2 (NOD2) is an intracellular PRR that is strongly associated with IBD susceptibility [562-564]. Expression of NOD2 variants in cDCs increases bacterial detection, cytokine production, and antigen presentation of microbiota antigens [565, 566]. Runt-related transcription factor 3 (RUNX3) is another common IBD associated gene that is involved in the regulation of immune responses [567]. Mice with cDC-specific loss of RUNX3 develop spontaneous early-onset colitis due to loss of intestinal tolerance to the microbiota. RUNX3 activates the expression of multiple genes associated with tolDC function, including genes that promote TGF-β signalling [568]. Loss of DC specific TGF-βR2 expression induces spontaneous multi-organ failure, including severe intestinal colitis due to loss of tolerance to the microbiome [569].

Current treatments for IBD involve the heavy use of non-specific immune suppressive drugs, which have been shown to decrease the activation of intestinal cDCs and promote a tolerogenic phenotype [570]. Adoptive transfer of moDCs conditioned with suppressive cytokines have also been shown to reduce inflammation in mouse models of IBD through the generation of Tregs [571, 572]. Further understanding of the regulatory mechanisms that mediate cDC activation or differentiation to tolerogenic phenotypes could lead to more treatments for IBD.

1.5 Cystic Fibrosis

Cystic Fibrosis (CF) is an incurable autosomal recessive genetic disease which is the most common inherited disorder among people of European heritage, affecting approximately 1 in 3000 Caucasians [573, 574]. CF is a multisystem disease primarily known for its effects on lung epithelial cells, causing obstruction of the airways and promoting colonization of pathogenic bacteria [573, 574]. CF also causes gastrointestinal pathology, including chronic inflammation in the intestinal tract that shares features with IBD [575]. CF also affects the function of various cells besides epithelial cells, including a previously unrecognized role in immune cell signalling [576]. Growing evidence suggests that CF causes dysfunctional immune cell function which alters responses to pathogenic bacteria and causes a loss of tolerance in the intestinal tract [575].

1.5.1 Cystic fibrosis overview

CF is caused by a variety of mutations in the cystic fibrosis transmembrane conductance regulator (*cftr)* gene, which encodes an ion channel whose expression on epithelial cells mediates chloride and bicarbonate transport across various epithelial surfaces [573, 574]. CFTR also regulates water efflux into the lumen via direct regulation of the activity of several sodium channels, causing dehydration of the cell surface layer of the lumen. Severity of CF disease is based on the particular CFTR mutation present but mainly causes the accumulation of mucus along epithelial surfaces, which leads to obstruction, infections, and inflammation [573]. Over 1900 different mutations of *CFTR* have been identified in human patients, comprising five different classes dictated by molecular mechanism of CFTR disruption [577]. Class 1 to 3 mutations result in total loss of CFTR function and cause severe disease phenotypes, whereas class 4 and 5 mutants are associated with reduced expression or function of CFTR and cause milder symptoms. The most clinically relevant mutation in human CF is a class 2 mutation resulting from deletion of a phenylalanine residue at position 508 (Δ508), which leads to protein misfolding and degradation via the proteasome. More then 70% of CF in humans is caused by a Δ508 mutation [573].

Pulmonary complications are the main cause of death in CF patients. Loss of CFTR function in the lungs causes an accumulation of viscous mucus which obstructs the airways. The viscous mucus also impairs mucoscilliary defense and leads to chronic infections from bacteria such as *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Although typically associated with lung pathology, CF also drastically affects the gastrointestinal system [578, 579]. Pathology of the intestinal tract is characterized by accumulation of viscous mucus that causes obstruction of the

small and large intestines, but also inhibits proper nutrient absorption [575, 580]. The abnormally viscous mucus contributes to an outgrowth of bacteria, particularly in the small intestine, which is referred to as small intestinal bacterial overgrowth (SIBO) [580]. The microbiota of CF patients is markedly disrupted, with an overall decrease in diversity and marked increase in the abundance of pro-inflammatory bacterial species such as *Escherichia coli* and *Enterobacter* [581].

In order to study the molecular and cellular mechanisms of CF pathogenesis, several mouse models have been generated. CFTR knockout mice (CFTR KO) and mice harboring the Δ508 mutation (CFTR F508) have been used extensively to evaluate novel therapies for CF [582-585]. Importantly, both CFTR KO and CFTR F508 develop intestinal pathology that is similar to what is observed in CF patients [575, 586]. Mice develop intestinal obstructions from viscous mucus accumulation, SIBO, and elevated intestinal inflammation [575, 586].

1.5.2 Cystic fibrosis as an inflammatory disease

The intestines of CF patients and mice are characterized by a substantial increase in inflammatory gene expression, which was initially thought to be due to outgrowth of bacteria [587-589]. However, antibiotic treatment does not lead to reduction in the inflammatory gene signatures observed in the intestines of CF patients [586, 589, 590]. The intestines of neonatal CF patients, which do not have SIBO, also show marked increases in inflammatory gene signatures [591]. Germ-free CFTR KO mice, which lack a microbiome, demonstrate similar histopathological signs of inflammation in the intestinal tract and increased numbers of pro-inflammatory $Th17^+$ T cells in the MLN [592]. Epidemiological studies on CF patients also show an increase in gut related inflammatory pathologies associated with loss of tolerance such as

CD, UC, and celiac disease [577, 593, 594]. This suggests dysregulation of the immune system in CF patients, leading to an increase in immune activation and loss of tolerance, while also failing to control infections [586, 590].

CFTR is functionally expressed in immune cells, including myeloid and lymphoid cells, and has been shown to regulate immune function [576, 587]. In many immune cells, CFTR acts as a negative regulator of immune cell activation by inhibiting various immune signalling cascades. In naïve $CD4^+$ T cells, CFTR is evenly expressed across the plasma membrane but quickly co-localizes with the TCR during immune synapse formation. CFTR-mediated efflux of chloride ions changes the membrane potential of $CD4^+$ T cells and inhibits the influx of Ca^{2+} needed for proper TCR signalling [595]. Loss of CFTR deregulates T cell signalling, causing overproduction of pro-inflammatory cytokines and hyperinflammatory immune responses [595]. CFTR loss also increases B cell proliferation and activation, though the mechanism has yet to be identified [596].

In innate immune cells, loss of CFTR has been shown to promote hyperresponsiveness to pro-inflammatory stimuli. Loss of CFTR in neutrophils and macrophages has been shown to increase production of IL-8, TNF-α, and proteases in response to activating stimuli [597, 598]. Loss of CFTR causes dysregulation of many innate immune signalling pathways, including PRR signalling and immune activation [576, 599, 600]. Macrophages from CFTR F508 mice show increased phosphorylation and activity of NF-κβ and MAPK , which potentiated pro-inflammatory cytokine secretion [601, 602]. CFTR inhibits the activity of NF-κβ by promoting the degradation of tumor necrosis factor receptor type 1-associated DEATH domain protein (TRADD), a key NF-κβ signalling intermediate during PRR signalling [603]. CFTR also indirectly

inhibits TLR4 trafficking to the plasma membrane, inhibiting its ability to signal in response to pro-inflammatory LPS [604]. Monocytes from children with CF expressed significantly more TLR4 unrelated to pulmonary infection and overproduced pro-inflammatory cytokines when stimulated *ex vivo* with LPS [604, 605]. Despite overactivation of the immune response, monocytes and macrophages derived from CF patients show reduced ability to kill internalized *P. aeruginosa* bacteria. This is due to loss of CFTR mediated influx of chloride ions into the phagolysosome, which is needed for proper killing of internalized pathogens by phagocytic cells [599, 606].

CFTR is also expressed on DCs but its role in regulating DC function is controversial [607, 608]. One group found that DCs isolated from the lungs of CFTR KO mice expressed reduced amounts of co-stimulatory markers and secreted less pro-inflammatory cytokines than control mice [607]. The same group showed that BMDCs from CFTR KO mice showed similar pro-inflammatory markers as control mice [609]. However upon infection with *P. aeruginosa*, DCs from CFTR KO mice expressed more co-stimulatory markers and secreted more pro-inflammatory cytokines compared to controls [610]. DCs have yet to be studied in the gastrointestinal tract of CF patients or in CFTR KO mice.

Preface to Chapter 2

The goal of this study was to characterize the potential role of microRNA-9 in the regulation of DC activation and function. MicroRNA-9 has previously been shown to be upregulated in human DCs stimulated with LPS and is predicted to target multiple negative regulators of immune cell function. Included in the list of putative targets is PCGF6, which we have previously shown to be a potent regulator of DC activation. We predicted that miR-9 contributed to the transition of DCs from the steady state to an activated state by repressing the expression of negative regulator of DC activation, including PCGF6.

Chapter 2: MicroRNA-9 fine tunes dendritic cell function by suppressing negative regulators in a cell-type-specific manner

Adapted from the published work:

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MicroRNA-9 fine tunes dendritic cell function by suppressing negative regulators in a cell-type-specific manner

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2.1 Short Summary

Dendritic cells, cells of the innate immune system, are found in a steady state poised to respond to activating stimuli. Once stimulated they rapidly undergo dynamic changes in gene expression to adopt an activated phenotype capable of stimulating immune responses. We find that the microRNA miR-9 is upregulated in both bone-marrow derived DCs and conventional DC1s but not in conventional DC2s following stimulation. miR-9 expression in BMDCs and conventional DC1s promotes enhanced DC activation and function, including the ability to stimulate T cell activation and control tumour growth. We find that miR-9 regulated the expression of several negative regulators of transcription, including the transcriptional repressor Polycomb group factor 6 (*Pcgf6*). These findings demonstrate that miR-9 facilitates the transition of DCs from steady state to mature state by regulating the expression of several negative regulators of DC function in a cell-type-specific manner.

2.2 Introduction

Dendritic cells (DCs) are cells of the innate immune system that regulate both innate and adaptive immune responses. DCs are found in the steady state in lymphoid and non-lymphoid tissues and their activity can be stimulated or suppressed by host factors such as cellular damage, cytokines, tumours and foreign substances including microbial factors and allergens. Microbial/pathogen/damage- associated molecular patterns (MAMPS/PAMPS/DAMPS) are recognized by pattern recognition receptors (PRRs) and lead to the activation or final maturation of DCs. DCs become differentially activated depending on

what type of stimuli they detect and, in doing so, are able to promote context-specific immune responses [611-614]. During activation, DCs undergo many phenotypic changes that enable activation of other cells of the immune system including T cells. These changes include increased expression of antigen presenting machinery, increased surface expression of co-stimulatory molecules such as CD80, CD86 and CD40 and increased secretion of pro-inflammatory cytokines including IL-12, IL-6, TNFα and Type 1 interferons (IFNs) [615, 616]. *In vivo* conventional DCs (cDCs) are comprised of two subsets: cDC1 and cDC2, which are distinct in terms of their differentiation and function. cDC1s are specialized cross-presenting DCs whereas cDC2s are specialized in $CD4^+$ T cell responses to extracellular agents [617-619]. cDC1s are essential for robust anti-tumour immune responses [620-622].

DCs are primed molecularly to respond rapidly to stimulation. A dynamic interplay among activating and repressive factors has been observed at the transcriptional and post-transcriptional level in as little as 30 minutes following PRR engagement [623, 624]. There is increasing appreciation that the maintenance of the steady state is an active process involving regulatory factors that temper or repress activating stimuli [357, 625, 626]. Full transition from steady state to the activated or mature state includes disabling these regulatory mechanisms by various strategies [627, 628]. For example, the transcriptional repressor Polycomb group factor 6 (PCGF6) restrains DC activation by repressing genes important for DC activation and function [357]. PCGF6 must be downregulated following activation for DCs to adopt a fully activated phenotype.

To determine whether microRNAs (miRNAs) contribute to downregulation of PCGF6 following Lipopolysaccharide (LPS) stimulation, we identified putative miRNAs that target *Pcgf6*

using TargetScan [629]. miRNAs are small RNA molecules that regulate gene expression through direct binding to RNA molecules and mediating translational inhibition, accelerated deadenylation and/or decay [630]. miRNAs are emerging as important regulators of immune function due to their fast action and ability to regulate programs of gene expression [376, 631, 632]. Recently, it has been shown that the cellular context affects the functional role of a microRNA in governing particular immunological processes such as activation or differentiation into specialized subsets [382].

MicroRNA-9 (miR-9) was among the conserved miRNAs identified to putatively target the 3'UTR of *Pcgf6*. The miR-9 family is comprised of three members, mir-9-1, miR-9-2 and miR-9-3, each encoded by a unique gene located on different chromosomes [633]. They have the same seed sequence, and thus similar targets, and are often collectively referred to as miR-9.

miR-9 was initially considered to be a neuro-specific miRNA but has recently been shown to regulate cellular function in other systems including cancer, fibrosis, and autoimmune and inflammatory conditions [440, 633-635]. Increased miR-9 expression has been identified in models of inflammatory diseases [636-639]. miR-9 is expressed in many types of immune cells in both the innate and adaptive immune systems [444, 638, 640]. The expression of miR-9 is dynamic and has been observed to be upregulated in human monocytes and neutrophils when stimulated with LPS [444, 641, 642]. In macrophages, inhibition of miR-9 leads to a reduction of organ damage in a model of severe LPS-induced inflammation [643]. Specifically in DCs, microRNA profiling of activated human DCs revealed that miR-9 is upregulated upon LPS stimulation [644]. Furthermore, the pri-miR-9 promoter region contains a putative nuclear factor κB (NF-κB) binding site, a transcription factor that is critical for DC activation [444, 645].

Together, these findings suggest that miR-9 may be important for regulating inflammatory responses.

We found that miR-9 is upregulated in both bone-marrow derived DCs (BMDCs) and cDC1s, but not cDC2s in response to activating stimuli. Overexpression of miR-9 in BMDCs led to enhanced phenotypic activation and increased secretion of pro-inflammatory cytokines. Inhibition of the function of miR-9 led to decreased activation of BMDCs. miR-9 expression in BMDCs improves their ability to activate both $CD4^+$ and $CD8^+$ T cells. Ectopic expression of miR-9 improved the ability of BMDCs to promote clearance of B16-melanoma tumors *in vivo.* The ability of miR-9 to promote DC activation and function is in part due to regulating the expression of PCGF6. Examination of a wider range of putative miR-9 targets revealed that miR-9 regulates the expression of several negative regulators of transcription but does so differently in cDC1s versus DC2s. Ectopic expression of miR-9 in cDC1s and not cDC2s led to increased phenotypic activation and increased antigen sensitivity. Together, these results demonstrate a key role for miR-9 in facilitating the activation and function of DCs by antagonizing the expression of negative regulators in a cell-type-specific manner.

2.3 Results

miR-9 Expression Is Upregulated upon Pro-inflammatory Stimulation of DCs

Analysis of miR-9 expression following LPS stimulation of BMDCs revealed that miR-9 expression increases early and decreases over the course of stimulation (**Figure 1A**). Because of sequence similarity, detection of the mature form of miR-9 cannot distinguish miR-9-1, miR-9-2

and miR-9-3. Therefore, we examined the expression of each individual gene by measuring the levels of pri-miRNA in BMDCs. Although all three pri-mir-9 genes are expressed to a similar extent in BMDCs, only pri-miR-9-1 was significantly upregulated following LPS stimulation (**Figure 1B**). To determine whether a similar pattern of miR-9 expression occurred in splenic DCs we sorted cDC1 (XCR1^{hi} CD172a^{lo} CD11c^{hi} MHCII^{hi} F4/80^{lo} CD64^{lo}) and cDC2 (XCR1^{lo} CD172a^{hi} CD11c^{hi} MHCII^{hi} F4/80^{lo} CD64^{lo}) from the spleens of mice injected with either PBS or LPS (**Figure S1**). At baseline, cDC2s expressed more miR-9 compared to in cDC1s. In response to LPS stimulation, mature miR-9 expression increased in cDC1s but not in cDC2s (**Figure 1C**). Despite increased mature miR-9 levels in cDC2s at steady state, lower levels of pri-miR-9 were detectable in cDC2s compared to cDC1s. Upon stimulation, pri-miR-9 transcripts increased in both cDC1s and cDC2 (**Figure 1D**). These data reveal differences in miR-9 expression patterns in cDC subsets and that increased mature miR-9 expression following LPS stimulation is a property of cDC1s and BMDCs.

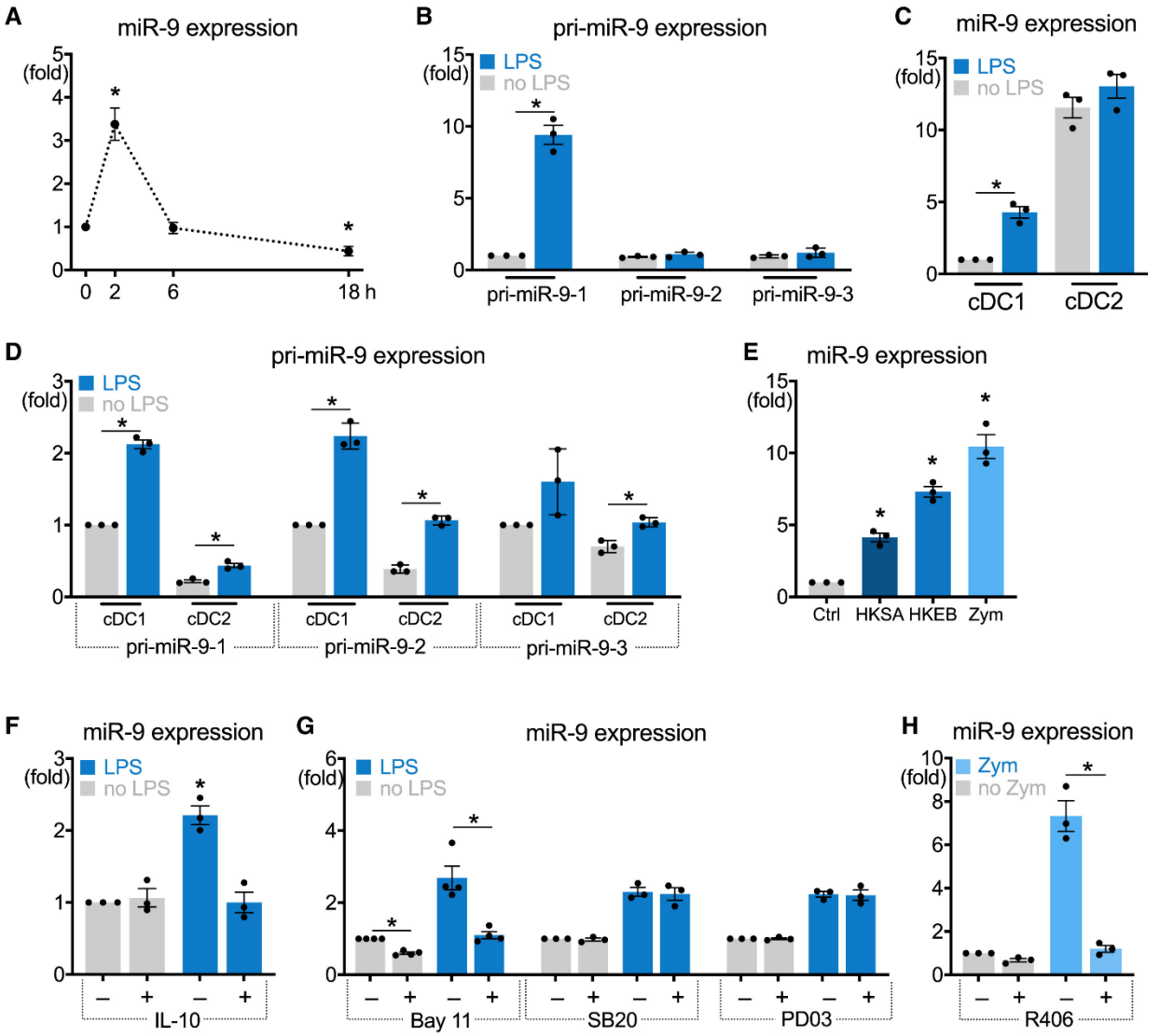


Figure 1. miR-9 Expression Is Upregulated through NF-κβ-Dependent Signaling.(A) Gene expression of mature miR-9 in BMDCs stimulated with 100 ng/mL LPS for the indicated time points.(B) Gene expression of primary miR-9-1, miR-9-2, and miR-9-3 in BMDCs at rest and after 1 h of LPS stimulation (100 ng/mL).(C) Gene expression of mature miR-9 in sorted cDC1s and cDC2s from spleens of mice 2 h post-injection with PBS or 1 μg of LPS.(D) Gene expression of primary miR-9-1, miR-9-2, and miR-9-3 in cDC1s and cDC2s from spleens of mice 2 h post-injection with PBS or 1 μg of LPS.(E) Gene expression of miR-9 in BMDCs stimulated with 2.5×10^7 cells/mL HKSA (heat-killed *Staphylococcus aureus*), 2.5×10^7 cells/mL HKEB (heat-killed *Escherichia coli B*), or 10 μg/mL Zymosan for 2 h. (F) Gene expression of miR-9 in BMDCs stimulated with 10 ng/mL recombinant IL-10 for 1 h followed with or without 100 ng/mL LPS for 2 h.(G) Gene expression of miR-9 in BMDCs treated with Bay117082 NF-κB inhibitor (10 μM), PD0325901 MEK inhibitor (5 μM), or SB203580 p38 MAPK inhibitor (5 μM) for 1 h followed with or without 100 ng/mL LPS for 2 h.(H) Gene expression of miR-9 in BMDCs treated with R406 Syk inhibitor (10 μM) for 1 h followed with or without 100 ng/mL Zymosan for 2 h. In all experiments, expression was measured by qRT-PCR and normalized to *SnoU6* control. Data points represent mean values of individual independent experiments (n = 3), and error bars represent SEM. Significance was determined through one-way ANOVA. *$p > 0.05$. See also Figure S1 and Table S1.

To determine whether other stimulants increased miR-9 expression, BMDCs were stimulated with other microbial agonists such as heat-killed *Staphylococcus aureus* (HKSA), heat-killed *Escherichia coli* (HKEB) and Zymosan (Zym). miR-9 expression was significantly upregulated upon stimulation with each of these agonists (**Figure 1E**). miR-9 expression was also evaluated following treatment with IL-10, a suppressive cytokine that is known to repress DC activation [646]. IL-10 on its own did not significantly alter the expression of miR-9 in BMDCs, however it did prevent LPS-induced upregulation of miR-9 (**Figure 1F**). Since IL-10 is known to inhibit TLR-induced activation of NF- κB [647, 648], we examined whether NF-κB was involved in the LPS-induced upregulation of miR-9 using the NF-κB inhibitor Bay11-7082 [649]. Bay11-7082 decreased the expression of miR-9 at steady-state and also following LPS-stimulation in BMDCs (**Figure 1G**). Treatment of DCs with either SB203580, a p38 MAPK inhibitor or PD0325901, a MEK inhibitor, did not affect LPS-induced upregulation of miR-9 (**Figure 1G**). Dectin-1 activates NF-κB downstream of Syk kinase signaling [650]. Pharmacologically inhibiting Syk signaling using R406 had no effect on miR-9 expression in the steady-state; however, it did inhibit Zymosan-induced upregulation of miR-9 (**Figure 1H**). Taken together, these results demonstrate that miR-9 expression is upregulated in DCs in an NF-κB dependent manner downstream of PRRs.

miR-9 Promotes the Activation and Function of DCs

To determine whether the upregulation of miR-9 expression upon stimulation was important for DC activation and function, miR-9 expression was manipulated using a miR-9

expression vector and a vector expressing a miR-9 sponge (miR-9-S) [440]. The sponge vector expresses GFP with a 3'UTR containing 8 tandem miR-9 binding sites [440]. These excess target sites sequester miR-9 from its targets, thus inhibiting its function. BMDCs transduced with miR-9 vector expressed significantly more miR-9 than empty vector controls **(Figure S2A)**. Expression of miR-9 or miR-9-S did not lead to any significant change in viability or the percentage of $CD11c^+$ cells in cultures (**Figures S2B and S2C**). miR-9 overexpression led to increased secretion of pro-inflammatory cytokines IL-12p40, IL-6, TNFα and IL-12p70 in steady-state and following 18 h of stimulation with LPS (**Figure 2A**). Overexpression of miR-9 in resting and LPS-activated BMDCs also led to increased surface expression of surface activation markers CD80 and CD86 (**Figure 2B**). To determine whether miR-9 regulates the ability of DCs to stimulate T cell responses, BMDCs overexpressing miR-9 were pulsed with whole ovalbumin (OVA) and co-cultured with OVA-specific $CD8^+$ or $CD4^+$ T cells from transgenic mice (OTI and OTII, respectively). miR-9-overexpressing BMDCs at steady state and after LPS stimulation were more efficient at inducing $CD8^+$ T cell activation (measured by expression of CD25, CD44 and CD69) and proliferation (**Figures 2C, 2D and S2D-S2F**). BMDCs overexpressing miR-9 also stimulated more IFNγ-producing $CD8^+$ T cells (**Figures 2E and S2G)**. BMDCs overexpressing miR-9 at steady state and after LPS stimulation were also more efficient at inducing expression of activation markers CD25, CD44 and CD69 on OTII $CD4^+$ T cells as well as increasing their proliferation compared to controls (**Figures 2F, 2G and S2H-J**). More IFNγ-producing cells were also observed when $CD4^+$ T cells were co-cultured with miR-9 expressing DCs compared to control DCs (**Figures 2H and S2K**).

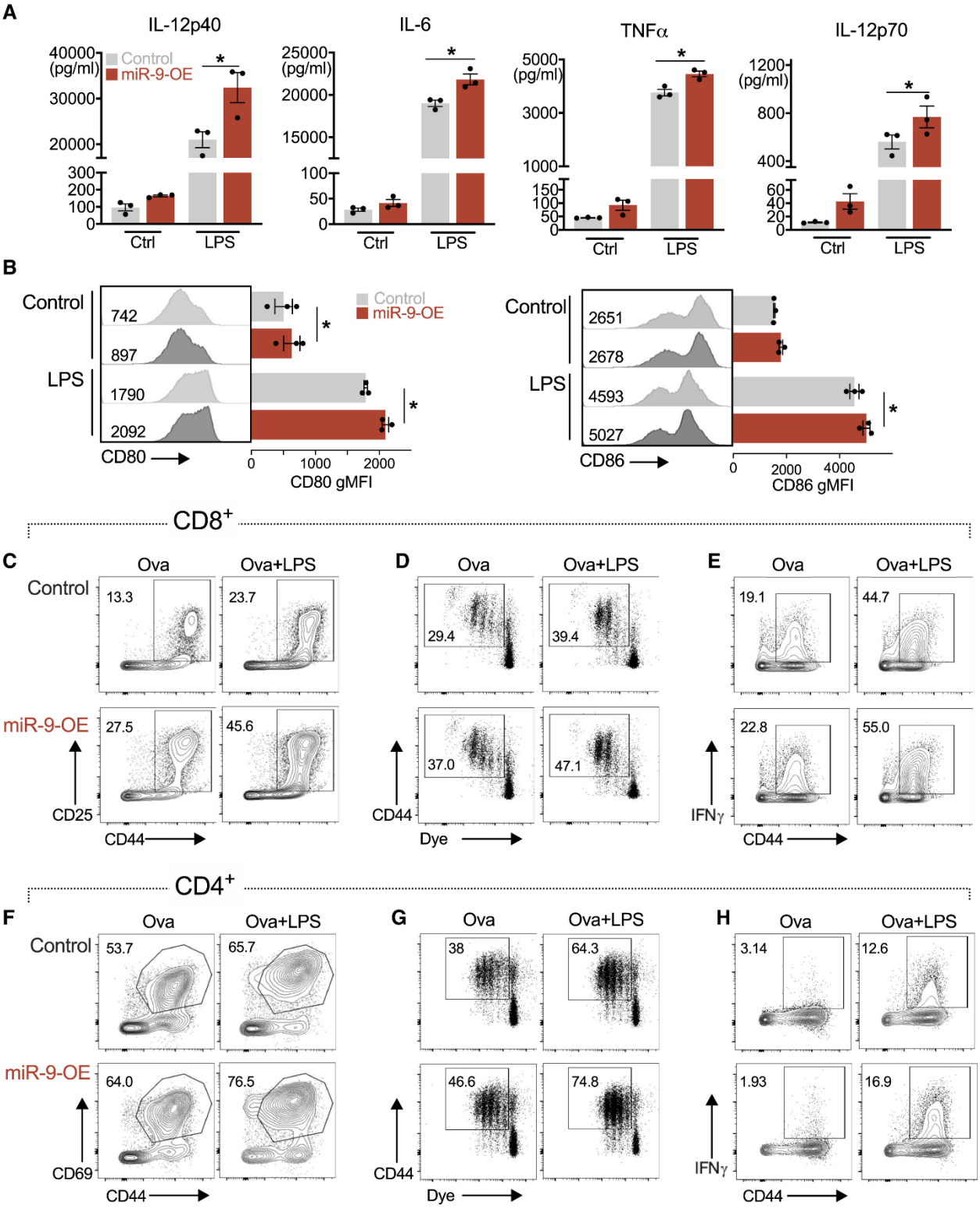

A
IL-12p40
IL-6
TNFα
IL-12p70
Control
miR-9-OE
(pg/ml)
Ctrl
LPS
B
Control
LPS
742
897
1790
2092
CD80
CD80 gMFI
2651
2678
4593
5027
CD86
CD86 gMFI
CD8+
C
D
E
Ova
Ova+LPS
13.3
23.7
27.5
45.6
CD25
CD44
29.4
39.4
37.0
47.1
Dye
19.1
44.7
22.8
55.0
IFNγ
CD4+
F
G
H
53.7
65.7
64.0
76.5
CD69
38
64.3
46.6
74.8
3.14
12.6
1.93
16.9

Figure 2. **miR-9 Overexpression Promotes the Activation and Function of DCs.** (A and B) Cytokine production (A) and cell surface marker expression (B) by BMDCs transduced with control vector or miR-9 examined at steady state or after 18 h of LPS treatment (100 ng/mL). Data points represent geometric mean fluorescence intensity (gMFI) of individual independent experiments (n = 3), and error bars represent SEM.(C–E) BMDCs transduced with miR-9 were pulsed with whole OVA protein with or without LPS stimulation for 6 h and co-cultured with $CD8^+$ OTI T cells labeled with proliferation dye. Three days after co-culture, T cell activation was measured via surface expression of (C) CD44 and CD25, and (D) proliferation by dye dilution. (E) On day 5 after co-culture, IFNγ production was measured via intracellular staining (ICS). See Figure S2 for quantitation of (C)–(H). Data shown are one representative of at least three independent experiments. (F–H) Experiments were performed as in (C)–(E), only $CD4^+$ OTII T cells were used for co-culture. Significance was determined through one-way ANOVA. $^*p < 0.05$.

In complementary experiments, LPS-activated DCs expressing the miR-9-S produced less of the proinflammatory cytokines IL-12p40, IL-6, TNFα and IL-12p70 (**Figure 3A**). Sequestration of miR-9 also led to reduced surface expression of CD80 and CD86 following LPS stimulation (**Figure 3B**). In T cell co-culture experiments, miR-9-S expressing BMDCs pulsed with whole OVA protein were less efficient at inducing T cell activation, measured by expression of CD25, CD44 and CD69 on co-cultured $CD8^+$ OTI cells (**Figures 3C, S3A and S3B**). Proliferation of $CD8^+$ T cells was also reduced and these cells produced less IFNγ compared to controls (**Figures 3D, 3E, S3C and S3D**). In similar experiments with $CD4^+$ T cells, co-culturing with miR-9-S expressing BMDCs decreased the surface expression of CD25, CD44 and CD69 and proliferation after LPS activation (**Figures 3F, 3G and S3E-S3G**). IFNγ expression was significantly decreased in OTII $CD4^+$ T cells co-cultured both with resting-state and LPS-stimulated miR-9-S-expressing BMDCs (**Figures 3H and S3H**). These results demonstrate that miR-9 expression promotes the activation and function of BMDC *in vitro*.

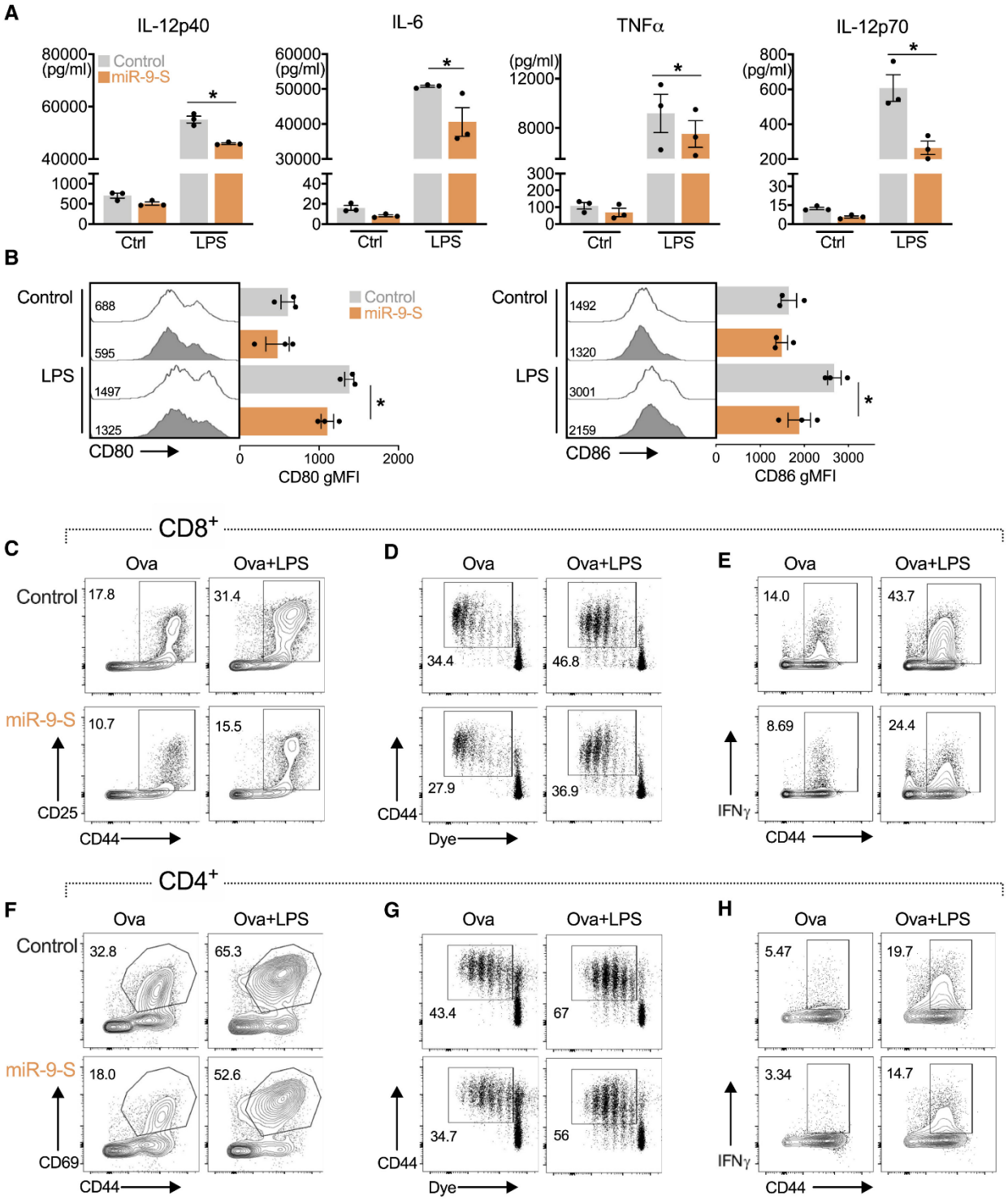


Figure 3. miR-9 Sequestration Diminishes DC Activation and Function. Experiments were performed as in Figure 2, only BMDCs were transduced with miR-9-S. (A and B) Data represent mean gMFI of individual independent experiments (n = 3), and error bars represent SEM. (C–H) Data shown are one representative of at least three independent experiments. See Figure S3 for quantitation. Significance was determined through one-way ANOVA. *$p < 0.05$.

miR-9 Overexpression in DCs Promotes Tumour Clearance *In Vivo*

To study the effects of miR-9 on the function of DCs *in vivo*, we used the B16 melanoma mouse model for which DCs are known to promote tumour clearance [651-653]. Mice were injected subcutaneously with B16-OVA cells and 3 days later LPS-stimulated DCs pulsed with whole OVA were transferred intravenously. Once tumours were palpable, tumour volume was measured every 2 days until endpoint was reached (2,000 mm^3). Tumours in mice treated with miR-9-overexpressing BMDCs had significantly decreased tumour volume and took longer to reach endpoint compared to mice injected with control BMDCs (**Figures 4A and 4B**). To measure OVA-specific $CD8^+$ T cell responses, 5 x 10^3 OTI cells were transferred 1 day prior to OVA-pulsed BMDC transfer and 5 days later, OVA-specific $CD8^+$ T cells in the spleen were detected using OVA-tetramer. Significantly more numbers of OVA-specific T cells were detected in tumour-bearing mice injected with miR-9 overexpressing BMDCs compared to controls (**Figure 4C)**. In complementary experiments, mice injected with miR-9-S expressing BMDCs had significantly larger tumours, reached endpoint faster and had less OVA-specific splenic T cells than mice injected with control BMDCs (**Figures 4D-4F**). These results demonstrate that miR-9 expression in BMDCs promotes the control of tumor growth, at least in part through stimulating T cell expansion.

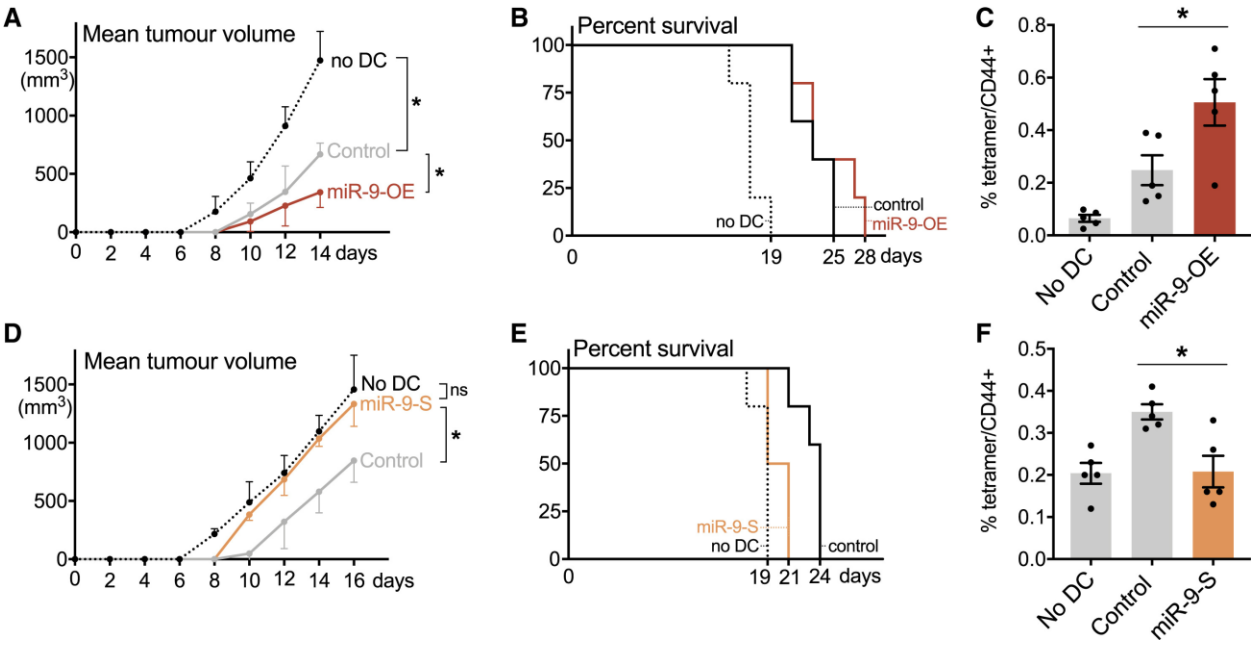


Figure 4. miR-9 Expression in BMDCs Promotes Control of Tumor Growth *In Vivo.* (A and B) Mice (n = 5/group) were injected subcutaneously on day 0 with B16-OVA melanoma cells. Three days later, BMDCs transduced with control or miR-9 vectors were pulsed with 1 mg/mL of whole OVA, stimulated for 6 h with 100 ng/mL of LPS, and injected via tail vein. (A) Palpable tumours were measured every 2 days to obtain mean tumour volume.(B) Survival curves of mice per group were plotted for each of the groups until one mouse reached endpoint (2,000 mm^3 tumor volume). (C) Mice were injected with B16-OVA cells as in (A). On day 1, 5 x 10^3 OTI T cells were transferred via tail vein. Three days later, BMDCs were prepared as in (A) and injected via tail vein. Spleens from tumor-bearing mice were stained on day 5 for OVA-specific $CD8^+$ T cells (tetramer) and CD44. (D–F) Experiments were performed as in (A)–(C), using BMDCs transduced with miR-9-S. Shown are mean tumour volume (D), survival curves (E), and percent tetramer positive/percent CD44 positive (F). Data shown are representative of at least two independent experiments and error bars represent SEM. Significance was determined through one-way ANOVA; *$p < 0.05$.

miR-9 Expression Reduces PCGF6 Expression

TargetScan analysis revealed that the transcriptional repressor PCGF6 is a putative target of miR-9. We have previously shown that PCGF6 restrains DC activation and is rapidly downregulated following activation of DCs by LPS [357]. To examine whether miR-9 targets PCFG6 to promote DC activation and function, we first performed a time course examining the expression of *Pcgf6* and miR-9 at early time points following LPS stimulation. miR-9 levels reproducibly increased as early as 10 min following stimulation and were significantly increased

following 30 minutes of stimulation (**Figure 5A**). Decreases in *Pcgf6* mRNA and protein levels were also consistently observed after 30 mins of LPS stimulation (**Figure 5A-5B**). *Pcgf6* expression was also measured in cells treated with the NF-κB inhibitor Bay11 and the Syk inhibitor R406. Both Bay11 and R406 inhibited *Pcgf6*-downregulation following PRR activation (**Figures 5C-5D**). To determine whether a similar pattern of *Pcgf6* expression occurred in *in vivo*-derived DCs, we sorted cDC1 and cDC2 from the spleens of mice 2 h after injection with PBS or LPS. *Pcgf6* expression decreased in response to LPS in cDC1s but not in cDC2s (**Figure 5E**). This is consistent with our findings that mature mir-9 expression increases in cDC1s but not cDC2s following LPS stimulation. These data reveal an inverse relationship between *Pcgf6* expression and mature miR-9 expression in BMDCs and cDC1s.

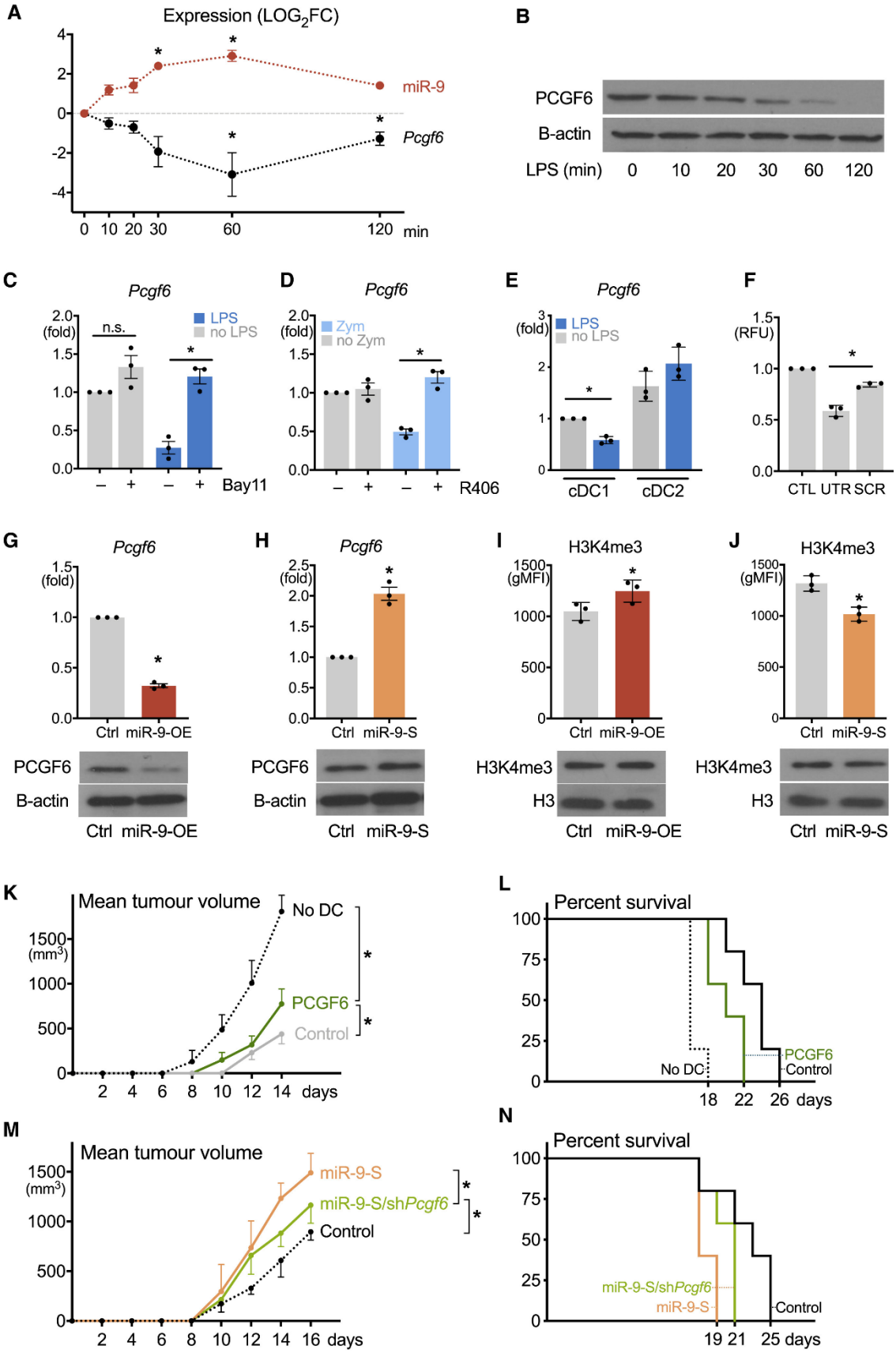

A
Expression (LOG2FC)
miR-9
Pcgf6
0 10 20 30 60 120 min
B
PCGF6
B-actin
LPS (min) 0 10 20 30 60 120
C
Pcgf6
LPS
no LPS
n.s.
Bay11
D
Pcgf6
Zym
no Zym
R406
E
Pcgf6
LPS
no LPS
cDC1
cDC2
F
CTL UTR SCR
G
Pcgf6
Ctrl miR-9-OE
PCGF6
B-actin
H
Pcgf6
Ctrl miR-9-S
I
H3K4me3
H3
J
K
Mean tumour volume
No DC
PCGF6
Control
days
L
Percent survival
No DC
PCGF6
Control
M
Mean tumour volume
miR-9-S
miR-9-S/shPcgf6
Control
N
Percent survival
miR-9-S/shPcgf6
miR-9-S
Control

Figure 5. miR-9 Reduces the Expression of Negative Regulators Including *Pcgf6* (A) Expression of miR-9 and *Pcgf6* in BMDCs stimulated with 100 ng/mL LPS for indicated time points by qRT-PCR. Data points represent mean fold expression of individual independent experiments (n = 3), and error bars represent SEM. (B) Expression of PCGF6 and beta-Actin in BMDCs stimulated with 100 ng/mL LPS for indicated time points by western blot. (C and D) Gene expression of *Pcgf6* in BMDCs treated with 10 μM Bay117082 NF-κB inhibitor (C) or 10 μM R406 Syk inhibitor (D) for 1 h followed with or without 2 h of 100 ng/mL LPS stimulation. (E) Gene expression of *Pcgf6* in cDC1s and cDC2s from spleen of mice 2 h post-injection with PBS or 1 μg of LPS. (F) Mouse embryonic fibroblasts were transfected with either empty PmiRGlo luciferase expression vector or constructs containing the 3′ UTR of *Pcgf6* (UTR) intact or with a mutation in the miR-9 binding site (SCR). Data shown are the best representative mean ratio of firefly luciferase/*Renilla* luciferase of one of three independent experiments, and error bars represent SD. (G and H) Expression of PCGF6 in BMDCs transduced with relevant control, miR-9 (G) or miR-9-S (H). (I and J) Global H3K4me3 levels measured by flow cytometry and western blot in relevant control, miR-9-expressing BMDCs (I) or miR-9-S expressing BMDCs (J). (K and L) Experiments were performed as in Figure 4 except mice were injected with BMDCs transduced with control or retroviral vectors overexpressing PCGF6. Shown are mean tumour volume (K) and survival curves (L). (M and N) Experiments were performed as in (K) except mice were injected with BMDCs transduced with control or miR-9-S or double-transduced with miR-9-S and a short hairpin RNA targeting PCGF6 (sh*Pcgf6*). Shown are mean tumour volume (M) and survival curves (N). Data points represent mean value from individual independent experiments (n = 3), and error bars represent SEM. All qRT-PCR data were normalized to *Hprt*. Western blot data shown are best representative of three independent experiments. Significance was determined through either one-way ANOVA (A, C–F, K, and M) or Student's t test (G–J). $^{*}p < 0.05$. See also Figure S4 and Table S1.

The putative miR-9 binding site in the *Pcgf6* 3'UTR was examined for its ability to regulate translation using the PmiRGlo reporter assay [654]. Addition of the *Pcgf6* 3'UTR to the firefly luciferase mRNA decreased the luciferase signal compared to control (**Figure 5F**). A mutation that scrambled the putative miR-9 target site in the 3'UTR of *Pcgf6* (SCR) prevented the decrease in firefly luciferase expression (**Figure 5F and S4A**). Expression of miR-9-S resulted in increased signal from the intact 3'UTR and not the mutated UTR indicating that the miR-9 sponge indeed functions to sequester miR-9 away from its binding site in the 3'UTR of *Pcgf6* **(Figure S4B)**. Together these findings suggest that the putative miR-9 binding site in the 3'UTR of *Pcgf6* has miR-9-dependent regulatory activity.

To determine whether miR-9 regulates *Pcgf6* expression, we examined PCGF6 levels following miR-9 and miR-9-S expression. Ectopic expression of miR-9 in BMDCs resulted in less PCGF6 expression, whereas expression of the miR-9-S resulted in more PCGF6 expression, at

both the transcript and protein levels (**Figures 5G-5H**). This decrease in expression remained consistent when the BMDCs were stimulated with LPS (**Figures S4C**). PCGF6 regulates the levels of the activating histone mark H3K4me3 in DCs [357], therefore we examined whether modulation of miR-9 expression would lead to corresponding changes in H3K4me3 levels. Global H3K4me3 levels were increased in miR-9-overexpressing BMDCs and decreased in miR-9-S-expressing BMDCs (**Figures 5I-5J**). Finally, to determine whether PCGF6-overexpressing BMDCs would, like miR-9-S expressing BMDCs, be less effective at promoting tumour clearance, BMDCs overexpressing PCGF6 were transferred into mice harboring B16-OVA tumors. Tumors in mice injected with BMDCs expressing PCGF6 had increased tumour volume and reached endpoint faster compared to mice injected with control BMDCs (**Figures 5K-5L**). We next examined whether reducing PCGF6 expression could rescue the defect in the ability of miR-9-S-expressing BMDCs to clear tumours by transducing BMDCs with both miR-9-S and a short hairpin RNA (shRNA) targeting *Pcgf6* (sh*Pcgf6*) [357]. Although, miR-9-S expressing BMDCs deficient in PCGF6 were better able to promote tumor clearance then miR-9-S expressing BMDCs, they were still significantly impaired relative to control BMDCs (**Figures 5M-N**). Furthermore, BMDCs expressing miR-9-S and deficient in PCGF6 expressed significantly less CD80, CD86, IL-6 and TNFα compared to control BMDCs but not significantly different expression compared to miR-9-S expressing BMDCs (**Figures S4D-S4E**). Double-transduced BMDCs expressed significantly less IL-12p40 than control BMDCs and miR-9-S expressing BMDCs. Double transduced BMDCs did not show significant change in IL-12p70 secretion compared to control BMDCs but showed significantly more secretion of IL-12p70 then miR-9-S

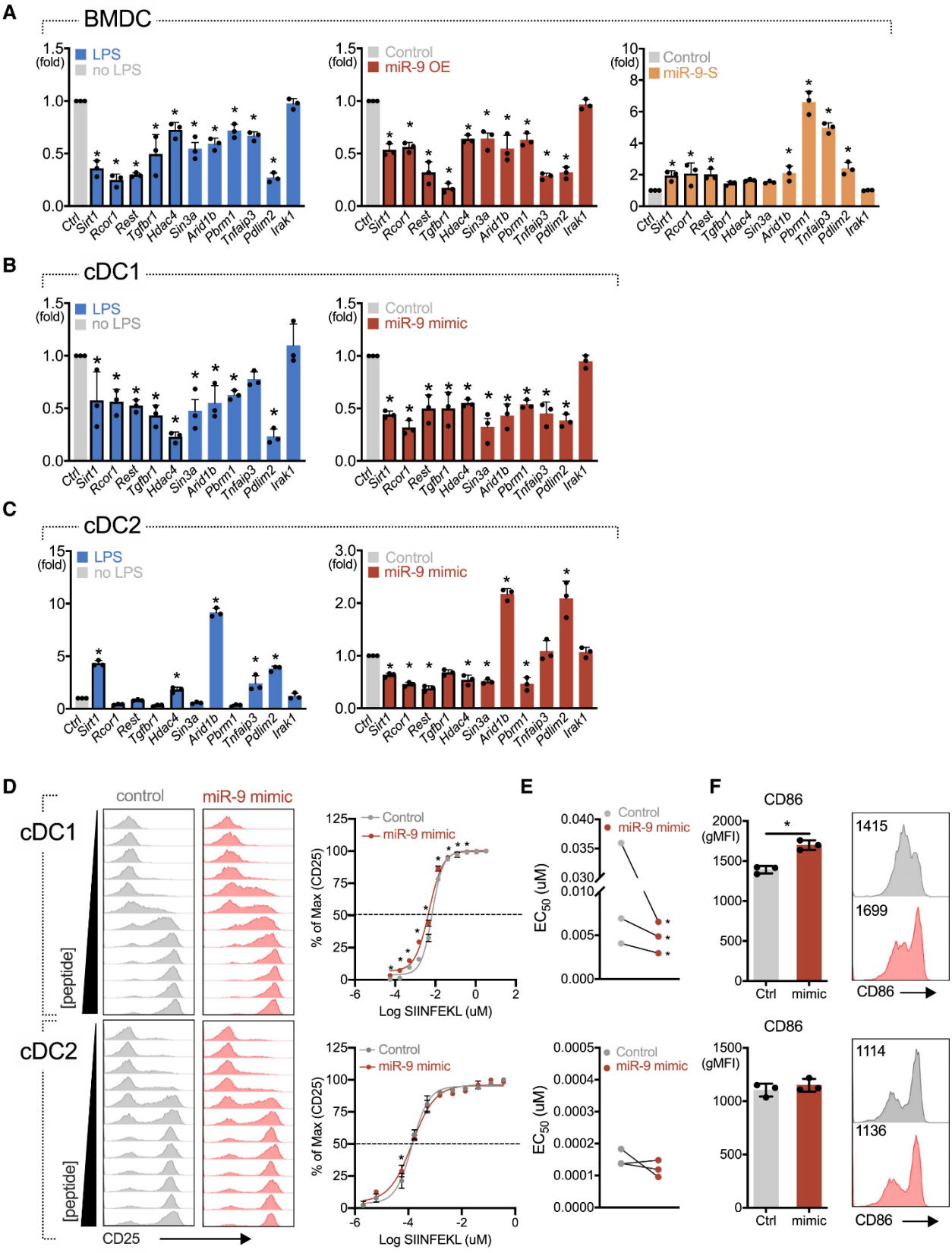
expressing BMDCs (**Figure S4E**). These results suggest that miR-9 promotes tumour clearance *in vivo* only in part through targeting PCGF6.

miR-9 Targets Negative Regulators

miRNAs can target many mRNAs simultaneously; thus, miR-9 is likely targeting many genes that collectively regulate DC function. Deletion of PCGF6 did not completely revert the phenotype of miR-9 sequestration, suggesting that other targets of miR-9 have a significant role in DC activation. The list of putative targets of miR-9 was curated from TargetScan and subjected to PANTHER Pathway analysis to determine whether any particular biological processes were enriched [629, 655]. This analysis revealed an enrichment for negative regulators of gene expression including negative regulation of cytoplasmic translation, regulation of gene silencing and regulation of mRNA stability (**Figure S5A).** Since we already identified the transcriptional repressor PCGF6 as a miR-9 target and miR-9 has previously been shown to target genes responsible for epigenetic silencing, we focused our analyses on the list of genes involved in regulation of gene silencing [656, 657]. Some of these genes, including Sirtuin 1 (*Sirt1*), Transforming Growth Factor Beta 1 (*Tgfβr1),* Polybromo1 (*Pbrm1*), TNF Alpha Induced Protein A (*Tnfαip3*), and Histone Deacetylase 4 (*Hdac4*), are already known to be negative regulators of innate immune signaling pathways and adaptive immune responses [643, 658-664].

We examined the expression of validated targets of miR-9 from this group, including *Tgfβr1, Hdac4, Sirt1,* REST Corepressor 1 (*Rcor1*) *and* RE1 Silencing Transcription Factor (*Rest*)

[434, 449, 665, 666]. Expression of predicted targets, including SIN3 Transcription Regulator Family A (*Sin3a),* AT rich Interaction Domain 1B (*Arid1b*)*, Pbrm1, TNFαip3* and PDZ and LIM 2 domain 2 (*Pdlim2*) were examined. Interleukin-1 receptor associated-kinase (*Irak1)*, which is neither decreased upon LPS stimulation nor a putative miR-9 target was used as a control. All of the examined targets were downregulated to some degree following LPS stimulation of BMDCs (coinciding with miR-9 upregulation) (**Figure 6A left panel**). Expression of the control *Irak1* did not change. Ectopic expression of miR-9 in BMDCs resulted in varying degrees of decreased expression of the examined targets (**Figure 6A middle panel**). Expression of miR-9-S led to increased expression of most targets, however *Sin3a, Tgfβr1* and *Hdac4* expression remained unchanged (**Figure 6A right panel**). Again, *Irak1* expression was not changed upon miR-9 modulation in BMDCs. To determine whether a similar pattern of expression occurred in *in vivo*-derived DCs we sorted cDC1 and cDC2 from the spleens of mice injected with either PBS or LPS. All of the examined miR-9 targets were significantly downregulated following LPS stimulation in cDC1s (**Figure 6B left panel**). In cDC2s, only *Sin3a*, *Tgfβr1*, *Pbrm1, Rcor1* and *Rest* were downregulated to some degree following LPS stimulation, whereas the other targets showed increased expression (**Figure 6C left panel**). *Irak1* expression was not changed following LPS stimulation in cDC1s or cDC2s (**Figure 6B and 6C)**.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BMDC
LPS
no LPS
Control
miR-9 OE
Control
miR-9-S
B
cDC1
LPS
no LPS
Control
miR-9 mimic
C
cDC2
LPS
no LPS
Control
miR-9 mimic
Ctrl
Sirt1
Rcor1
Rest
Tgfbr1
Hdac4
Sin3a
Arid1b
Pbrm1
Tnfaip3
Pdlim2
Irak1
D
control
miR-9 mimic
cDC1
cDC2
[peptide]
CD25
Control
miR-9 mimic
% of Max (CD25)
Log SIINFEKL (uM)
E
Control
miR-9 mimic
EC50 (uM)
F
CD86
(gMFI)
Ctrl
mimic
1415
1699
1114
1136
CD86

Figure 6. miR-9 Reduces the Expression of Negative Regulators (A) Gene expression levels of *Sin3a*, *Tgfbr1*, *Arid1b*, *Hdac4*, *Pbrm1*, *Tnfaip3*, *Pdlim2*, *Sirt1*, *Rcor1*, *Rest*, or *Irak1* in BMDCs with or without 100 ng/mL LPS stimulation for 2 h, transduced with relevant control (Ctrl), miR-9 or miR-9-S. (B and C) Gene expression of *Sin3a, Tgfbr1, Arid1b, Hdac4, Pbrm1, Tnfaip3, Pdlim2, Sirt1, Rcor1, Rest,* or *Irak1* in sorted (B) cDC1s or (C) cDC2s from mice injected with PBS or 1 μg of LPS for 2 h or transfected with miR-9 mimic or control mimic. Data points represent mean fold expression from individual independent experiments (n = 3), and error bars represent SEM. All qRT-PCR data were normalized to Hprt. Black-bordered columns indicate validated miR-9 targets. (D) Percentage of CD25+ CD8+ OT1 T cells co-cultured with cDC1s (top panels) or cDC2s (bottom panels) pulsed with titrated concentration of SIINFEKL peptide. Data were normalized to the proportion of CD25+ cells at saturating peptide concentration (10 μM) and are best representative of three individual experiments. Significance was determined through two-way ANOVA. $*p < 0.05$. (E) EC50 for CD25+ of CD8+ OT1 T cells co-cultured with cDC1s (top panel) or cDC2s (bottom panel). Data in (E) show EC50 values for three individual experiments. Significance achieved for individual experiments is denoted by an asterisk (∗); however, significance was not achieved combining all experiments due to the variable EC50 in each experiment. (F) CD86 surface expression of cDC1s (top panels) or cDC2s (bottom panels) transfected with miR-9 mimic or control mimic examined at steady state. Data points represent mean gMFI of biological replicates (n = 3), and error bars represent SEM. Significance was determined through one-way ANOVA (A–C), two-way ANOVA (D), or Student's t test (E and F). $*p < 0.05$. See also Figure S5 and Table S1.

In order to ectopically express miR-9 in splenic cDC1s and cDC2s, we utilized a miR-9 mimic. We first transfected BMDCs with the miR-9 mimic to verify that it could reproduce the phenotype observed with transduction of miR-9-OE. miR-9 mimic transfected BMDCs expressed significantly more miR-9 then BMDCs transfected with a control mimic (**Figure S5B**). miR-9 mimic transfected BMDCs showed no significant change in viability or CD11c expression compared to controls (**Figures S5C and S5D**). BMDCs transfected with miR-9 mimic showed increased expression of surface activation marker CD80 (**Figure S5E).** To determine whether miR-9 regulates the expression of the targets examined in BMDC, splenic cDC1 and cDC2s were sorted and transfected with miR-9 mimic. Both cDC1s and cDC2s transfected with miR-9 mimic showed significantly increased expression of miR-9 compared to controls (**Figures S5F and 5G**). cDC1s transfected with miR-9 showed significant reduction of all targets examined (**Figure 6B right panel**). However, cDC2s transfected with miR-9 showed decreased expression of most of the examined targets except *Pbrm1*, *Arid1b* and *TNFαip3*. *Irak1* expression was unchanged in miR-9 mimic transfected cDC1s or cDC2s (**Figure 6C right panel**).

To determine whether ectopic expression of miR-9 led to increased function of cDC1s and cDC2s, we performed antigen sensitivity assays using $CD8^+$ OT1 T cells [667, 668]. cDC1s transfected with miR-9 resulted in increased antigen sensitivity of $CD8^+$ OT1 T cells (measured by CD25 expression) compared to control cDC1s, based on the peptide concentration required to elicit 50% of maximum CD25 expression **(Figure 6Dand 6E, top panels)**. Ectopic expression of miR-9 in cDC2s did not lead to significant changes in antigen sensitivity of $CD8^+$ OT1 T cells **(Figure 6D-6E, bottom panels)**. cDC1s transfected with miR-9 showed increased expression of the surface activation marker CD86 **(Figure 6F, top panel)**. cDC2s on the other hand did not show consistent significant changes in surface CD86 expression when transfected with miR-9 **(Figure 6F, bottom panel)**. Together our results demonstrate that miR-9 expression in BMDCs and cDCs decreases the expression of several negative regulators of transcription and leads to increased activation and function.

2.4 Discussion

DCs respond rapidly to environmental cues in order to mount an efficient response to pathogens. The transition of DCs from steady state to the mature or activated state is tightly controlled to limit activation only in response to appropriate stimuli. Poised at the cellular level, DCs are also poised at the molecular level to rapidly and precisely regulate gene expression [623, 624, 669]. In several contexts miR-9 expression has been associated with inflammation [636, 637, 639]. Here we show that miR-9 is upregulated in response to pro-inflammatory stimuli in BMDCs and cDC1s, but not cDC2s. miR-9 promotes BMDC and cDC1 activation and

their ability to stimulate T cell responses. Sequestration of miR-9 restrained BMDC activation and reduced the ability of BMDCs to promote T cell responses. Mechanistically, we found that miR-9 promotes DC function by regulating the expression of negative regulators of transcription, including the epigenetic regulator PCGF6. These observations demonstrate that miR-9 facilitates DC activation by targeting negative regulators in a cell-type-specific manner.

It has been demonstrated that genes important for DC activation are primed to respond rapidly to activating transcription factors [357, 623, 624]. Activating signals, often downstream of PRRs or cytokine/chemokine receptors, are dynamic and balanced by mechanisms that restrain DC activation. Downregulation or disabling repressive mechanisms may facilitate DC activation; however, the mechanisms by which they are downregulated are not well understood. Here, we identify miR-9 as one mechanism for their downregulation. Several putative miR-9 targets, including those downregulated in our study, have been implicated as regulators of DC function. For example, deletion of TNFAIP3, which directly inhibits MyD88-mediated pro-inflammatory signalling, leads to spontaneous DC activation in both inflammatory bowel disease model and a systemic lupus erythematosus (SLE) model [625, 670]. Similarly, DC-specific deletion of PRDM1 in female mice led to an increase in anti-double stranded DNA autoantibodies, leading to a SLE-like phenotype [671]. Deletion of $\alpha_v\beta_8$ integrin, which promotes signalling through the TGF-β signaling cascade, has also been shown to promote spontaneous activation of DCs and an increase in autoimmune colitis [672]. These studies underscore the importance of active restraint of DC activation to prevent spontaneous and potentially pathological inflammation. We show that miR-9 targets several of these genes and therefore may play a significant role in these processes.

The *ex vivo* generation of DCs from bone-marrow cells cultured in granulocyte-macrophage colony stimulating factor (GM-CSF) is often used as a model system to study DC biology. These cultures are comprised of a heterogeneous population of DCs and macrophages [673], limiting the interpretation of some of our data to effects in this model system. It should be noted that GM-BMDCs express markers of the cDC2 lineage such as CD11b and lack expression of XCR1 which marks cDC1 *in vivo*. Even so, in the context of these studies, miR-9 regulation and function were comparable in BMDCs and cDC1s, but not cDC2s. This is not entirely surprising since BMDCs are known to have functional qualities similar to cDC1s, such as cross-presentation and IL-12 production. We found that both splenic cDC1s and BMDCs upregulate the expression of miR-9 in response to LPS. These data suggest that there are similarities in the biochemical wiring of cDC1s and BMDCs, though whether these similarities are specific to a subpopulation in the BMDC culture is unknown. Further, we did not determine which population of cells in the BMDC cultures are responsible for promoting OT-I and OT-II responses *in vitro* and anti-tumour immunity *in vivo*. However, in line with data presented by Helft et. al, it is likely that DCs within the BMDC culture are inducing T cell responses to whole antigen and stimulating anti-tumour immunity.

We consistently observed more mature miR-9 expression in cDC2 compared to cDC1s. However, miR-9 expression did not increase in cDC2s following LPS stimulation. Yet pri-miR-9 levels in cDC2s were both lower than cDC1s and were induced upon LPS stimulation. Differences in miR-9 processing may contribute to these differences, either at the level of the individual miR-9 paralogues or in a cell-type-specific manner [674-676]. Furthermore, differences in miR-9 stability, turnover and/or post transcriptional modifications may be

different in cDC1s and cDC2s. It is also possible that the net abundance of miR-9 targets in cDC1s and cDC2s is not equal leading to different regulation dynamics in the two cell types. Likewise, increased miR-9 expression will affect targets differently, depending on their relative abundance [677]. Potentially, the expression of lncRNAs that act as miR-9 sponges, such as NEAT1, TUG1 and CircMTO1, or the presence of LIN28A also mediate differences between cDC1 and cDC2s [676, 678-680].

We also consistently observed differences between cDC1s and cDC2s in the expression dynamics of known and putative miR-9 targets. For these studies, we focussed on regulators of transcription based on our previous studies on PCGF6 regulating the chromatin landscape in DCs [357]. Of the selected validated and putative miR-9 targets, most were downregulated in response to either LPS stimulation or miR-9 overexpression similarly in cDC1s and BMDCs. However, the expression of these genes in cDC2s was not similar. In particular *Sirt1*, *Hdac4, Arid1b*, *Tnfαip3* and *Pdlim2* were increased in cDC2s in response to LPS and over-expression of miR-9 did not decrease the expression of *Arid1b*, *TNFαip3* and *Pdlim2.* miR-9 has been previously shown to specifically target *Sirt1* in macrophages, preventing *Sirt1* from deacetylating and inhibiting NF-κB, possibly leading to a feed forward loop, increasing miR-9 expression [643]. Other studies have linked miR-9 with regulation of the chromatin landscape in neural progenitors through its action on *Rest*, and its corepressor *Rcor1*, which were also regulated by miR-9 in DCs [656, 657]. Further work will determine whether miR-9 governs the chromatin landscape in DCs as it has been shown to in neural progenitors [656, 657] and whether it does so differently in cDC1s and cDC2s.

Although miRNAs only represent a small percentage of the genes transcribed in mammals, they have been shown to have vast regulatory effects on the genome [395, 681]. Sixty percent of the genome has computationally been shown to be regulated by miRNAs, with many ancient miRNAs predicted to target upwards of 1,000 different genes [629, 682]. Many of these targets may share a common pathway or protein complex, implying that a single miRNA could influence an entire cellular process or program. miR-16 has been demonstrated to regulate the G0/G1 to S cell cycle transition through coordinated repression of key genes governing this process, providing the first evidence of a single miRNA regulating a specific program or process in mammalian cells [683]. This form of regulation was further confirmed through a statistical analysis of target predictions against functional annotations, where coordinated repression of multiple genes in a process was found to be prevalent in mammalian genomes [684]. Single miRNAs have since been implicated in governing various developmental programs and angiogenesis [685-687]. miRNAs have also been implicated in the governance of immune response programs, where miR-181 has been shown to regulate the sensitivity of T cell activation through targeting multiple phosphatases which attenuate T cell activation [688]. Our study adds to the growing evidence that miRs contribute to cellular responses by regulating programs of gene expression. Additionally, our results demonstrate that miR-9 expression increases very quickly at very early time points following PRR stimulation and that several validated and putative miRNA targets are concomitantly downregulated. An exhaustive analysis of miRNA expression dynamics, particularly at early time points, could uncover other miRNAs that regulate coordinated molecular changes required for DC function.

We found that increased miR-9 expression in BMDCs, both *in vitro* and *in vivo,* improves their ability to stimulate $CD4^+$ and $CD8^+$ T cell proliferation and IFNγ production in response to whole protein antigen. In BMDCs, miR-9 expression altered Signal 2 (CD80, CD86 expression) and Signal 3 (cytokine production) and possibly Signal 1 (antigen presentation). Changes in these signals individually or collectively could result in increased T cell responses. In cDCs, miR-9 was upregulated in cDC1s but not cDC2s and increasing miR-9 expression in cDCs only affected the ability of cDC1s to stimulate $CD8^+$ T cell responses. cDC1s are effective at promoting Type 1 immune responses suggesting a relationship between miR-9 upregulation in response to stimuli and induction of Type 1 immune responses. Further work is necessary to determine whether miR-9 differentially regulates Signals 1, 2 and 3 in cDC1s and cDC2s.

We also consistently observed an increase in antigen sensitivity of $CD8^+$ T cells when stimulated with cDC2s compared to cDC1s. cDC1s have been demonstrated to be more efficient at cross-presentation and stimulation of $CD8^+$ T cells than cDC2s [618, 619]. However, the comparative ability of cDC1 and cDC2 to stimulate $CD8^+$ T cells to endogenous derived MHCI antigens has not been adequately explored. It is possible that the increased miR-9 levels observed in cDC2s participates in the increased antigen sensitivity of $CD8^+$ T cells. Because Locked nucleic acids (LNAs) themselves activate DCs (data not shown) we were unable to test whether loss of miR-9 in cDC2s changes antigen sensitivity of T cells. Further work will reveal the significance of increased antigen sensitivity of T cells stimulated by cDC2s.

Our data reveal that the expression of negative regulators can be coordinately restrained by miR-9. Their coordinated downregulation may be necessary to permit DC activation. Thus, change in expression of miR-9 alters the balance between positive and

negative regulators to fine-tune DC responses. We also show that despite miR-9 being expressed in cDC1s and cDC2s, its kinetics and function appear to differ between subtypes. Thus, miR-9 contributes to the coordinated effort that enables DCs to respond rapidly to environmental stimuli and engage in protective immunity.

2.5 Author contributions

Conceptualization, C.M.K., S.M.S, and B.C.; Methodology, C.M.K., B.C., S.M.S, S.G, R.G.J. and D.G.R.; Validation, B.C., P.J., M.C. and O.L.; Formal Analysis, B.C.; Investigation, B.C., P.J., G.M.B., M.C. and O.L.; Resources, S.M.S., R.G.J. and D.G.R.; Data curation, B.C.; Writing – Original Draft, C.M.K. and B.C.; Writing- Review and Editing, C.M.K., B.C., K.W., G.M.B., D.G.R., R.G.J., S.G., and S.M.S.; Visualization, C.M.K., B.C. K.W., and D.G.R.; Supervision, C.M.K. and S.G.; Project Administration, C.M.K., B.C. and G.M.B.; Funding Acquisition, C.M.K.

2.6 Experimental procedures

Experimental Model and Subject Details

Female C57BL/6N mice were purchased from Charles Rivers Laboratories at 6-8 weeks of age (Montreal, QC Canada) and bred in house. OTI and OTII transgenic mice were purchased from the Jackson Laboratory (Bar Harbor, ME USA) and bred in house. Animals were maintained in a specific pathogen-free environment. All experiments were conducted following the guidelines of the Canadian Council of Animal Care, as approved by the animal care committee of McGill University.

Method Details

Bone-marrow Derived DC Culture: Bone marrow was extracted and cultured in RPMI media (Corning) with 1% Penicillin-Streptomycin (Wisent), 10% fetal calf serum (HyClone/Wisent), 1% L-glutamine (Wisent), 0.1 % β-Mercaptoethanol (Gibco) and 20 ng/ml of granulocyte/macrophage colony stimulation factor (GM-CSF; Peprotech) in 6-well non-tissue culture treated plates. DCs were cultured as suspended colonies for 8-9 days at 37°C and 5% CO_2. Non-adherent cells were collected and plated at $1x10^6$ cells/ml into 12 well non-tissue culture treated plates and stimulated as indicated. DC activators included LPS (*Escherichia coli* serotype 0111:B4, Sigma-Aldrich), heat-killed *Staphylococcus aureus* (Invivogen) , heat-killed *Escherichia coli* serotype 0111:B4 (Invivogen) and Zymosan (Invivogen). Bay117082 NF-κB inhibitor, PD0325901 MEK inhibitor, SB203580 p38 MAPK inhibitor and R406 Syk inhibitor were purchased from SelleckChem.

Vector Construction: A 1000bp region containing the miR-9-1 gene was cloned into a MSCV-Ef1a-Thy1.1 (MeT) retroviral vector using HindIII and XhoI restriction enzymes (New England BioLabs). For the miR-9 sponge vector, the sponge-containing region from the pBabe-puro-miR-9 sponge vector (Bob Weinberg- Addgene plasmid # 25040), was sub-cloned into MeT retroviral vector using HindIII and XhoI restriction enzymes (NEB). For the luciferase assay, the 3'UTR sequence of *Pcgf6*, with miR-9 target sequence intact or scrambled, was cloned into a PmirGlo luciferase-expressing vector (Promega) using XhoI and XbaI restriction enzymes (NEB). All

vectors were sequence verified using Genome Quebec Nanuq Services. Plasmids were purified using cesium chloride gradient.

Virus Production and Transduction: 293T cells were transfected with 5 μg Helper plasmid and 12 μg of MSCV-based vector in optiMEM media (LifeTech) containing Lipofectamine 2000 (LifeTech) as described [689]. Briefly, on day 2 of culture, half the DC media was replaced with a solution containing virus supplemented with β-Mercaptoethanol (Lifetech), polybrene (Lifetech), Hepes (Multicell) and L-Glutamine (Multicell). DCs were transduced by spin infection for 90 min. at 2500rpm, at 30°C. The media was then replaced with complete DC media and the DCs were collected for RNA extraction/flow cytometry on day 8 or 9 of culture.

Luciferase Reporter Assay: 1 x 10^5 MEFs were plated in 6-well tissue culture treated plates per well. The following morning the complete DMEM media was replaced with DMEM containing 10% FCS. In optiMEM media (Lifetech) containing lipofectamine (Lifetech), 12 μg of PmirGlo vector (Promega), PmirGlo-PCGF6 3'UTR or PmirGlo-PCGF6 with scrambled miR-9 site were incubated for 1 hr before the addition of the solution. The Dual Luciferase Assay System (Promega) was used as per manufacturer's instruction. The firefly luciferase and renilla luciferase signals were measured using a multimode plate reader (EnSpire), with four measurements being recorded over 5 minutes. The relative fluorescent units were obtained by taking the ratio of firefly luciferase expression and renilla luciferase expression.

Flow Cytometry: Cells were stained with FITC or PE-CD80 16-10A, PeCy7-CD86-GL1, PerCP-Cy5.5-CD11c N418, APC-CD40 IC10 and APC-Cy7-MHC class II M5.114.15.2, e450 or PerCP-Cy5.5 CD4 RM4-5,APC-cy7 or e450 CD8 53.7, PE CD25 PC61.5, PerCP-Cy5.5 or Pe-Cy7 CD44 IM7 ,FITC CD69 H1 2f3 and FITC or APC IFNγ XMG1.2 (eBiosciences and BD Biosciences) and Alexafluor-647 H3K4me3 mAbcam1012 (Abcam).To determine transduction efficiency of DCs, cells were also stained with FITC or PE Thy1.1/CD90.1 OX-7 for detection of cells containing MIT or MET vectors. Samples were collected on BD Biosciences flow cytometer, FACS CantoII or Fortessa and analyzed using FlowJo.

Western Blot: Cells were lysed in CHAPS buffer (150mM KCl, 50mM HEPES, 0.1% CHAPS) supplemented with protease inhibitor cocktail (Fisher) and Sodium orthovanadate (Sigma). Lysates were sonicated for 10 minutes (30 seconds on, 30 seconds off) at 40% amplitude (qSONICA) and then cleared. Lysates were separated on SDS-PAGE gels and transferred to nitrocellulose membranes, blocked in 5% skim milk or BSA and probed with primary antibody Anti-PCGF6 (Abcam Ab192395) or anti-beta-Actin (Cell Signaling, 13E5) or anti-H3K4me3 (Abcam Ab8580) or anti-H3 (Abcam Ab1791), followed by horseradish peroxidase (HRP) conjugated anti-rabbit or anti-mouse antibodies before addition of ECL (Amersham).

Quantitative Reverse Transcription Polymerase Chain Reaction: RNA was extracted from DCs using Trizol (Life Tech). The High-Capacity cDNA Reverse transcription kit (Applied Biosystems) was used for cDNA synthesis for downstream detection of *Pcgf6, Sin3a, TGF-βr1, Arid1b, Hdac4,*

Pbrm1, TNFαip3, Pdlim2, Sirt1, Rcor1, Rest, Irak1 and *Hprt*. MiScript II RT Kit (Qiagen) was used for miRNA-specific cDNA synthesis for downstream analysis of mature miR-9 and snoRNU6 (U6) using the spec buffer and pri-miR-9-1, pri-miR-9-2, pri-miR-3, and *Hprt* using the flex buffer. qPCR reactions were performed using SensiFAST^{TM} SYBR Green Supermix (FroggaBio). Data was normalized to *Hprt* for *Pcgf6, Sin3a, TGF-βr1, Arid1b, Hdac4, Pbrm1, TNFαip3, Pdlim2, Sirt1, Rcor1, Rest, Irak1* pri-miR-1, pri-miR-2 and pri-miR-9-3. Data was normalized to *SnoRNU6* for miR-9. Relative fold change was calculated using the Livak-method. Primers listed in Table S1.

DC-T cell Co-Culture: 1 x10^4 steady-state or LPS activated (3 ng/ml) transduced DCs were pulsed with whole Ovalbumin (OVA) protein (1 mg/ml) per well in 96-well plates. 6 hours following stimulation, 1x10^5 sorted $CD4^+$ T cells or $CD8^+$ T cells from OTII or OTI mice, respectively were added (1:10 ratio). On day 3, T cells were examined for proliferation (pre-labeled with e450 Proliferation Dye) and expression of cell-surface activation markers using flow cytometry. Alternatively, T cells were stimulated with phorbol 12-myristate 13-acetate (PMA) (50 ng/ml) and ionomycin (500 ng/ml) for 4 hours, and then cytokine production was examined using flow cytometry.

B16-Melonama Tumour Model: On day 0, three groups of five C57BL/6 mice were injected subcutaneously with 1x10^6 B16-melanoma tumour cells which express the OVA antigen [690]. Three days later, 2x10^5 transduced DCs were pulsed with OVA and stimulated for 6 hours with 100 ng/ml of LPS. The three groups of mice were injected via tail vein with either PBS, control

DCs or DCs transduced with retroviral vectors. On day 7, tumours are palpable and tumour volume was measured every two days until tumour volume reached endpoint (2000 mm^3).

Alternatively, on day 1 post-inoculation with $1x10^6$ B16-melanoma tumour cells which express the OVA antigen, $5x10^3$ OT1 T cells were injected via tail vein into three groups of C57/BL6 mice. The next day, $2x10^5$ transduced DCs were pulsed with OVA and stimulated for 6 hours with 100 ng/ml of LPS. The three groups of mice were injected via tail vein with either PBS, control DCs or DCs transduced with retroviral vectors. On day 7, mice were sacrificed, and spleens were removed from all three groups of mice and digested in HBSS +/+ with phenol red (Wisent) supplemented with 1mg/ml of collagenase D (Roche) and 1ug/ml of DNase 1 (Roche). Splenic cells were then stained with APC H-2Kb bound to SIINFEKL Antibody (Biolegend) before being stained with PerCP 5.5-CD3 17A2, FITC-NK1.1 PK136, FITC-B220 RA3-6B2, PE CD25 PC61.5, Pe-Cy7 CD44 IM7 , e450 CD4 RM4-5, APC-cy7 CD8 53.7 and e506 Fixable viability dye and e450 CD11c N418 (eBioscience).

DC Subset Phenotyping and Sorting: Single-cell suspensions of spleens from C57BL/6 mice injected via tail vein with PBS or 1μg of LPS were produced by digestion in HBSS +/+ with phenol red (Wisent) supplemented with 1 mg/ml of collagenase D (Roche) and 1 ug/ml of DNase 1 (Roche). Spleen suspensions were enriched for DC populations with a Pan-DC enrichment kit (Miltenyi). cDC1s and cDC2s were then phenotyped or isolated via flow sorting with antibodies against FITC-CD3 17A2, FITC-NK1.1 PK136, FITC-B220 RA3-6B2, PE-CD80 16-10A, PeCy7-CD86-GL1, APC-CD172a P84, Brilliant Violet 650- XCR1 ZET, Brilliant Violet 711- CD64 X54-5/7.1,

PE/Dazzle 594- F4/80 BM8, APC-Cy7-MHC class II M5.114.15.2, e506 Fixable viability dye and e450 CD11c N418 (eBioscience). Gating strategy shown in **Figure S1**.

miR-9 Mimic Transfection

On day 8 of culture, BMDC media was replaced with a solution 40nM of miR-9 mimic (IDT) or 40nM control mimic (IDT) in optiMEM (Lifetech) containing Lipofectamine (Lifetech) for four hours. The media was then replaced with complete DC media and the BMDCs were collected for RNA extraction/flow cytometry. cDC1 and cDC2 subsets were sorted via flow cytometry and plated at $1x10^6$ cells/ml in 12 well non-tissue culture treated dishes. Cells were then transfected as above for four hours. The media was then replaced with complete DC media and one hour later the cDCs were collected for RNA extraction.

Antigen Sensitivity Assay

$1 x10^4$ steady-state miR-9 mimic or control transfected cDC1s or cDC2s were pulsed with Ovalbumin peptide (SIINFEKL) titrations for 6 hrs followed by addition of $25x10^3$ sorted $CD8^+$ T cells from OTI mice (1:5 ratio). On day 2, $\%CD25^+$ T cells was determined via flow cytometry and normalized to top dose of peptide (10 uM).

Quantification and Statistical Analysis: Statistics were performed on Prism software (GraphPad). Significance was determined using one-way ANOVA or Student T-test. Data was

presented as mean showing SEM or SD as indicated. Statistical significance is represented as *$p<0.05$.

2.7 Acknowledgements

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2.8 Supplemental Information

Supplemental information inventory

Key Resources Table

- A table containing detailed information about all reagents used in this study

Supplemental figures 1-5

-Further data to support the main text figures and results, the figures are titled as follows:

Figure S1, related to Figure 1

Figure S2, related to Figure 2

Figure S3, related to Figure 3

Figure S4, related to Figure 5

Figure S5, related to Figure 6

Key Resources Table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
FITC-CD80 16-10A	eBioscience	Cat# 11-0801-82; RRID: AB_465133
PE-CD80 16-10A	eBioscience	Cat# 12-0801-82 RRID: AB_465752
PeCy7-CD86-GL1	eBioscience	Cat# 25-0862-82 RRID: AB_2573372
PerCP-Cy5.5-CD11c N418	eBioscience	Cat# 45-0114-82 RRID: AB_925727
APC-CD40 IC10	eBioscience	Cat# 17-0401-82 RRID: AB_469386
APC-Cy7-MHC class II M5/114.15.2	eBioscience	Cat# 47-5321-82 RRID: AB_1548783
e450-CD4 RM4-5	eBioscience	Cat# 48-0042-82 RRID: AB_1272194
PerCP-Cy5.5 CD4 RM4-5	eBioscience	Cat# 45-0042-82 RRID: AB_1272194
APC-Cy7 CD8 53.7	eBioscience	Cat# 47-0081-82 RRID: AB_1272185
e450 CD8 53-6.7	eBioscience	Cat# 48-0081-82 RRID: AB_1272198
PE CD25 PC61.5	eBioscience	Cat# 12-0251-83 RRID: AB_465608
PerCP-Cy5.5 CD44 IM7	eBioscience	Cat# 45-0441-82 RRID: AB_925746
Pe-Cy7 CD44 IM7	eBioscience	Cat# 25-0441-82 RRID: AB_469623
FITC CD69 H1 2f3	eBioscience	Cat# 11-0691-82 RRID: AB_465119
FITC IFNγ XMG1.2	eBioscience	Cat# 11-7311-41 RRID: AB_10718840
APC IFNγ XMG1.2	eBioscience	Cat# 17-7311-82 RRID: AB_469504
Alexafluor-647 H3K4me3	Abcam	Cat# mAbcam1012 RRID: AB_442796

REAGENT or RESOURCE	SOURCE	IDENTIFIER
FITC Thy1.1/CD90.1 OX-7	BD	Cat# 554897 RRID: AB_395588
PE Thy1.1/CD90.1 OX-7	BD	Cat# 554898 RRID: AB_395589
e450 Proliferation Dye	eBioscience	Cat# 65-0842-85
APC H-2Kb bound to SIINFEKL	Biolegend	Cat# 141606 RRID: AB_11219595
PerCP 5.5-CD3 17A2	Biolegend	Cat# 100218 RRID: AB_1595492
FITC-NK1.1 PK136	eBioscience	Cat# 11-5941-85 RRID: AB_465319
FITC-B220 RA3-6B2	eBioscience	Cat# 11-0452-85 RRID: AB_465055
e506 Fixable viability dye	eBioscience	Cat# 65-0866-18
e450 CD11c N418	eBioscience	Cat# 48-0114-80 RRID: AB_1548665
FITC-CD3 17A2	eBioscience	Cat# 11-0032-82 RRID: AB_2572431
APC-CD172a P84	eBioscience	Cat# 17-1721-82 RRID: AB_10733158
Brilliant Violet 650- XCR1 ZET	Biolegend	Cat# 148220 RRID: AB_2566410
Brilliant Violet 711- CD64 X54-5/7.1	Biolegend	Cat# 139311 RRID: AB_2563846
PE/Dazzle 594- F4/80 BM8	Biolegend	Cat# 123146 RRID: AB_2564133
Anti-PCGF6	Abcam	Cat# Ab192395 RRID:N/A
anti-beta-Actin	Cell Signaling	Cat# 4970S RRID: AB_2223172
anti-H3K4me3	Abcam	Cat# Ab8580 RRID: AB_306649
anti-H3	Abcam	Cat# Ab1791 RRID: AB_302613
Chemicals, Peptides, and Recombinant Proteins		
Lipopolysaccharide, Escherichia coli, serotype 0111:B4	Sigma-Aldrich	L4391-1MG
Heat-killed Staphylococcus aureus	Invivogen	tlrl-hksa

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Heat-killed Escherichia coli serotype 0111:B4	Invivogen	tlrl-hkeb2
Zymosan	Invivogen	tlrl-zyn
Bay117082 NF-κB inhibitor	SelleckChem	S2913
PD0325901 MEK inhibitor	SelleckChem	S1036
SB203580 p38 MAPK inhibitor	SelleckChem	S1076
R406 Syk inhibitor	SelleckChem	S1533
Whole Ovalbumin	Worthington	LS003056
Ovalbumin peptide (SIINFEKL)	Biosynth	FO73537
phorbol 12-myristate 13-acetate (PMA)	Calbiochem	524400
Ionomycin	Sigma-Aldrich	10634
Experimental Models: Cell Lines		
293T cells	ATCC	From R. Jones
B16-OVA melanoma tumour cells	As previously described (Aitken et al., 2018)	
Experimental Models: Organisms/Strains		
Mouse: C57BL/6N	Charles Rivers Laboratories	027
Oligonucleotides		
5’ miR-9 mimic: rUrCrUrUrUrGrGrUrUrArUrCrUrArGrCrUrGrUrArUrGrA	IDT	N/A
3’ miR-9 mimic: rArUrArArArGrCrUrArGrArUrArArCrCrGrArArArGrU	IDT	N/A
5’ control mimic: rArGrUrArUrGrUrCrGrArUrCrUrArUrUrGrGrUrUrUrCrU	IDT	N/A
3’ control mimic: rUrGrArArArGrCrCrArArUrArGrArUrCrGrArArArUrA	IDT	N/A
Pcgf6_F: Ggagaagcaactatcgggca	IDT	N/A
Pcgf6_R: Ccagtaagtgatccccacaga	IDT	N/A
Hprt_F: Ctccgccggcttcctcctca	IDT	N/A
Hprt_R: Acctggttcatcatcfctaatc	IDT	N/A
Pri-miR-9-1_F: ggcggggttggttgttatct	IDT	N/A
Pri-miR-9-1_R: Aacctttgaaggcgacgagt	IDT	N/A

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Pri-miR-9-2_F: ccttgtgagggaagcgagtt	IDT	N/A
Pri-miR-9-2_R: Cgttcctcggtgaccttgaa	IDT	N/A
Pri-miR-9-3_F: aggtcggatacctggtccc	IDT	N/A
Pri-miR-9-3_R: Ggcccgtttctctctttggt	IDT	N/A
Primers for miR-9, SnoRNU6, Sin3a, Tgfbr1, Arid1b, Hdac4, Pbrm1, Tnfaip3, Pdlim2, Sirt1, Rcor1, Rest, Irak1, see Table S1	IDT	N/A
Recombinant DNA		
MSCV-Ef1a-Thy1.1 (MeT) retroviral vector	This paper	N/A
miR-9 sponge vector	Addgene plasmid -Bob Weinberg	# 25040
PmirGlo luciferase-expressing vector	Promega	E1330
Software and Algorithms		
GraphPad Prism8	Graphpad	N/A
FlowJo_V10	FlowJo	N/A

2.8.1 Supplemental Figures

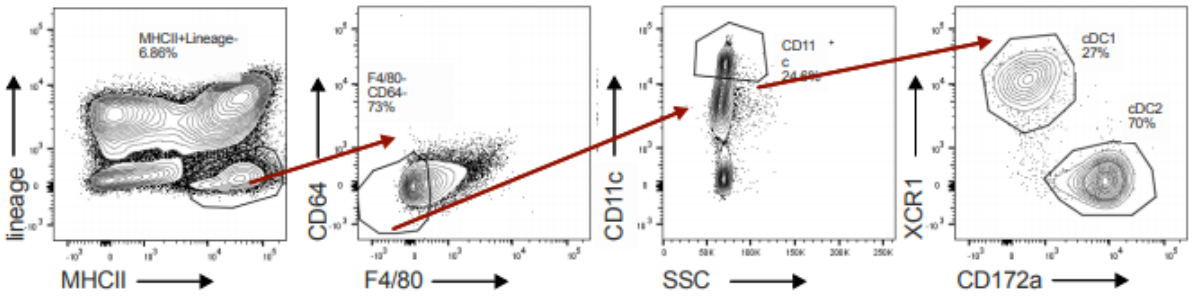

Figure S1: Related to Figure 1. Gating strategy used for sorting cDC1s and cDC2s from mouse spleens. Cells were first gated using FSC-H vs SSC-H, followed by single cells using FSC-A vs FSC-H. Next the lineage negative, MHCII+ cells were gated (see methods for antibody list), followed by CD64- F480- cells and then gated on CD11c hi cells. Finally, XCR1 vs CD172a were used to identify cDC1s and cDC2s as indicated.

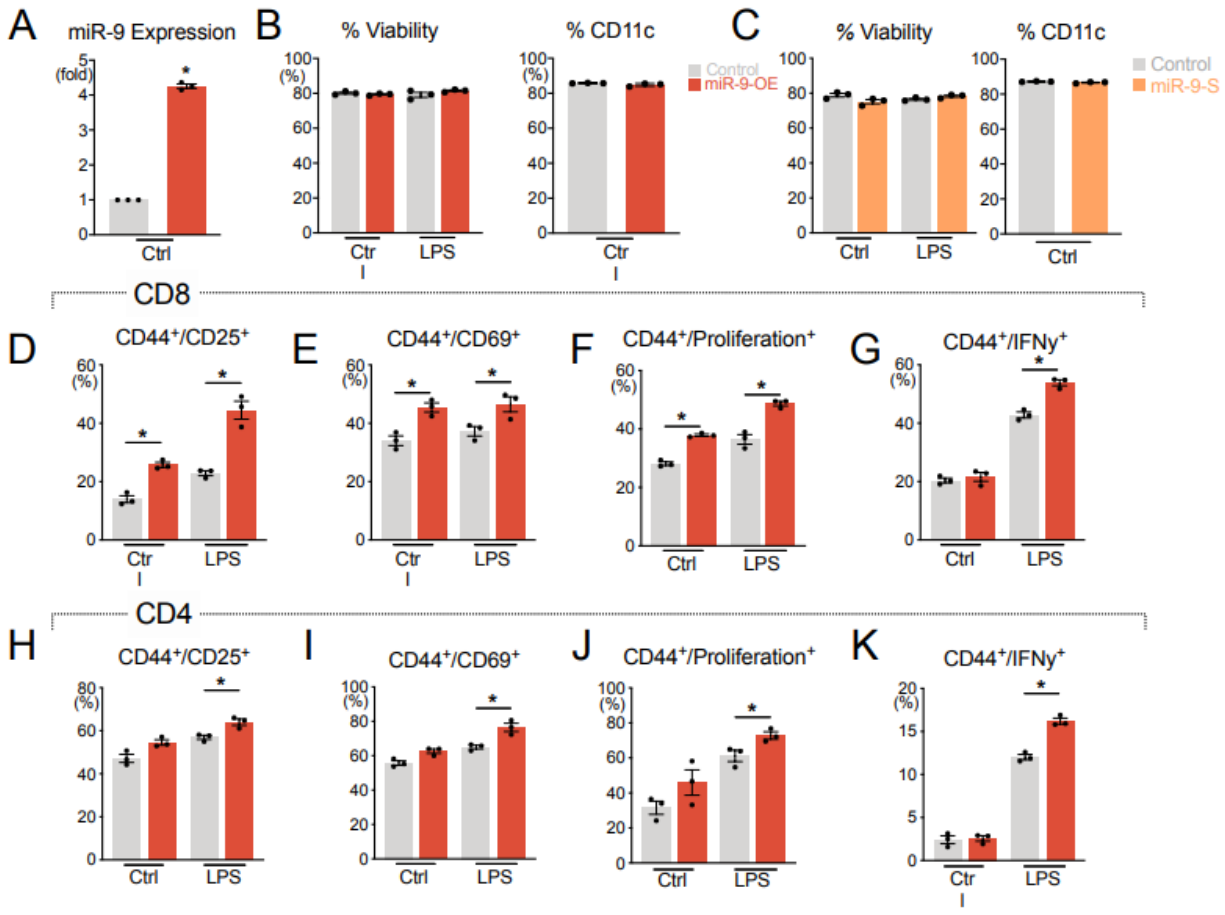

Figure S2: Related to Figure 2 A) Gene expression of mature miR-9 in BMDCs transduced with control vector (ctrl) or miR-9 overexpression vector (miR-9) via qRT-PCR. Data points shown were normalized to SnoU6 loading control and represents the mean of three independent experiments(n=3). B-C) Percentage of viable cells measured through 7-AAD staining and %CD11c+ positive cells in BMDCs transduced with control or (B) miR-9 or (C) miR- 9-S with or without 100 ng/ml of LPS stimulation for 18hours measured by flow cytometry. Data represents the mean percentage of cells that were negative for 7-AAD staining or positive for CD11c staining. Data points represent mean gMFI from individual independent experiments (n=3). D-K) BMDCs transduced with miR-9 were pulsed with whole OVA protein with or without LPS stimulation for 6 hours as in Figure 2. Following stimulation BMDCs were co-cultured with CD8+ OTI or CD4+ OTII T cells labelled with proliferation dye. Three days after co-culture, surface expression of CD44 and CD25 (D,H) or CD69 (E,I) or proliferation with e450 dye (F,J) or IFNγ were measured (G,K). Data points represent the mean % positive population (D-F, H-J) or gMFI (G,K) of individual independent experiments (n=3) and error bars represent SEM. Significance was determined through one-way ANOVA *p<0.05

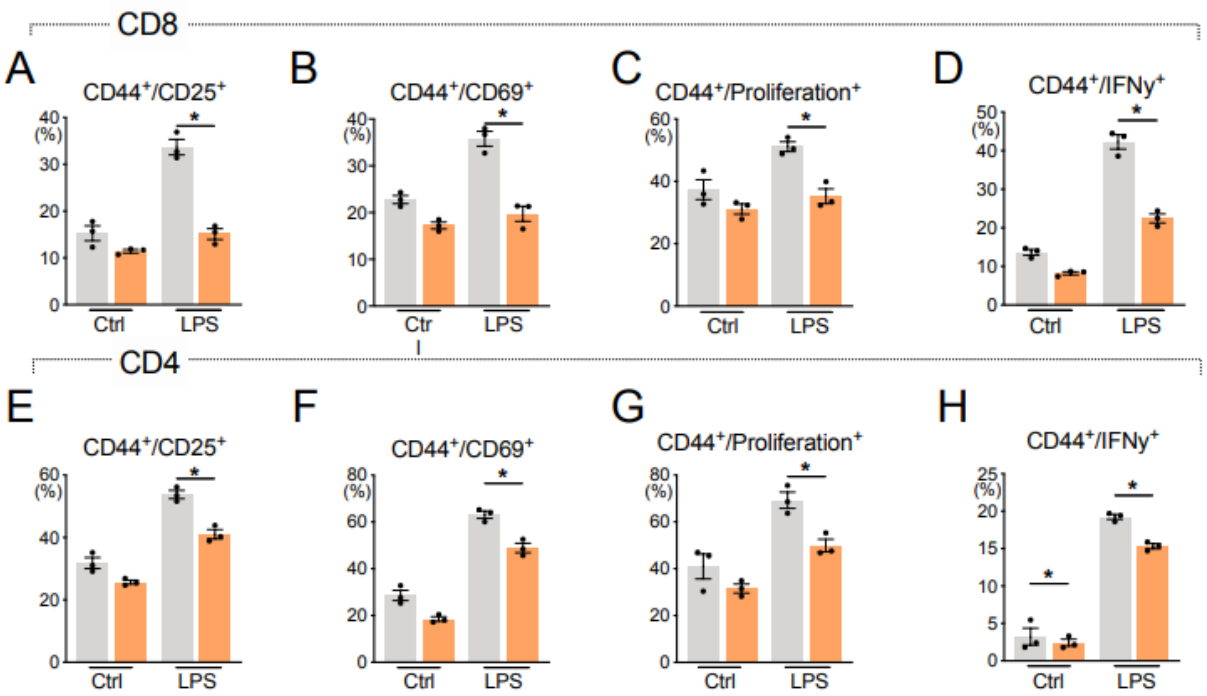

Figure S3: Related to Figure 3 Experiments were performed as in Figure S2 only BMDCs were transduced with either control vector or one expressing miR-9-S. Data points represent the mean % positive population (A-C,E-G) or gMFI (D,H) of individual independent experiments (n=3) and error bars represent SEM. Significance was determined through one-way ANOVA *p<0.05

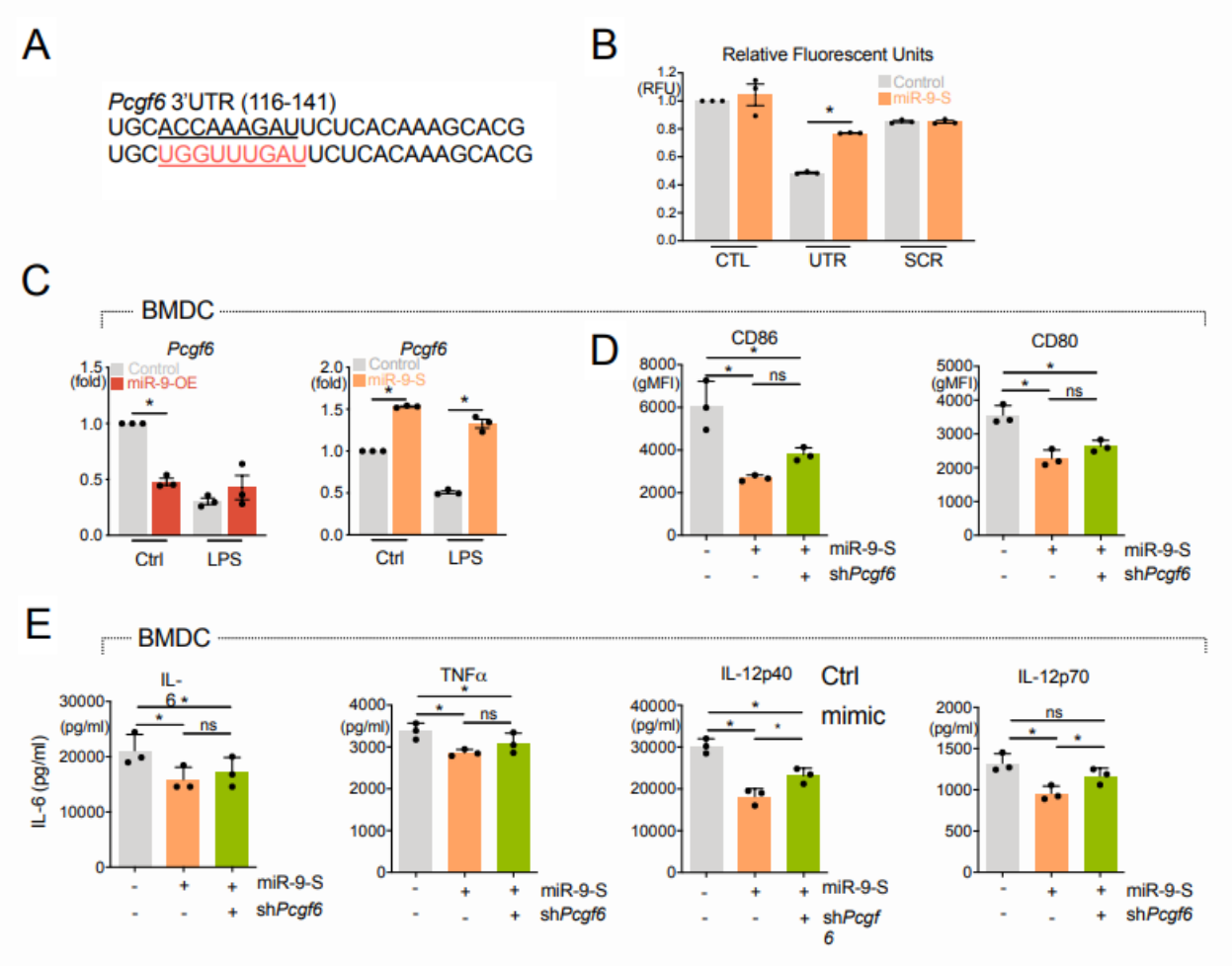

Figure S4: Related to Figure 5 A) Sequence of Pcgf6 3'UTR containing the putative miR-9 binding site (underlined). The sequence of the mutated miR-9 binding site is shown in red. B) Mouse embryonic fibroblasts were first transfected with miR-9-S or control vector and then transfected with either empty PmiRGlo luciferase expression vector or constructs containing the 3'UTR of Pcgf6 (UTR) intact or with a mutation in the miR-9 binding site (SCR). Data shown is the mean for the luciferase/renilla ratio for one of three independent experiments and error bars represent SD. C) Pcgf6 expression of BMDCs transduced with miR-9 or miR-9-S with or without 100 ng/ml LPS stimulation. qRT-PCR data was normalized to Hprt. Data points represent the mean fold expression of individual independent experiments (n=3) and error bars represent SEM. D-E) Cell surface marker expression and cytokine production (E) by BMDCs transduced with control vector or miR-9-S or double-transduced with miR-9-S and shPcgf6 examined at resting or after 18 hours of LPS stimulation (100 ng/ml). Data points represent mean gMFI of individual independent experiments (n=3) and error bars represent SEM. Significance was determined through one-way ANOVA *p<0.05

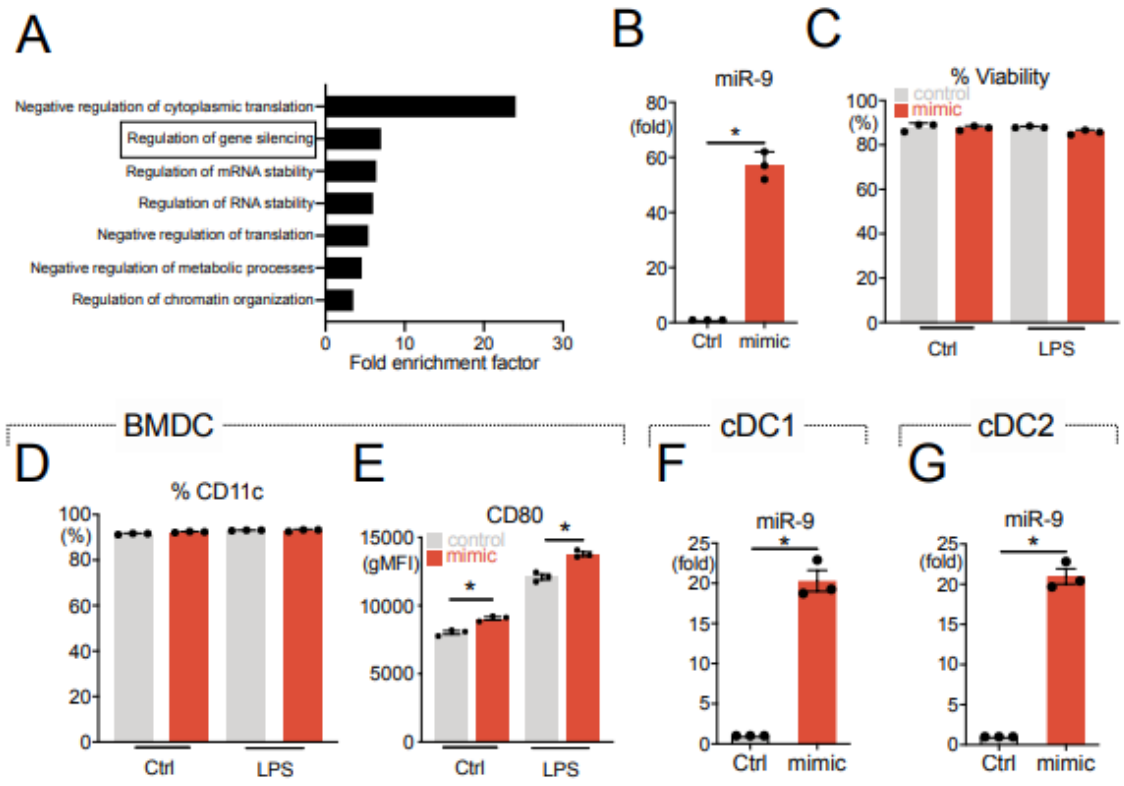

Figure S5: Related to Figure 6 A) PANTHER pathway analysis of putative targets of miR-9 curated from Targetscan. B-G) BMDCs were transfected with miR-9 mimic or control mimic and B) gene expression of mature miR-9 was measured via qRT-PCR, C) percentage of viable cells measured through 7-AAD staining and D) percentage of CD11c+ positive cells were assessed. E) CD80 expression by BMDCs transfected with miR-9 mimic or control mimic examined at steady state or after 18 hours of LPS (100 ng/ml). F-G) Gene expression of mature miR-9 in F) cDC1s and G) cDC2s transfected with miR-9 mimic or control mimic, via qRT-PCR. All qRT-PCR data was normalized to SnoRNU6. Data points represent the mean fold expression (B,F-G) or the mean % positive population (C-D) or gMFI (E) of biological replicates (n=3) and error bars represent SEM. Significance was determined either through one-way ANOVA (C-E) or student t- test (B,F and G) *p<0.05

Preface to Chapter 3

In Chapter 2, we examined a novel mechanism of regulation of DC activation by miR-9. In this chapter, we found evidence of a previously unknown role of the cystic fibrosis transmembrane conductance regulator (CFTR) as a negative regulator of DC activation and function. As discussed in Chapter 1, loss of CFTR function causes the incurable genetic disease Cystic fibrosis (CF). The lungs and gastrointestinal tracts of CF Patients display an increase in inflammation and also show an increased risk for multiple autoimmune and inflammatory conditions. Intestinal conventional DCs (cDCs) are potent regulators of intestinal tolerance and dysregulated intestinal cDC function has shown to promote intestinal inflammation in both humans and mice. Since DCs express CFTR, we predicted that loss of CFTR function may be dysregulating DC activation and promoting the steady state inflammation seen in CF patients.

Chapter 3 : Loss of CFTR function promotes susceptibility to *Citrobacter rodentium* infection and immune dysregulation in the gastrointestinal tract

Loss of CFTR function promotes susceptibility to *Citrobacter rodentium* infection and immune dysregulation in the gastrointestinal tract

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3.1 Short Summary

Cystic fibrosis is an incurable genetic disease caused by loss of function of the cystic fibrosis transmembrane conductance regulator (CFTR) protein. CFTR loss leads to the creation of an environment suitable for colonization by various pathogenic bacteria in both the lungs and the intestines. Patients display an increase in inflammatory signalling that is cell intrinsic and independent of bacterial colonization while also showing an increased risk for multiple autoimmune and inflammatory conditions. We found that CFTR mutant and knockout mice are significantly more susceptible to *Citrobacter rodentium* infection and that this susceptibility is independent of CFTR loss in intestinal epithelial cells. The gastrointestinal tract of CFTR mutant mice is significantly more inflamed than controls with increased frequency of myeloid cells and $Th17^{+}$ $CD4^{+}$ T cells. We also observed that activation of intestinal dendritic cells (DCs) was potentiated by loss of CFTR function. DCs express CFTR and potently mediate the balance of tolerance and immune activation in the intestinal tract. Mice with CFTR knocked out specifically in CD11c-expressing cells showed a defect in early immune responses towards *C. rodentium*. CD11c-specific loss of CFTR also increased the activation of intestinal DCs and promoted the activation of $Th17^{+}$ $CD4^{+}$ T cells. Finally, we found that loss of CFTR inhibits anti-inflammatory PI3K-Akt signaling in DCs. Together, these results demonstrate that CFTR regulates the activation of DCs in the gastrointestinal tract and loss of CFTR promotes immune responses driven potentially by dysregulated DCs.

3.2 Introduction

Cystic fibrosis (CF) is an incurable autosomal recessive genetic disease and is the most common inherited disorder among people of European heritage, affecting approximately 1 in 3000 Caucasians [573, 574]. CF is caused by a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which encodes a phosphorylation-regulated chloride/bicarbonate channel [573, 574]. CFTR expressed on exocrine epithelial cells regulates salt and water secretion across various epithelial surfaces, particularly in the respiratory and gastrointestinal tracts. Partial or total loss of CFTR results in classical CF pathology including increased chloride concentration in sweat, male sterility, and the accumulation of dehydrated, viscous mucus in the lungs and gastrointestinal tract [573, 574]. Over 1900 different mutations of *Cftr* have been described in human patients, comprising five different classes dictated by molecular mechanism of CFTR disruption [577]. The most clinically relevant mutation in human CF is a mutation resulting from deletion of a phenylalanine residue at position 508 (ΔF508), which leads to protein misfolding and degradation via the proteasome. The ΔF508 mutation contributes to more then 70% of CF in humans [573].

CF is typically associated with lung pathology, as the accumulation of viscous mucus not only obstructs the airways but also impairs mucoscilliary defense, which promotes colonization of the lung with bacteria, including *Staphylococcus aureus* and *Pseudomonas aeruginosa* [573, 574]. CF also causes considerable gastrointestinal pathology, as CFTR-mediated anion secretion contributes significantly to bile formation, pancreatic function, and salt and water secretion across the intestinal epithelium [578, 579]. Much as in lung pathology, accumulation of viscous mucus in the intestinal tract causes obstruction in the distal small intestine and inhibits

absorption of nutrients, contributing to malabsorption and growth retardation in CF patients [575, 580]. The abnormally viscous mucus also creates a niche for small intestinal bacterial overgrowth (SIBO) [575, 580]. The microbiota of CF patients is heavily disrupted, with an overall decrease in diversity and marked increase in the abundance of pro-inflammatory bacterial species such as *Escherichia coli* and *Enterobacter* [581]. *E. coli* is responsible for persistent infections in CF patients and contributes to a large burden of intestinal and genitourinary disease in humans [691].

Multiple mouse models have been created to study the pathogenesis of CF disease. CFTR knockout (CFTR KO) mice and mice which have had the ΔF508 mutation genetically introduced (ΔF508 mice) both develop CF disease that is similar to what is observed in CF patients [575]. The intestines of CF mice recapitulate many features associated with human CF pathology including viscous, dehydrated mucus and bacterial overgrowth [575, 586]. CF mice must also be weaned on to water that is supplemented with laxatives to prevent fatal intestinal obstruction. Due to these similarities, CF mice are relevant models for studying CF pathogenesis in the intestinal tract.

The intestines of both CF patients and mice are characterized by a substantial increase in inflammatory gene expression, which is thought to be due to SIBO [587-589]. However, the intestines of neonatal CF patients, who do not yet have SIBO, also show marked increases in inflammatory gene signatures [591]. Antibiotic treatment also does not lead to reduction in the inflammatory gene signatures observed in the intestines of CF patients [586, 590]. Germ-free CFTR KO mice still exhibit intestinal pathology and inflammation, with increased T cells and Th17 cells present in the mesenteric lymph nodes [692]. Epidemiological studies on CF patients

also show an increase in gut-related immune pathologies such as Crohn's disease, ulcerative colitis, and celiac disease [577, 593, 594]. This suggests dysregulation of the immune system in CF patients, which is promoting an increase in immune activation and immune pathology.

CFTR is functionally expressed in immune cells, including myeloid and lymphoid cells, and has been shown to regulate immune function [576, 587]. In innate immune cells, loss of CFTR has been shown to promote hyperresponsiveness to pro-inflammatory stimuli, which leads to exaggerated production of IL-8, TNF-α, and proteases [597, 598]. Loss of CFTR causes dysregulation of many innate immune signalling pathways, including PRR signalling and immune activation [576, 599, 600]. Together, these findings suggest dysregulated mucosal immunity contributes to the inflammation observed in CF patients and mice.

Dendritic cells (DCs) are innate immune cells that are critical for regulating immune homeostasis, especially in the intestinal tract. DCs express CFTR but its role in regulating DC function is controversial [607, 610]. One group found that DCs isolated from the lungs of CFTR KO mice expressed reduced amounts of co-stimulatory markers and secreted less pro-inflammatory cytokines than control mice [607]. It has also been shown that upon infection with *P. aeruginosa*, DCs from CFTR KO mice expressed more co-stimulatory markers CD86 and CD80, and secreted more pro-inflammatory IL-6 and IL-12 compared to controls [610]. DCs have yet to be studied in the gastrointestinal tract of CF patients or in CFTR KO mice.

In order to study defects in immunity in the gastrointestinal tract, we infected CFTR KO mice with *Citrobacter rodentium (C. rodentium)*, a natural murine enteric pathogen which is used as a model for human enterohaemorrhagic *E. coli* (EHEC) and enteropathogenic *E. coli*

(EPEC) infection [693, 694]. *C. rodentium* induces attaching and effacing (A/E) lesions during colonization, in a mechanism similar to EHEC and EPEC [693]. A/E lesions are characterized by close adhesion of the bacteria to host colonocytes, and localized loss of microvilli [693]. *C. rodentium* is consequently used to study host-microbial interaction in *in vivo* models of infectious colitis by A/E pathogens [694] . Furthermore, Th17 responses that are induced by *C. rodentium* infection have been shown to be altered in the intestines of CF mouse models and in CFTR patients [695-697].

We found that CFTR mutant and knockout mice are significantly more susceptible to *C. rodentium* infection and that this susceptibility is not due to loss of CFTR function in intestinal epithelial cells. The colons and small intestines of CFTR mutant mice are significantly more inflamed than littermate controls both at steady state and after infection, with increased infiltration of myeloid cells, increased frequency of $Th17^{+}$ $CD4^{+}$ T cells, and increased activation of intestinal DCs. Loss of CFTR specifically in LysM-expressing cells does not lead to susceptibility to *C. rodentium* infection. However, mice deficient in CFTR in CD11c-expressing cells showed a defect in early immune responses towards *C. rodentium*. CD11c-specific loss of CFTR increased the activation of intestinal DCs and promoted the activation of $Th17^{+}$ $CD4^{+}$ T cells. Finally, we found that CFTR loss inhibits phosphorylation of Akt and S6 in bone-marrow derived DCs (BMDCs). Together, these results demonstrate that CFTR regulates the activation of DCs in the gastrointestinal tract and loss of CFTR promotes dysregulated immune responses driven potentially by dysregulated DCs.

3.3 Results

CFTR mutant and knockout mice are significantly more susceptible to *C. rodentium* infection than WT mice

To evaluate potential defects in mucosal immunity caused by loss of CFTR function, we characterized the overall response of FVB wildtype (WT), FVB ΔF508, and BALB/C CFTR KO mice to infection with *C. rodentium* via oral gavage. CFTR KO, ΔF508, and WT littermates were weaned on water containing a laxative as intestinal obstructions are common in mice with CFTR defects. WT mice infected with *C. rodentium* displayed few signs of overt disease and survived the infection. However, ΔF508 mice began to succumb to infection within 5 days and reached 85% mortality by 12 days post-infection (DPI) (**Figure 1A**). Similarly, CFTR KO mice were significantly more susceptible to *C. rodentium* infection than WT controls, reaching 70% mortality by 12 DPI (**Figure 1B**). Assessment of bacterial loads showed a significant increase in bacterial burden in the small intestine, colon, and stool of ΔF508 mice at 4 DPI (**Figure 1C-E**). Systemic dissemination of *C. rodentium* was also significantly higher in the ΔF508 mice than their WT littermate controls, as measured through increased CFU in the spleens (**Figure 1F**)**.** Of note, the increased bacterial burden in the small intestine is indicative of altered localization of *C. rodentium* infection in ΔF508 mice, as the infection is typically limited to the cecum and colon in WT mice [538].

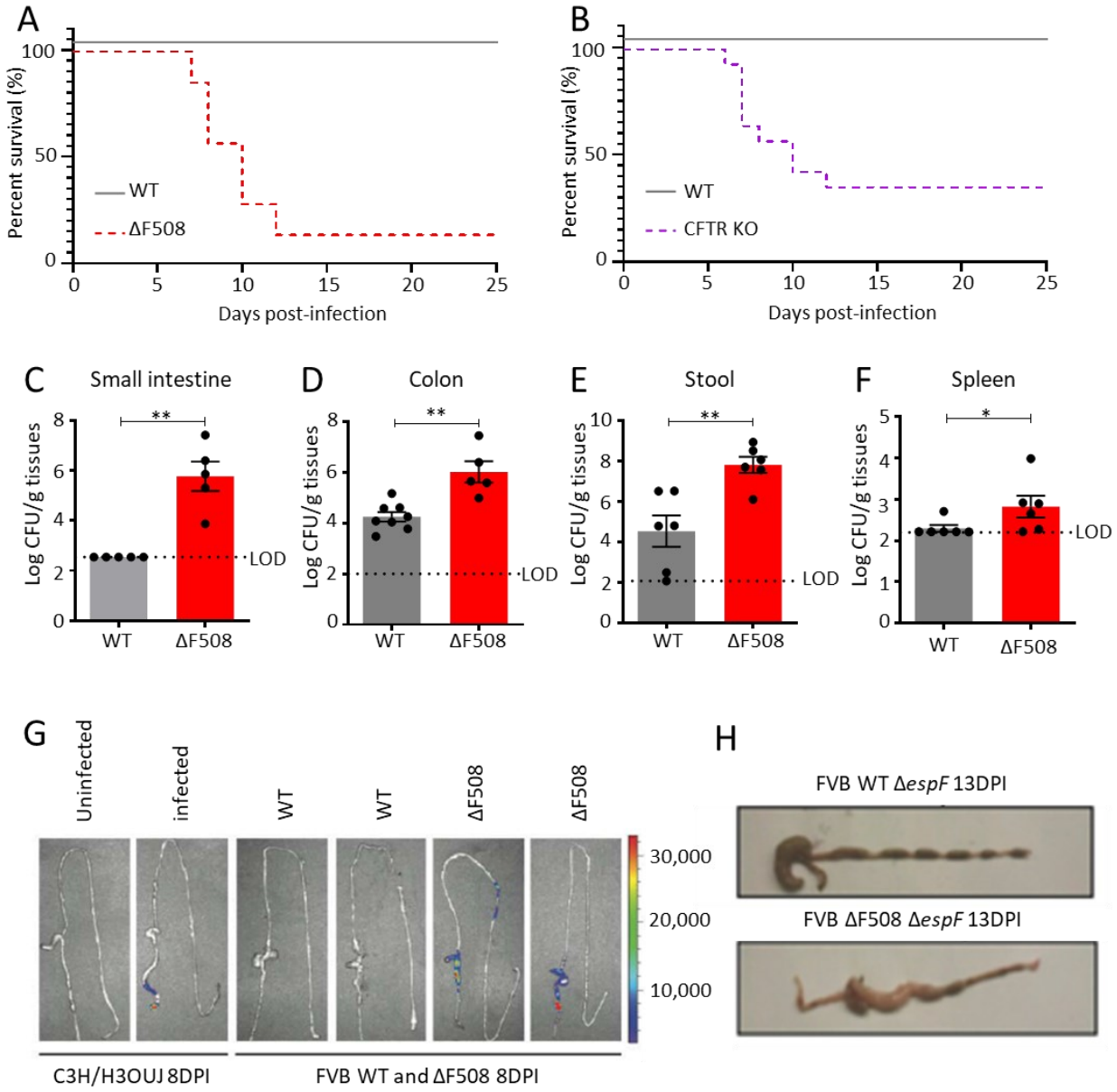

Figure 1: CFTR mutant and knockout mice are significantly more susceptible to *C. rodentium* infection
(A-B) Percent survival of (A) ΔF508 (n=7) and (B) CFTR KO (n=14) mice and associated controls following oral infection with *C. rodentium.* (C-F) Bacterial burden in (C) small intestine, (D) colon, (E) Stool and (F) Spleen of WT (n=5) and ΔF508 (n=5) mice at 4DPI with *C. rodentium*. Data points represent individual independent experiments and error bars represent SEM. Dotted lines represent the limit of detection. Significance was determined by two tailed unpaired Student's t-test. *p < 0.05 **p< 0.01 (G) In-vivo imaging of WT, ΔF508 mice and C3H/HeOuj susceptible mice 8 DPI with a luciferase expressing strain of *C. rodentium*. (H) Microscopic imaging of the large intestine of WT and ΔF508 mice at 13 DPI with Δ*espF* C. *rodentium*.
See also Figure S1

To further characterize the altered localization of infection in ΔF508 mice, we infected WT and ΔF508 with luminescent *C. rodentium* [698] and visualized the gastrointestinal tract 8 DPI via *in vivo* imaging. We also infected C3H/HeOuJ mice, which are known to be susceptible to *C. rodentium* infection and have well characterized responses to infection [699]. Luminescent signal, corresponding to *C. rodentium,* was localized to the distal colon in infected C3H/HeOuJ mice (**Figure 1G**). In contrast, *C. rodentium* was localized to the jejunum, cecum and proximal colon in ΔF508 mice, and was below the limit of detection in infected WT littermate mice (**Figure 1G**). To further characterize the altered localization of *C. rodentium* in the ΔF508 mice, we performed immunofluorescent staining in WT and ΔF508 proximal colon tissues. Staining with *C. rodentium* lipopolysaccharide (LPS)-specific antibodies demonstrated that *C. rodentium* directly interacts with the epithelial surface of the proximal colon of ΔF508 mice despite the altered localization (**Figure S1A**). In contrast, *C. rodentium* was below the limit of detection in WT mice, consistent with the lower CFU counts (**Figure S1A**).

Loss of CFTR drastically changes the lumenal environment in the intestines and may expose *C. rodentium* to different metabolites that could enhance its growth. Therefore, we assessed *ex vivo* growth of *C. rodentium* in diluted mucus and lumenal contents from the jejunum, cecum, and proximal colon of WT and ΔF508 mice and found no significant differences (**Figure S1B-D**). These results indicate that differences in the lumenal environment of ΔF508 mice are unlikely to be causing the observed changes in *C. rodentium* localization and growth.

To characterize the tissue response to *C. rodentium* infection in ΔF508 mice as compared to WT mice, we performed Hematoxylin and Eosin (H&E) staining at 8 DPI in the jejunum, cecum, and proximal colon. H&E staining showed markedly higher tissue hyperplasia

and immune cell infiltration in the jejunum, cecum, and colon of ΔF508 mice compared to WT mice (**Figure S1E-G**). Tissue sections from the proximal colon and cecum of infected ΔF508 mice analyzed showed significantly higher depletion of goblet cells, surface epithelial injury, edema, and immune cell infiltration (**Figure S1E-G**). To evaluate the tissue response at later timepoints, we infected WT and ΔF508 mice with an attenuated strain of *C. rodentium* that lacked the critical effector molecule *EspF* (Δ*EspF*). WT mice infected with Δ*EspF C. rodentium* cleared the infection quickly and did not display signs of disease (data not shown). Conversely, ΔF508 mice were heavily colonized by *ΔEspF C. rodentium* and began to succumb from infection at 13 DPI (**Figure 1H**). ΔF508 mice showed significant tissue hyperplasia, especially in the proximal colon, compared to WT mice (**Figure 1H**). These results shows that loss of CFTR function significantly increases susceptibility to *C. rodentium* infection and elicits greater infection- induced tissue responses.

Increased inflammatory cytokine profile and myeloid cell infiltration in the colon and small intestine of ΔF508 mice after infection with *C. rodentium* and at steady state

Our results show that the intestines of *C. rodentium* infected ΔF508 mice showed increased hyperplasia and immune infiltration compared to WT mice, suggesting an exaggerated immune response against *C. rodentium* was present in these mice. To examine the inflammatory phenotype in the intestine of ΔF508 mice, we first performed qRT-PCR to measure pro-inflammatory gene expression in total colon lysates from infected ΔF508 and WT mice. We found significantly elevated mRNA levels of the pro-inflammatory cytokines *Tnfα*, interferon-γ (*Ifnγ*), interleukin 1β (*IL1β*), the neutrophil-attracting chemokine *Cxcl1*, and

members of the bactericidal activity conferring Reg3 family in total colon lysates of ΔF508 mice when compared to WT animals at 4 DPI (**Fig. 2A**).

To determine whether increased inflammatory cytokine secretion affected immune cell infiltration, we analyzed the recruitment of neutrophils and monocytes in the colon and small intestine of ΔF508 and WT mice by flow cytometry (**Figure S2A**). *C. rodentium* infection led to increased neutrophil frequency and total numbers in both the small and large intestines of ΔF508 mice when compared to WT animals at 4 DPI (**Fig. 2B-C**). We also observed an increase in the frequency and total number of $CD11b^{+}$ $Ly6C^{+}$ monocytes in the large but not small intestine of ΔF508 mice when compared to WT animals at 4 DPI (**Figure S2B-S2C**).

CF in human patients is associated with a general pro-inflammatory phenotype, which includes heightened production of cytokines such as CXCL1 and tumor necrosis factor alpha (TNFα) in the serum and increased inflammatory myeloid cell infiltrates in the lungs [573, 574]. In order to determine whether the intestines of ΔF508 mice were more inflamed in the absence of infection, we quantified pro-inflammatory gene expression in total colon lysates from both ΔF508 and WT mice at steady state. We observed elevated *Tnfα, Reg3β,* and *Reg3γ* in total colon lysates of ΔF508 mice compared to WT mice, at steady state (**Figure 2E**). There were no differences in the frequency or total number of neutrophils in the colons of WT and ΔF508 mice at steady state, but we did observe a significant increase in the number of monocytes (**Figure 2F and S2D**). In the steady state small intestine, there was a significant increase in the frequency and total numbers of neutrophils but not monocytes in ΔF508 mice compared to WT (**Figure 2G and S2D**). These results indicate that even in steady state conditions, the gastrointestinal tract of CF mutant mice is more inflamed than that of WT mice.

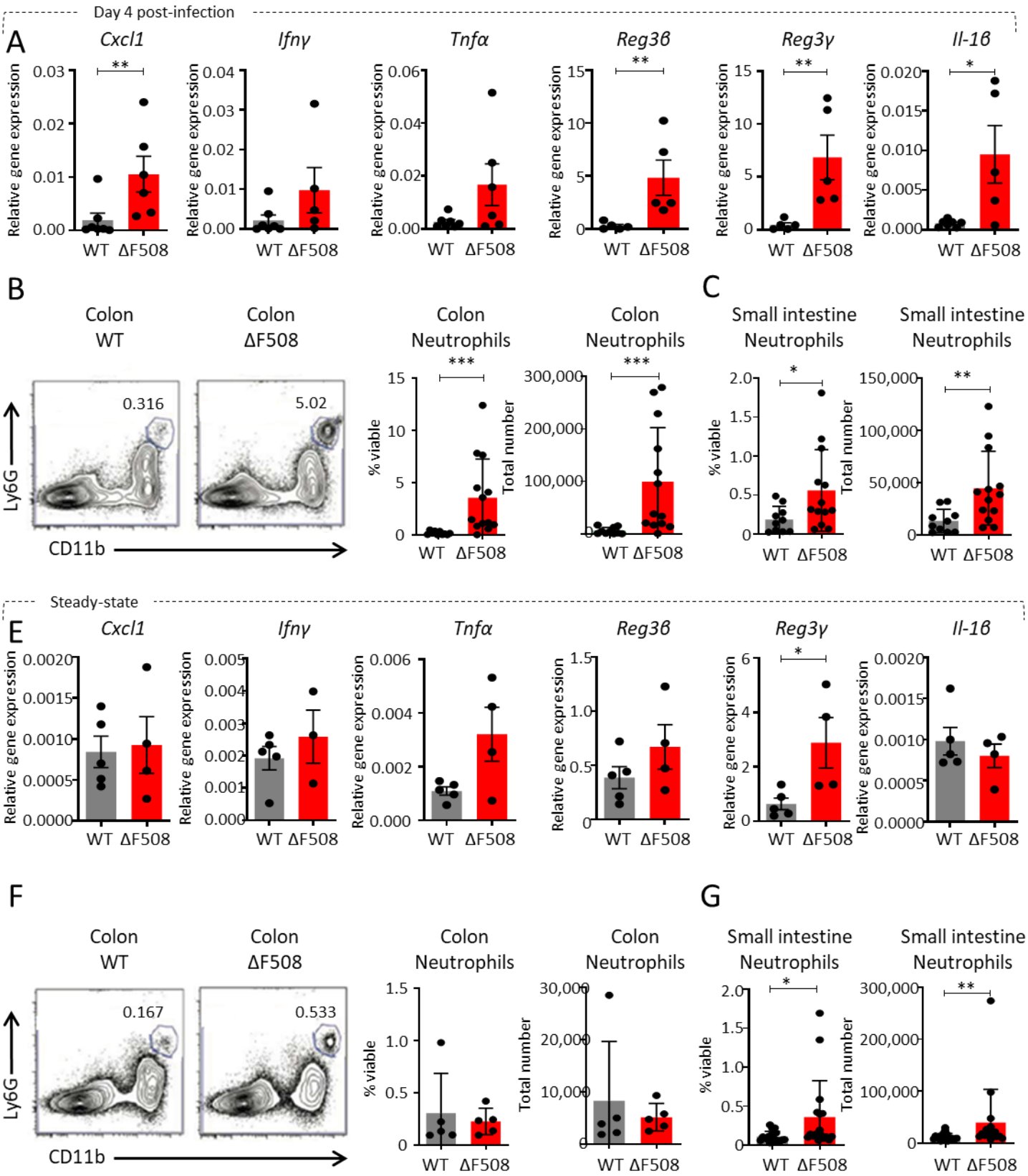
Day 4 post-infection
A
Cxcl1
Ifnγ
Tnfα
Reg3β
Reg3γ
Il-1β
Relative gene expression
WT ΔF508
B
Colon WT
Colon ΔF508
0.316
5.02
Ly6G
CD11b
Colon Neutrophils
% viable
Total number
C
Small intestine Neutrophils
Steady-state
E
F
0.167
0.533
G

Figure 2: CFTR deficiency promotes inflammation in the gastrointestinal tract after infection with *C. rodentium* and at steady-state (A) Gene expression levels of *cxcl1*, *ifnγ*, *tnfα Reg3β*, *Reg3γ* and *Il-1β* in the proximal colon of WT (n=7) and ΔF508 (n=5) mice at 4DPI with *C. rodentium*. Data represents mean gene expression relative to GAPDH of individual independent experiments and error bars represent SEM. Significance was determined through two-tailed unpaired Student t-test. *p<0.05 **p<0.01 (B) Representative contour plot, frequencies and total numbers of viable $CD11b^+$ $Ly6G^+$ neutrophils in the colon lamina propria of WT and ΔF508 mice at 4 DPI with *C. rodentium* (C) Frequency and total number of $CD11b^+Ly6G^+$ neutrophils in the small intestine of WT (n=10) and ΔF508 (n=13) mice. Data points represent individual independent experiments and error bars represent SEM. (E-G) Experiments were performed as in (A-C) but on tissues from WT (n=5) and ΔF508 (n=4) mice at steady-state. Significance was determined through two-tailed unpaired Mann-Whitney test. *p<0.05 **p<0.01
See also Figure S2

Susceptibility to *C. rodentium* infection in CF mice is independent of loss of CFTR in intestinal epithelial cells and myeloid cells.

Since multiple cell types express CFTR, dissecting the exact cause of the increased susceptibility to *C. rodentium* infection is difficult in full body CFTR KO and ΔF508 mice. Conditional *Cftr* knockout mice allow for cell type specific deletion of *Cftr* and would allow us to determine which cell-types are responsible for susceptibility to *C. rodentium*. We first assessed whether Meox2-driven Cre *Cftr*-floxed (MeoxCre) C57BL/6 mice, which express Cre in all cells developed from embryonic stem cells, would recapitulate our findings in CFTR KO and ΔF508 mice upon *C. rodentium* infection. In agreement with our previous findings, MeoxCre mice infected with *C. rodentium* are significantly more susceptible to infection (**Figure 3A left panel**). Assessment of bacterial burden also shown a significant increase in bacterial CFUs in MeoxCre mice at 3 DPI and 6 DPI compared to controls (**Figure 3B right panel**).

CFTR KO, ΔF508, and MeoxCre mice develop intestinal pathology that is similar to what is observed in human CF patients, including accumulation of viscous mucus and intestinal obstruction, mediated by loss of CFTR function in intestinal epithelial cells [575, 586]. To address whether loss of CFTR function specifically in intestinal epithelial cells was responsible

for the increased susceptibility to *C. rodentium* infection in CF mice, we characterized *Cftr*-floxed mice that express Villin-driven Cre (VillinCre).We found that VillinCre mice were not susceptible to infection with *C. rodentium* and presented with similar fecal bacterial loads as litter mate controls at 3 DPI and 6 DPI (**Figure 3B)**. To validate CFTR deletion in intestinal epithelial cells in these mice, we measured CFTR function by stimulating tissues with forskolin, which specifically induces CFTR-mediated chloride ion secretion and induces a short-circuit current response which can be quantified. The ileum, cecum, and proximal and distal colons of VillinCre mice produced significantly reduced short-circuit current responses compared to control mouse tissues when stimulated with forskolin, indicating CFTR was non-functional in intestinal epithelial cells in VillinCre mice (**Figure S3A**).

In complementary experiments, we utilized CFTR KO mice which harbor a human WT *Cftr* transgene driven by an intestinal promoter (FABP2) to correct CFTR loss in only intestinal epithelial cells. These gut-corrected mice (GC) mice do not have many of the intestinal phenotypes of CF, including no intestinal obstruction or accumulation of viscous mucus, and are not required to be maintained on water containing laxative. Upon infection with *C. rodentium,* GC mice were susceptible to infection with similar bacterial burden and mortality to ΔF508 mice (**Figure 3C left panel**). We observed significantly more bacterial burden in the feces 3 DPI in GC mice compared to WT but no significant difference at 6 DPI (**Figure 3C right panel**). However, FABP2 expression is known to decrease distally along the GI tract, so we assessed the degree of CFTR correction in these mice. The ileum of gut-corrected CF mice produced short-circuit current responses that were significantly increased compared to ΔF508 mice, indicating partial CFTR correction in this tissue (**Figure S3B**). However, the change in current in the cecum,

proximal colon, and distal colon of the gut-corrected CF mice was comparable to ΔF508 mice levels, indicating a lack of functional CFTR correction in these tissues. Nevertheless, when taken together with the results of the VillinCre mice, these results indicate that neither intestinal epithelial cells or laxative administration are responsible for the increased susceptibility and bacterial burdens observed in CF mice.

Despite the increase in pro-inflammatory cytokine expression and increased infiltration of myeloid cells, ΔF508 mice are more susceptible to infection with *C. rodentium* infection. The immune response required for clearance of *C. rodentium* involves recruitment of myeloid cells, particularly neutrophils, which are a critical first step in controlling spread of the pathogen [700]. Since it has previously shown that loss of CFTR negatively affects neutrophil function [701], we hypothesized that dysfunctional neutrophils in CF mice intestines might be responsible for the susceptibility to *C. rodentium* infection. Similar to what was observed in ΔF508 mice, steady state colons from MeoxCre mice showed increased frequency and total numbers of neutrophils and monocytes compared to control mice (**Figure S3C-D**). To determine whether loss of CFTR in myeloid cells was contributing to the increased susceptibility to *C. rodentium* infection in CF mice, we utilized myeloid cell specific *Cftr* conditional knockout (LysMCre) mice. We first validated that LysMCre mice lacked *Cftr* expression specifically in monocytes and neutrophils and not in other immune cells such as $CD8^+$ T cells (**Figure S3E**). We then infected LysMCre mice with *C. rodentium* but loss of *Cftr* specifically in LysM-expressing cells did not induce susceptibility to infection (**Figure 3D**).

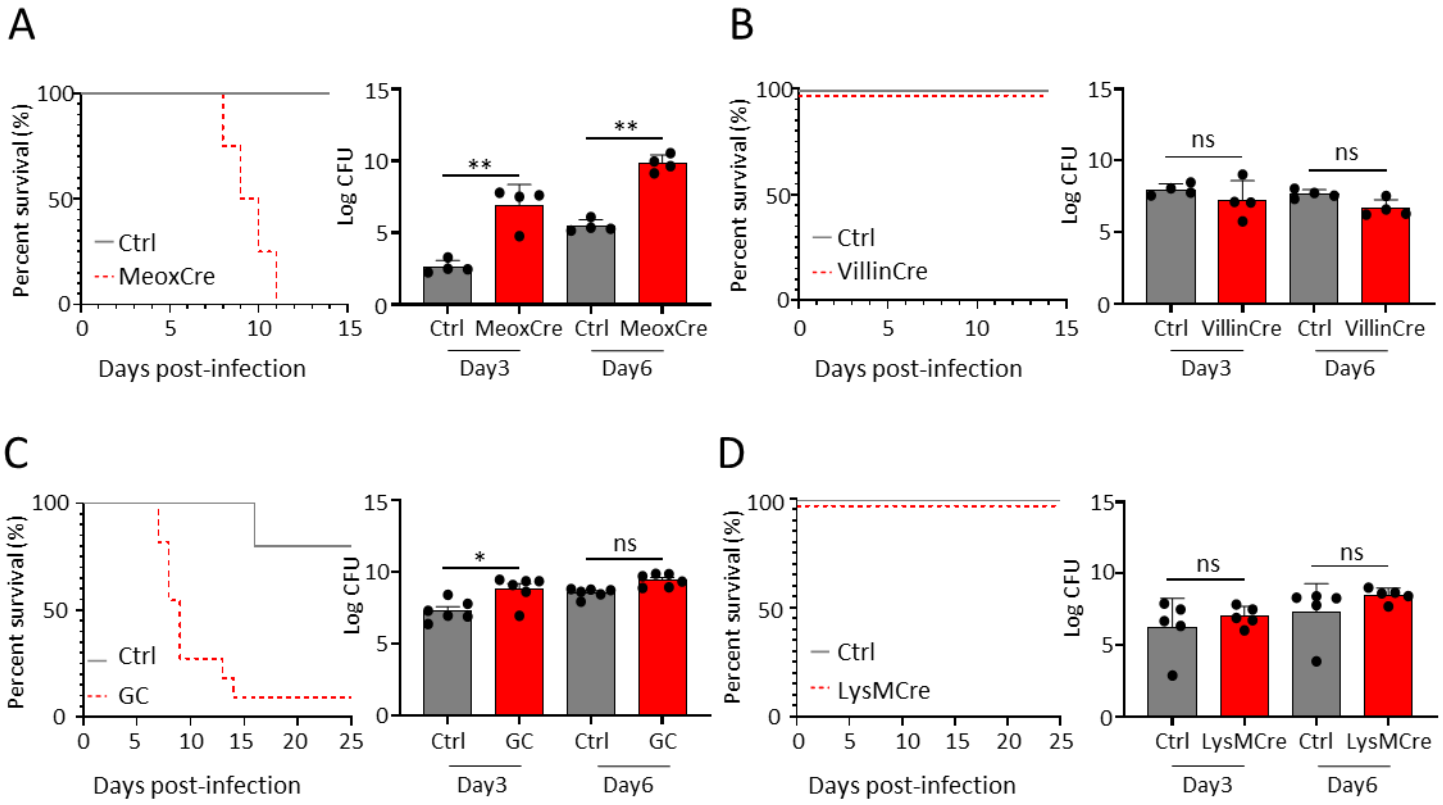

Figure 3: Susceptibility to *C. rodentium* infection is independent of loss of CFTR in intestinal epithelial cells or myeloid cells (A) MeoxCre (n=4), (B) VillinCre (n=4), (C) gut-corrected mice (n=4), (D) LysMCre (n=5) and related control mice were orally infected with *C. rodentium* and assessed for percent survival (left panels) and fecal burdens (right panels) measured at 3DPI and 6DPI. Data shown are representative of at least two independent experiments and error bars represent SEM. Significance was determined by one-way ANOVA followed with Sidak's multiple comparison's test. *$p < 0.05$ **$p< 0.01$
See also Figure S2

CFTR-deficiency promotes dendritic cell and CD4$^+$ T cell activation at steady state

Since CFTR loss in myeloid cells did not lead to susceptibility to *C. rodentium* infection, we continued to examine the intestinal immune system in steady state CFTR KO mice but focused on lymphoid cell populations. CD4$^+$ T cells are critical for clearance of *C. rodentium* and have been shown to be dysregulated in CF patients [695, 702], and so we analyzed CD4$^+$ T cells in both control and MeoxCre colons at steady state by flow cytometry (**Figure S4A**). We found no significant difference in frequency and total numbers of CD4$^+$ T cells in steady state MeoxCre and control colons (**Figure S4B**). However, the frequency of effector CD4$^+$ T cells (CD62L$^-$CD44$^+$) was significantly increased in the colons of MeoxCre mice compared to controls at steady state (**Figure 4A**). The intestinal immune response to *C. rodentium* involves both IFNγ and IL-17 production from effector CD4$^+$ T cells, so we analyzed the production of IFNγ and IL-17 in both MeoxCre and control colon CD4$^+$ T cells upon restimulation with PMA/ionomycin. Strikingly, we observed a significant increase in the frequency of IL-17$^+$ CD4$^+$ T cells but a significant reduction of IFNγ$^+$ CD4$^+$ T cells in MeoxCre colons compared to control colons (**Figure 4C**). These results suggest that CFTR deficiency is promoting the activation of CD4$^+$ T cells and skewing their responses towards a Th17 response in steady-state MeoxCre mice colons.

Our analysis of colons from CFTR-deficient mice revealed an increase in both innate and adaptive immune activation at steady state, suggesting that loss of CFTR was potentially disrupting immune homeostasis. Conventional DC (cDC) subsets are central antigen presenting cells and are potent mediators of both innate and adaptive immune responses. *In vivo* cDCs are

composed of two subsets: cDC1 and cDC2, which are distinct in terms of differentiation and function in most tissues[21]. Intestinal cDC1s and cDC2s are particularly critical for the maintenance of tolerance but are also important for the clearance of intestinal pathogens, including *C. rodentium* [532]. Since dysfunctional cDC activation has been shown to induce inflammatory disorders, including inflammatory bowel disease [453, 556, 557], we hypothesized that CFTR deficiency in cDCs was promoting aberrant immune responses in the intestinal tract of CF mice. To test this, we used flow cytometry to phenotype the two major cDC subtypes, cDC1s and cDC2s, in the colons of MeoxCre mice and controls. We found no difference in the frequency and total number of cDC1s and cDC2s in the colon (**Figure S4D-E**). However, we observed that both cDC1s and cDC2s expressed significantly more CD86, a surface activation marker, in MeoxCre colons compared to control mice (**Figure 4C**). In order to determine if CFTR deficiency specifically in DCs was responsible for susceptibility to *C. rodentium* infection, we infected CD11c-specific CFTR knockout (CD11cCre) mice with *C. rodentium* and characterized their response. CD11cCre mice showed no difference in mortality following *C. rodentium* infection compared to control mice (**Figure 4D**). Interestingly, unlike what was observed for VillinCre or LysMCre mice infected with *C. rodentium*, CD11cCre mice showed increased bacterial burdens at 3 DPI but not differences at 9 DPI compared to control mice (**Figure 4D**).

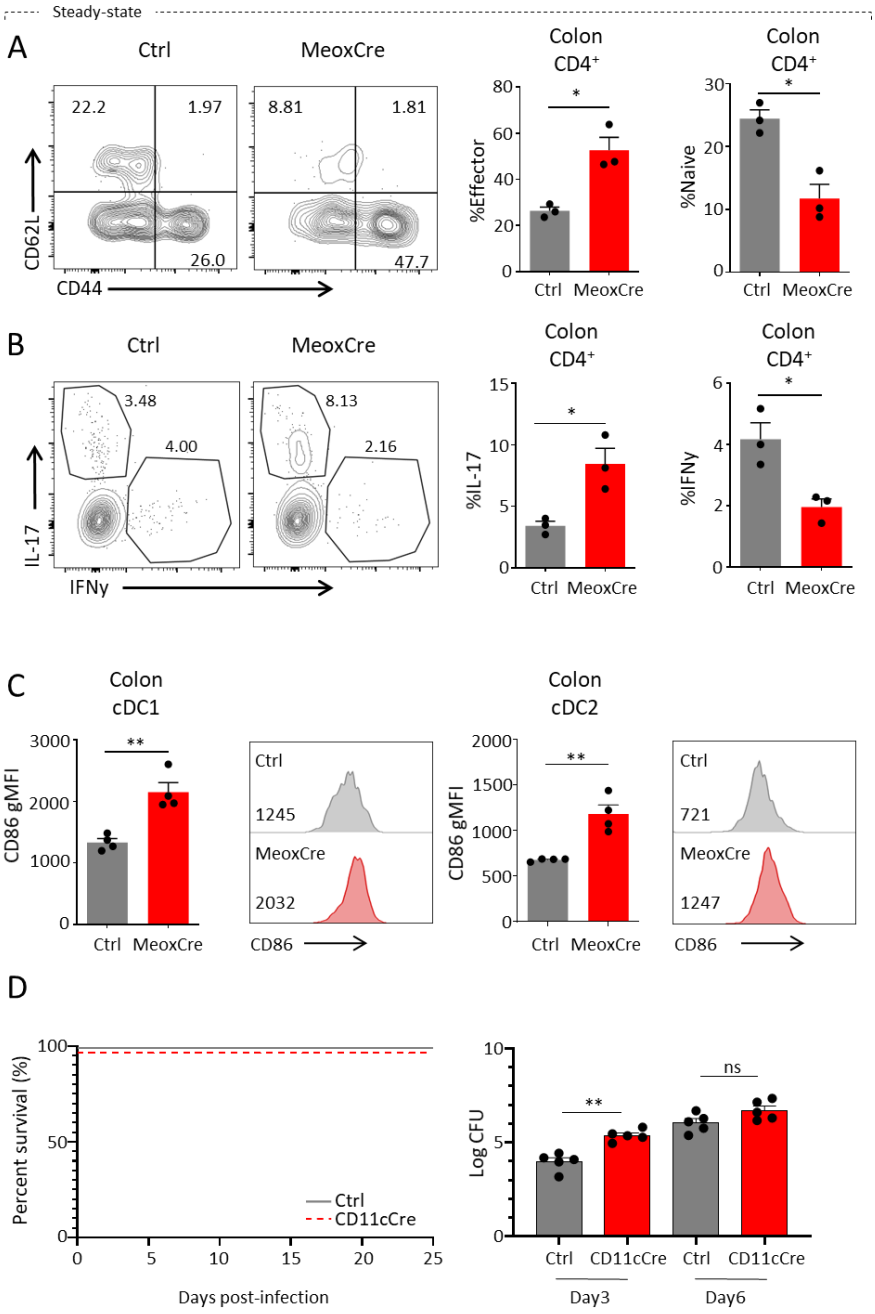

Figure 4: CFTR-deficiency promotes dendritic cell and CD4+ T cell activation at steady state (A) Representative contour plot and frequencies of effector ($CD62L^{-}CD44^{+}$) and naïve ($CD62L^{+}CD44^{-}$) colon $CD4^{+}$ T cells from control and MeoxCre mice at steady state. (B) Representative contour plot and frequencies of IL-17^{+} and $IFNy^{+}$ colonic $CD4^{+}$ T cells from Control and MeoxCre mice at steady state. (C) Cell surface expression of CD86 in colonic cDC1s and cDC2s from (left panel) control and (right panel) MeoxCre mice at steady state. (D) Control (n=5) and CD11cCre (n=5) mice were orally infected with *C. rodentium* and (left panel) percent survival was assessed, and fecal burdens (right panel) were measured 3DPI and 6DPI. Data shown are best representative of individual independent experiments (n=3) and error bars represent SEM. Significance was determined through (B-C, E-F) two-tailed unpaired Student's t-test and (G) one-way ANOVA followed with Sidak's multiple comparison's test. *p<0.05 **p<0.01 See also Figure S4

CD11c-specific CFTR deletion promotes DC activation and adaptive immune responses in the gastrointestinal tract

Although no CD11cCre mice succumbed to *C. rodentium* infection, we observed an increase in bacterial burden early in the infection which was similar to what was seen in GC mice. These results suggest that loss of CFTR is dysregulating the immune response generated by DCs in the intestinal tract. We thus performed similar immunophenotyping experiments on the large intestine of CD11cCre mice to determine if CFTR deficiency in intestinal cDC subsets was promoting dysfunctional intestinal immune responses at steady state. In agreement with our findings in MeoxCre mice, cDC1s and cDC2s from the colons of CD11cCre mice expressed significantly more surface activation marker CD86 than controls (**Figure 5A**). We next examined if the increased activation of cDCs promoted aberrant T cell activation by characterizing the $CD4^+$ T cell response in the large intestine of CD11cCre mice. We observed a significant increase in the frequency of effector $CD4^+$ T cells in the large intestine of CD11cCre mice (**Figure 5B**). Since IL-17^+ RORγt^+ $CD4^+$ effector T cells are known to be generated from aberrant DC responses in the intestinal tract, and the adaptive immune response was polarized towards Th17 responses in MeoxCre mice, we further characterized the $CD4^+$ T cells in the CD11cCre mice to determine whether DC-specific loss of CFTR was promoting Th17 responses. In the colons of CD11cCre mice, there was a significant increase in the frequency of IL-17^+ and RORγt^+ expressing CD4+ effector T cells compared to controls (**Figure 5C-D**). No significant difference in the production of the Th1-related IFNγ was measured in the colon of CD11cCre mice compared to controls (**Figure S5A**). These results indicate that the enhanced Th17 $CD4^+$ T cell response in MeoxCre mice can be recapitulated in CD11c-specific knockout mice, suggesting that the

dysregulated adaptive immune response observed in those mice is likely due to CFTR loss in DCs.

cDCs in the small intestine also critically govern the balance of tolerance and inflammation against intestinal microbes. We hypothesized that, like cDCs in the large intestine, CD11cCre small intestine cDC subsets would also be dysregulated and promoting inflammation. In complementary experiments, both cDC1s and cDC2s from the small intestine of CD11cCre mice were more activated than controls (**Figure 5E**). In agreement with previous results in the large intestine, we observed a significant increase in the frequency of effector $CD4^+$ T cells in CD11cCre mice compared to controls (**Figure 5F**). We saw a trend towards an increase in IL-17^+ $CD4^+$ T cells but a significant increase in RORγt-expressing $CD4^+$ T cells in CD11cCre mice compared to controls (**Figure 5G-H**). There was no significant difference in IFNγ-expressing $CD4^+$ T cells in CD11cCre mice (**Figure S5B**). These results show that cDC activation and function is dysregulated throughout the gastrointestinal tract in CD11cCre mice.

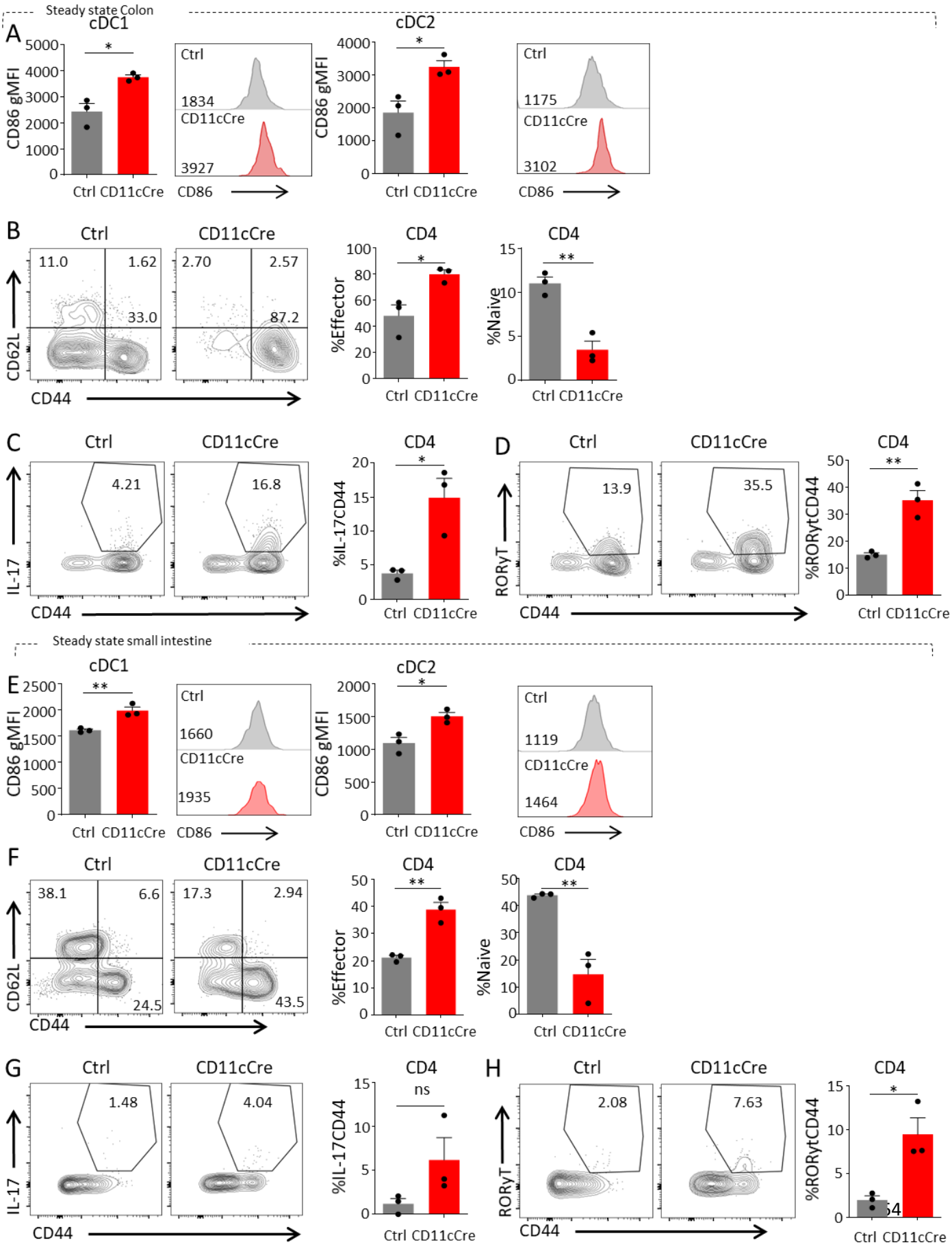
Steady state Colon
A
cDC1
CD86 gMFI
Ctrl
CD11cCre
1834
3927
CD86
cDC2
1175
3102
B
Ctrl
CD11cCre
11.0
1.62
33.0
2.70
2.57
87.2
CD62L
CD44
CD4
%Effector
%Naive
C
4.21
16.8
IL-17
%IL-17CD44
D
13.9
35.5
RORγT
%RORγtCD44
Steady state small intestine
E
1660
1935
1119
1464
F
38.1
6.6
24.5
17.3
2.94
43.5
G
1.48
4.04
ns
H
2.08
7.63

Figure 5: DC-intrinsic CFTR deletion promotes adaptive immune responses in the gastrointestinal tract
Colons from control and CD11cCre mice at steady state were digested and either (A-B) stained for extracellular markers or (C-D) were stimulated with cell stimulation cocktail for 3 hours and then stained for (D) IL-17 and (E) RORγt via ICS. (A) Cell surface expression of CD86 in colonic (left panel) cDC1s and (right panel) cDC2s from control and CD11cCre mice at steady state. (B-D) Representative contour plots and frequencies of (B) viable effector ($CD62L^{-}CD44^{+}$), naïve ($CD62L^{+}CD44^{-}$), (C) $IL\text{-}17^{+}CD44^{+}$ and (D) $ROR\gamma t^{+}$ $CD44^{+}$ colonic $CD4^{+}$ T cells from control and CD11cCre mice at steady state. (E-H) Experiments were performed as in (A-D) but with small intestinal $CD4^{+}$ T cells from control and CD11cCre mice. Data shown are best representative of individual independent experiments (n=3) and error bars represent SEM. Significance was determined through two-tailed unpaired Student's t-test. *p<0.05 **p<0.01 See also Figure S5

We also characterized the immune response in the spleens of CD11cCre mice to determine whether any defects observed were specific to the intestinal tract. In the spleens of CD11cCre mice, cDC1s expressed more CD86 than control mice. However, splenic cDC2s expressed similar amounts of CD86 as control mice (**Figure S5C**). There was also a significant increase in the frequency of effector CD4+ T cells and IL-17 expressing $CD4^{+}$ T cells in the spleens of CD11cCre mice compared to controls (**Figure S5D-E**). There was no significant difference in $ROR\gamma t^{+}$ or $IFN\gamma^{+}$ expressing $CD4^{+}$ T cells in CD11cCre mice compared to controls (**Figure S5F-G**). These results show that cDCs are more activated in tissues other than the intestinal tract, but do not promote aberrant T cell responses to the same extent as what was observed in the small and large intestine.

Loss of CFTR promotes the activation and function of DCs

Since intestinal cDCs subsets are rare and difficult to culture, we utilized *in vitro* BMDC cultures to allow us to fully characterize the effects of CFTR loss on DC function. We first confirmed that CFTR was absent in BMDCs generated from CD11cCre mice by Western blot (**Figure S6A**). Loss of CFTR did not lead to any significant change in viability or percentage of $CD11c^{+}$ cells in culture (**Figure S6B**). Loss of CFTR in resting and lipopolysaccharide (LPS)-activated BMDCs led to significantly increased surface expression of the activation markers

CD80, CD86, and MHC2 (**Figure 6A**). In a follow-up experiment, we stimulated BMDCs with other toll-like receptor (TLR) agonists such as zymosan (Zym) and house dust mite (HDM) and found that no matter the TLR agonist used, CD11cCre BMDCs expressed more CD86 than controls (**Figure S6C**). Resting and LPS-activated CD11cCre BMDCs secreted significantly more TNFα and IL-23 but significantly less IL-12p70 and IL-10 (**Figure 6B**). To determine whether CFTR loss regulates the ability of DCs to stimulate T cell responses, BMDCs from CD11cCre mice were pulsed with whole ovalbumin (OVA) and co-cultured with OVA-specific $CD4^+$ T cells from transgenic OTII mice. BMDCs from CD11cCre mice at steady state and after LPS stimulation were more efficient at inducing $CD4^+$ T cell activation (measured by expression of CD25 and CD44) and proliferation (**Figure 6C-D**). OTII $CD4^+$ T cells co-cultured with CD11cCre BMDCs expressed significantly more IL-17 and RORγt but less IFNγ compared to cells co-culture with control BMDCs (**Figure 6E-F and S6D**). Resting and LPS-activated BMDCs from CD11cCre mice were also able to induce increased antigen sensitivity of co-cultured $CD4^+$ OTII T cells (**Figure S6E-F**). This data shows that BMDCs from CFTR KO mice are more activated, more functional, and skew $CD4^+$ T cells towards a Th17 immune response, consistent with our results observed *in vivo*.

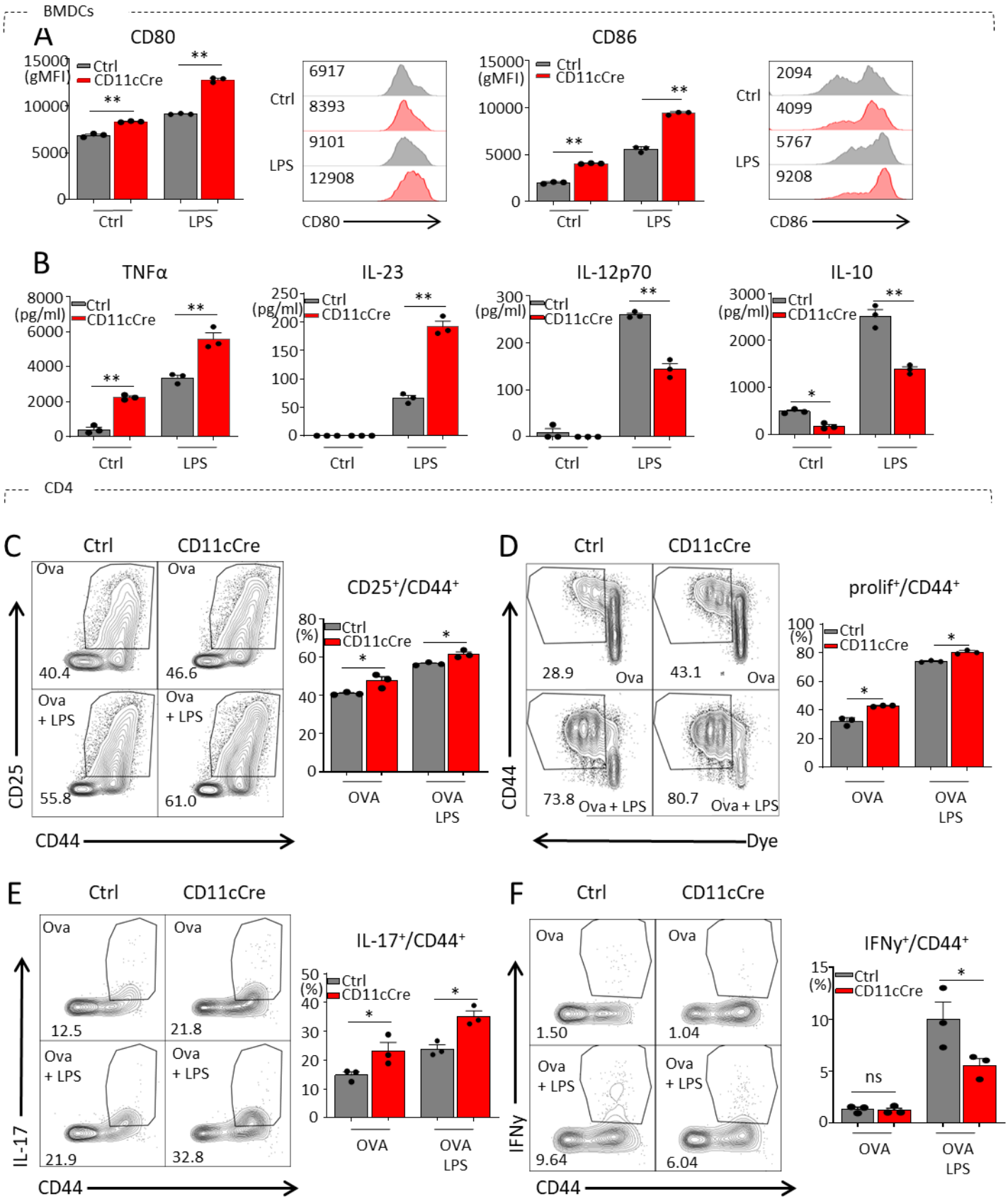
BMDCs
A
CD80
15000
(gMFI)
10000
5000
0
Ctrl
CD11cCre
**
Ctrl
LPS
6917
8393
9101
12908
CD80
CD86
2094
4099
5767
9208
CD86
B
TNFα
8000
(pg/ml)
6000
4000
2000
IL-23
250
200
150
100
50
IL-12p70
300
IL-10
3000
1000
*
CD4
C
Ctrl
CD11cCre
Ova
40.4
46.6
Ova + LPS
55.8
61.0
CD25
CD44
CD25⁺/CD44⁺
80
(%)
60
40
20
OVA
OVA LPS
D
28.9
43.1
73.8
Ova + LPS
80.7
Dye
prolif⁺/CD44⁺
100
E
12.5
21.8
21.9
32.8
IL-17
IL-17⁺/CD44⁺
50
F
1.50
1.04
9.64
6.04
IFNγ
IFNγ⁺/CD44⁺
15
ns

Figure 6: Loss of CFTR promotes the activation and function of DCs
(A-B) Cell surface expression (A) and cytokine production (B) by BMDCs generated from control and CD11cCre mice examined at steady state or after 18 h of LPS treatment (100 ng/mL). Data represents (A) gMFI or (B) pg/ml of secreted cytokine of best representative of individual independent experiments (n = 3), and error bars represent SEM. (C–F) BMDCs generated from control and CD11cCre mice were pulsed with whole OVA protein with or without LPS stimulation for 6 h and co-cultured with $CD4^+$ OTII T cells labeled with proliferation dye. Three days after co-culture, T cell activation was measured via surface expression of (C) CD44 and CD25, and (D) proliferation by dye dilution. (E-F) On day 5 after co-culture, (E) IL-17 and (F) RORγt production and was measured via intracellular staining (ICS). Data shown are one representative of at least three independent experiments (n=3). Significance was determined through one-way ANOVA followed with Sidak's multiple comparison's test. $*p < 0.05$. **p<0.01 See also Figure S6

Loss of CFTR promotes the function of BMDCs potentially through reducing Akt signaling

Since multiple TLR agonists induced increased activation in CD11cCre BMDCs, we suspected that CFTR may be regulating a general activation pathway in DCs. Innate immune recognition of microbes by TLRs activates the PI3K-Akt pathway which has been shown to serve as an anti-inflammatory negative feedback loop to dampen the activation of proinflammatory pathways [703, 704]. Recently, it has been shown that anti-inflammatory PI3K-Akt signaling in epithelial cells and macrophages is suppressed in CF, thereby leading to chronic inflammation and altered bacterial clearance [705-707]. To determine if CFTR loss was similarly affecting PI3K-Akt signalling in BMDCs, we analyzed the phosphorylation status of Akt and downstream ribosomal protein S6 (S6) by intracellular flow cytometry. BMDCs from control mice that were stimulated with LPS demonstrated a strong induction of PI3K-Akt signaling, with a significant increase in mean fluorescence intensity (MFI) levels of p-Akt and p-S6, while BMDCs from CD11cCre mice failed to induce p-Akt and p-S6 (**Figure 7A**). We then examined whether the addition of the Akt activator SC79, which increases the phosphorylation of Akt in the cytoplasm, can restore p-Akt and p-S6 activation. SC79 restored p-Akt and p-S6 activation in CD11cCre BMDCs (**Figure 7A-B**). We next tested whether the addition of SC79 could reduce the activation

and function of CD11cCre BMDCs by performing DC-$CD4^+$ T cell co-culture assays. Upon addition of the Akt activator SC79, the elevated $CD4^+$ T cell activation (measured by CD44 and CD25 expression) in CD11cCre BMDCs was restored to levels observed with control BMDCs (**Figure 7C-D**), demonstrating that CFTR-mediated alterations of the PI3K-Akt pathway in DCs affect $CD4^+$ T cell activation levels. Collectively our data suggest that CFTR-deficiency in BMDCs alters the PI3K-Akt signalling pathway, which leads to elevated DC and $CD4^+$ T cell activation.

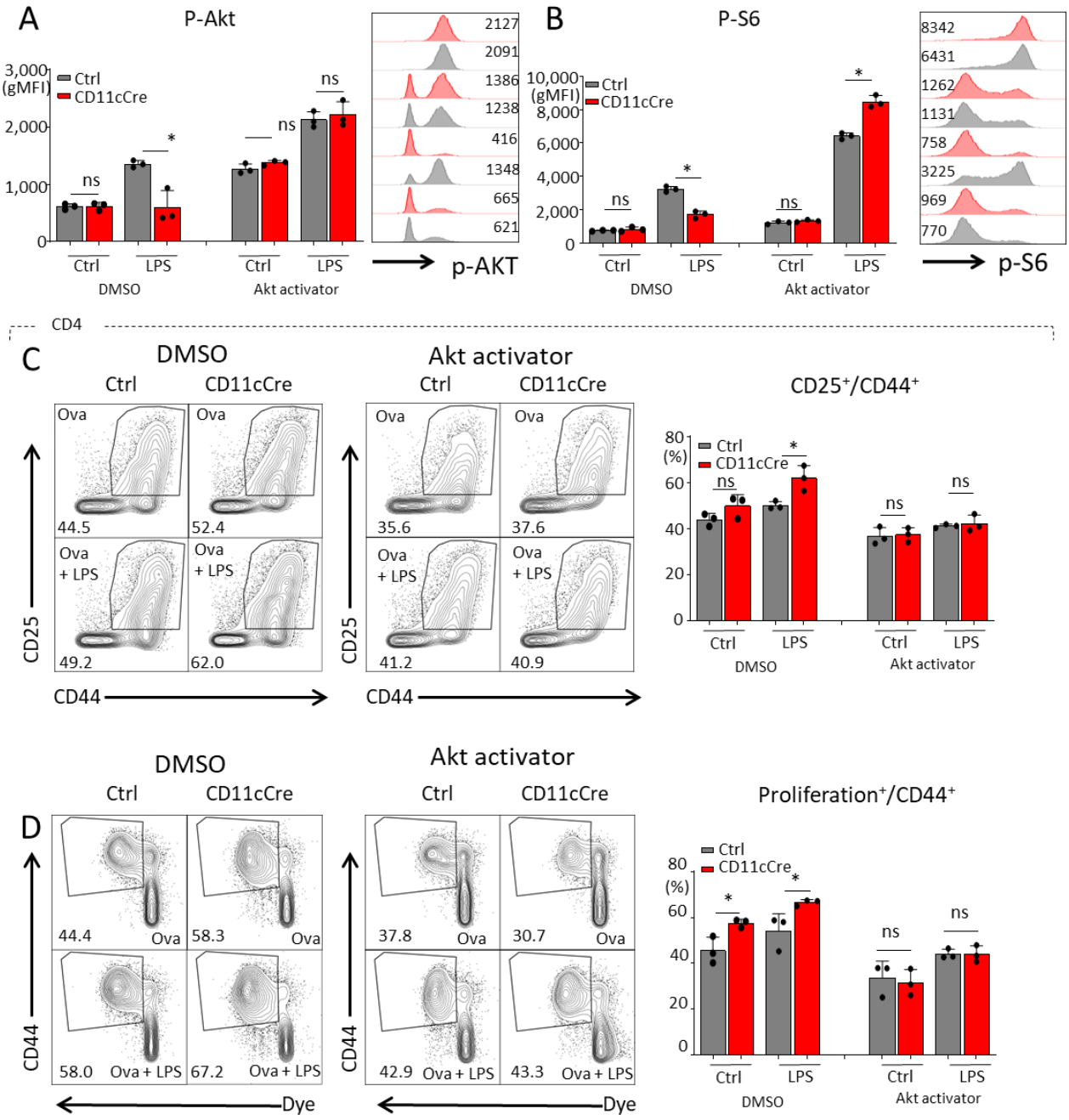

Figure 7: Loss of CFTR promotes the function of BMDCs potentially through reducing AKT signaling
(A-B) BMDCs generated from control and CD11cCre mice were pre-treated with DMSO or SC73 AKT activator for one hour followed with or without 100 ng/mL of LPS for 18 h and then stained for (A) phospho-AKT and (B) phospho-S6 via ICS. Data represents geometric mean fluorescence intensity (gMFI) of best representative of individual independent experiments (n = 2), and error bars represent SEM. (C–D) BMDCs generated from control and CD11cCre mice were pre-treated with DMSO or SC73 AKT activator, washed with PBS and then pulsed with whole OVA protein with or without LPS stimulation for 6 h and co-cultured with CD4+ OTII T cells labeled with proliferation dye. Three days after co-culture, T cell activation was measured via surface expression of (C) CD44 and CD25, and (D) proliferation by dye dilution. Data shown are one representative of at least two independent experiments. Significance was determined through one-way ANOVA followed with Sidak's multiple comparison's test. $*p < 0.05$.

3.4 Discussion

Although CF disease is primarily associated with airway obstruction, loss of CFTR has been shown to promote increased inflammation in the lung and gastrointestinal tract [586, 589, 590]. Since this increased inflammation is present even in the absence of bacterial colonization, which indicates a potential dysregulation of the immune system in these tissues [591]. It is currently unclear how CFTR loss of function affects the cellular and immunological mechanisms that lead to immunopathologies in the gastrointestinal tract of CF patients. In the present study, we used *C. rodentium* as a model to study immune responses in the gastrointestinal tract of CF mouse models and found that loss of CFTR led to susceptibility to *C. rodentium* infection. CFTR deficiency promoted inflammation in the gastrointestinal tract with increased inflammatory signaling and immune cell infiltration. We found that the susceptibility in CF mice was not due to the effects of CF pathology on the lumenal microenvironment, or loss of CFTR in intestinal epithelial cells or LysM-expressing cells. Loss of CFTR function in DCs exacerbated the early stages of *C. rodentium* infection and promoted aberrant Th17 $CD4^+$ T cell effector responses at steady state. Mechanistically, we found that loss of CFTR promoted DC activation and function, possibly by reducing anti-inflammatory PI3K-Akt signaling. These observations demonstrate that CFTR plays an important functional role in regulating DC function and that CF disease promotes dysregulated inflammation due to direct effects on immune cells.

In this work, we show for the first time that CF mice are much more susceptible than WT littermates to infection with *C. rodentium*, a common murine model of intestinal infection of attaching and effacing bacteria [540]. We show that loss of functional CFTR in C57BL/6

(MeoxCre), BALBC (CFTR KO) and FVB (ΔF508) mouse backgrounds promotes susceptibility to *C. rodentium* infection, suggesting that genetic background is not responsible for our findings.

The kinetics of *C. rodentium* infection are well documented in most inbred mouse strains [535, 708, 709]. *C. rodentium* infection in WT mice is normally initiated in the cecum but then migrates to colon, with the highest levels of colonization in the distal colon [709]. Here we show that in CF mice, *C. rodentium* was present in the small intestine, cecum and proximal colon but also in the spleen, suggesting not only an altered localization but also systemic spread. Although GC mice only partially corrected CFTR function, restricted only to the small intestine, these mice do not suffer from intestinal obstruction and do not require laxatives to survive. Since GC mice are still susceptible to *C. rodentium* infection like CF mice, this suggests that susceptibility is not the result of increased intestinal mucus, intestinal obstruction, or the laxative water.

It is well documented that the gut microbiota can influence immune homeostasis in the gastrointestinal tract and affect the susceptibility of mice to intestinal infection, including by *C. rodentium* [710-712]. Segmented filamentous bacteria have been shown to cause a shift towards a Th17 $CD4^+$ T cell response through direct actions on intestinal epithelial cells [713]. Microbiota dysbiosis has been reported in CF mice and human patients [714, 715]. CF patients and mice show increased colonization by microbiota species such as *Lactobacillus*, *Bifidobacterium* and *E. coli*, and depletion of these species has been shown to influence steady state inflammation in the CF gut [715, 716]. To account for changes in the microbiome, our CF mice are co-housed with control litter mates to minimize differences in microbiota between our experimental groups. Furthermore, we show susceptibility to *C. rodentium* infection in multiple

different genetic backgrounds, which all differ in their microbiota composition [717, 718]. To completely rule out the possibility that microbiome dysbiosis is responsible for are observed phenotypes, it would be interesting to perform microbiome screening through 16S rRNA sequencing to provide us with a complete picture of any differences in microbiota composition between susceptible mice and controls.

Our study is the first to show that CFTR tempers DC activation. Previous studies on the effects of CFTR loss on DCs in CF mice have shown conflicting results [607, 610], with some groups showing a negligible effect on DC activation and others showing a reduction in activation. These previous studies looked at cDC populations in the lungs of CF mice, which do not totally recapitulate the inflammatory phenotype observed in lungs from CF patients [582, 719]. Another factor that may contribute to our differing results is the development of new gating strategies that reduce contamination when analyzing cDC populations *in vivo.* Until recently, common gating strategies for cDC subsets in mouse and human tissues have been contaminated with macrophages and monocytes [68]. Recent advances in single cell sequencing have led to the creation of gating strategies that can analyze cDCs in a variety of mouse and human tissues, while also excluding macrophages and other contaminating cells, using lineage and ontogeny markers [70]. Using this gating strategy, we analyzed cDC subsets from the spleen, small intestine and large intestine of CF mice and have shown that both cDC1s and cDC2s are significantly more activated in all of these tissues. The effect of CFTR loss in splenic cDC subsets was more subtle compared to intestinal cDC susbets, suggesting that our phenotype may be more pronounced in the intestinal tract compared to other tissues, which

may also explain why loss of CFTR function in lung cDCs showed no appreciable effect on activation in CF mice [607, 610].

Intestinal cDCs potently mediate the balance between maintenance of tolerance to the commensal microbiota and immune activation against intestinal pathogens like *C. rodentium.* Intestinal cDCs are required for the induction of Th17 effector T cell responses necessary for the clearance of *C. rodentium* [500, 542, 543]. Dysregulation of cDC activation has been shown to induce susceptibility to *C. rodentium* infection in previously resistant mouse strains [534]. Our results show that CD11c-specific CFTR knockout mice have an increased burden of *C. rodentium* in the early stages of infection but showed no difference in mortality compared to littermate controls. Immunophenotyping of the intestinal tract revealed that CD11c-specific CFTR knockout greatly promoted effector $CD4^+$ T cell frequencies. Further analysis of these $CD4^+$ T cell populations indicated a bias in differentiation towards a Th17 effector response and a reduced Th1 effector response at steady state. *C. rodentium* induces an early Th1 response followed by a robust Th17 response, both of which are required for *C. rodentium* clearance [540-542]. Failure to produce Th1 related cytokines early in infection has been shown to result in acute susceptibility to many intestinal pathogens, including *Helicobacter pylori* and *C. rodentium* [720-722]. We speculate that loss of CFTR in DCs is promoting a dysfunctional Th1 response at steady state that is promoting early spread of *C. rodentium* infection but the enhanced Th17 response may compensate adequately for early-infection defects.

Our results indicate that CFTR-loss promotes dysregulated cDC activation and function, which induces an inflammatory state in the steady-state gastrointestinal tract. Dysfunctional cDC activation is particularly pathogenic in the intestinal tract, where breaks in tolerance

towards the microbiota are associated with multiple inflammatory diseases of the intestinal tract, including Inflammatory bowel disease [537-594]. Epidemiological studies on CF patients show an increase in gut related inflammatory pathologies associated with loss of tolerance such as Crohn's disease, ulcerative colitis, and celiac disease [577, 593, 594]. Our results suggest that loss of CFTR-mediated regulation of DC activation could be responsible for the increased occurrence of inflammatory pathology in CF patients.

Since we consistently observed significantly more expression of surface activation markers in CFTR deficient BMDCs stimulated with different TLR agonists, we suspected that CFTR was regulating a general TLR activation pathway in DCs. TLR signaling results in the activation of multiple pathways in DCs, including PI3K-Akt signaling [703]. PI3K-Akt signaling in various innate immune cells serves as an anti-inflammatory negative feedback loop that prevents overactivation upon stimulation. In intestinal cDCs, PI3K-Akt signaling negatively regulates the secretion of pro-inflammatory IL-12 but promotes secretion of anti-inflammatory IL-10 [723, 724]. Our results show that PI3K-Akt signaling is reduced in CFTR-deficient BMDCs. We also found that the addition of an Akt activator (SC79) can not only rescue the defect in Akt phosphorylation but also return to baseline the ability of CFTR-deficient BMDCs to activate $CD4^+$ T cells. These results warrant further study on the crosstalk of CFTR with PI3K-Akt signaling and its impact on gastrointestinal immunopathologies.

Despite all our evidence that CFTR regulates immune responses in the intestinal tract, we have not yet determined what cell type was responsible for the mortality following *C. rodentium* infection observed in CF mice. We first focused on evaluating innate immune cells as being responsible for the increased susceptibility, as innate immune responses are critical for

clearance of *C. rodentium* infection and CFTR has previously been shown to regulate neutrophil function [701]. However, neither LysMCre nor CD11cCre mice showed increased mortality following infection with *C. rodentium.* CD11cCre mice showed an increase in *C. rodentium* bacterial burden early in infection and a significant increase in the activation of $CD4^+$ T cell responses. Since it has previously been shown that adaptive immune responses are essential for clearance of *C. rodentium* [702] and that $CD4^+$ T cells are dysfunctional in CF patients [595, 695, 725], we speculate that loss of CFTR in $CD4^+$ T cells may be responsible for the increased susceptibility to infection. Various $CD4^+$ T cell-specific Cre lines are available, including CD4-Cre and Lck-Cre, which can be used to specifically knock out CFTR in T cells and evaluate their susceptibility to infection. Alternatively, it is possible that loss of CFTR in multiple immune cell types may be responsible for the increased susceptibility. To answer this, we plan to infect hematopoietic cell-specific CFTR knockout mice (Vav-Cre *cftr*-floxed mice) to determine if CFTR loss in only the hematopoietic cell compartment will lead to increased susceptibility.

Our data reveal that loss of CFTR promotes susceptibility to *C. rodentium* infection by dysregulating the intestinal immune response. We also show that CFTR is a potent negative regulator of DC activation, potentially through regulation of anti-inflammatory PI3K-Akt signaling. Loss of CFTR in DCs promotes activation and induces significant effector CD4+ T cell responses in steady state intestines. Our work highlights the potential role for CFTR loss in inducing dysregulated DC activation, which may be promoting the increased inflammatory conditions observed in CF patients. We also demonstrate that use of Akt activators as a potential treatment for chronic inflammation in CF patients warrants further study.

3.5 Author contributions

Conceptualization, S.G., J.F, and B.C.; Methodology, S.G., J.F., B.C., J.H., C.M.K. and L.Z.; Validation, B.C., T.A., M.Y., D.J., and B.M.; Formal Analysis, B.C. and T.A.; Investigation, B.C., T.A., M.Y., D.J., B.M., and L.Z; Resources, J.H.; Data Curation, B.C.; Writing- Original Draft, B.C.; Writing- Review and Editing, S.G., J.F., and B.C.; Visualization, S.G., J.F., and B.C.; Supervision, S.G., J.F. and C.M.K; Project Administration, S.G., J.F., and B.C. ; Funding Acquisition, S.G. and J.F.

3.6 Experimental procedures

Experimental Model and Subject Details: BALB/c.129P2-*Cftr*^{tm1Unc} (CFTR KO) and FVB/N.129P2-*Cftr*^{tm1Eur} (ΔF508) mice were generously provided by Dr. John Hanrahan (McGill University, QC). B6.129P2-*Cftr*^{tm1Unc}. FVB-Tg(FABPCFTR)1Jaw/J (Gut-corrected), B6.129P2-Lyz2$^{tm1(cre)Ifo}$/J (LysMcre), B6.129S4-*Meox2*$^{tm1(cre)Sor}$/J (MeoxCre)and B6.Cg-Tg(Vil1-cre)10000Gum/J (Vil1-Cre) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). B6.Cg-Tg(Itgax-cre)1-1Reiz/J (CD11cCre) were obtained from Dr. Judith Mandl (McGill University, QC) . B6.129-*Cftr*^{tm1Cwr}(*cftr*-floxed) mice were obtained from Dr. Mitchell Drumm (Case Western Reserve University, OH). LysMcre *Cftr*-floxed mice were generated by crossing *Cftr-floxed* mice with the F1 progeny of LysMcre X *Cftr*-floxed. CD11cCre *Cftr-floxed* mice were generated by crossing *Cftr-floxed* mice with the F1 progeny of CD11cCre X *Cftr-floxed*. Presence of the LysMcre transgene, CD11cCre transgene and floxed *Cftr* gene was validated by PCR. OTI and OTII transgenic mice were purchased from the Jackson Laboratory (Bar Harbor, ME USA) and bred in house. Animals were maintained in a specific pathogen-free environment. Mice were

transferred to Biohazard Level 2 rooms for infection. CFTR KO, ΔF508 and MeoxCre mice were kept on polyethylene glycol iin the form of CoLyte (69.5g CoLyte/L containing 60g polyethylene glycol/L) (Pendopharm) at weaning to prevent intestinal obstruction. All experiments were conducted following the guidelines of the Canadian Council of Animal Care, as approved by the animal care committee of McGill University.

Method Details

Infection with *C. rodentium*: Six to eight week old mice were infected via oral gavage with 4 x10^8 CFU of chloramphenicol -resistant *C. rodentium*. For bioluminescent imaging, a bioluminescent strain of *C. rodentium* DBS100 expressing the lux-CDAB# operon from *Photorhabdus luminescens* was obtained from Dr. Bruce Vallance (University of British Columbia, BC) [726]. Bacterial burden was monitored by collecting faeces from each mouse, weighed, dissociated in PBS, serial diluted and played on chloramphenicol MacConkey agar petri dishes. To determine tissue-associated *C. rodentium* infection, tissues were weighed, dissociated in PBS, diluted, and plated on chloramphenicol MacConkey agar petri dishes. Petri dishes were then incubated at 37C overnight and colonies were counted the following day. Final counts were measured as CFU/g faeces and plotted on a log-scale.

Bioluminescent imaging: 8 days post-infection with a bioluminescent strain of *C. rodentium* DBS100 expressing the lux-CDAB# operon from *Photorhabdus luminescens* [698], mice were dissected to remove the gastrointestinal tract and spleen. Tissues were imaged using a Caliper IVIS-100 (Xenogen).

Histological staining: Tissue sections were fixed in 10% buffered formalin, paraffin-embedded, sectioned at 4 µm, and either stained with hematoxylin and eosin (H&E) or left unstained. Unstained sections were deparaffinized with xylene and rehydrated through an ethanol gradient to water. Antigen retrieval was achieved by boiling sections in a 0.1M citric acid 0.1M trisodium citrate solution for 10 minutes, followed by cooling to room temperature. Sections were blocked using 10% fetal calf serum (FCS; Wisent) in PBS containing 3% bovine serum albumin (BSA; Sigma) and 0.2% Tween 20 (BioShop) for 1 hour at 37°C. Primary antibodies were diluted in PBS containing 3% BSA and 0.2% Tween 20 and incubated for 3 hours at 4°C. The primary antibodies used were rabbit anti-*E.coli* Poly D8 LPS (1:100; Mast Group) and rabbit anti-Muc2 (1:100; Santa Cruz Biotech) followed by incubation for 1 hour at 37°C using an AlexaFluor 488-conjugated goat anti-rabbit IgG secondary antibody (1:100; Molecular Probes/Invitrogen) diluted in PBS containing 3% BSA and 0.2% Tween 20. Following application of 4', 6'-diamidino-2-phenylindole (DAPI; Sigma) for DNA staining, sections were mounted using ProLong Gold Antifade reagent (Molecular Probes/Invitrogen). Sections were imaged at 350 and 488 nm on a Zeiss Axiovert 200M microscope and images were obtained using a Hamamatsu Monochrome camera operating through AxioVision software (Version 3.0).

RNA extraction and quantitative RT-PCR: Total RNA from the proximal colon was isolated using TRIzol (Invitrogen) according to the manufacturer's instructions. The purity of RNA was assessed by a spectrophotometer; all samples had a 260/280 absorbance ratio between 1.8 and 2.0. Complementary DNA was synthesized from 1 ug of RNA with ProtoScript II reverse transcriptase (NEB) and random primers (Invitrogen) using an Eppendorf PCR thermal cycler. Expression levels of *Reg3B* and *Reg3y* were measured using TaqMan Gene Expression Assays

(Applied Biosystems) and expression levels of *KC, IFNy, IL-1B,* and *TNFa* were measured using SYBR Green PCR Master Mix (Applied Biosystems) on a StepOnePlus Real-Time PCR system (Applied Biosystems). Analysis was performed according to the comparative CT method using *Gapdh* as the housekeeping gene. Primer pairs for SYBR Green assays are listed in Table 1.

Isolation of intestinal lamina propria cells: Intestinal lamina propria cells from mice were isolated using a modified version of a previously described method [727]. In brief, the small intestines and colons were removed and placed in cold calcium- and magnesium-free Hanks balanced salt solution (HBSS; Gibco) supplemented with 2% heat-inactivated FCS and 15 mM HEPES (Gibco). Intestines were cut open longitudinally, washed thoroughly, cut into 2 cm pieces, and incubated with shaking in EDTA buffer (HBSS supplemented with 2% FCS, 15 mM HEPES, and 5 mM EDTA) for 60 minutes at 37°C to remove epithelial cells. After removing the supernatant, tissue pieces were incubated in RPMI-1640 (Sigma) supplemented with 10% FCS, 15 mM HEPES, 100 ug/ml DNase I (Roche) and 200 ug/ml collagenase type IV (Sigma) for 40 minutes at 37°C. Cell suspension was filtered through a 70 um cell strainer (Sigma), washed, and resuspended in FACS buffer (1X PBS supplemented with 2% FBS and 0.5M EDTA) before proceeding with antibody staining.

Bone-marrow Derived DC Culture: Bone marrow was extracted and cultured in RPMI media (Corning) with 1% Penicillin-Streptomycin (Wisent), 10% fetal calf serum (HyClone/Wisent), 1% L-glutamine (Wisent), 0.1 % β-Mercaptoethanol (Gibco) and 20 ng/ml of granulocyte/macrophage colony stimulation factor (GM-CSF; Peprotech) in 6-well non-tissue culture treated plates. DCs were cultured as suspended colonies for 8-9 days at 37°C and 5% CO_2. Non-adherent cells were collected and plated at $1x10^6$ cells/ml into 12 well non-tissue

culture treated plates and stimulated as indicated. DC activators included LPS (*Escherichia coli* serotype 0111:B4, Sigma-Aldrich), heat-killed *Staphylococcus aureus* (Invivogen) , heat-killed *Escherichia coli* serotype 0111:B4 (Invivogen), Zymosan (Invivogen), Zymosan-depleted (Invivogen) and house dust mite (CiTeq). SC79 Akt phosphorylation activator were purchased from SelleckChem.

Flow Cytometry: Cells were washed and stained in FACS buffer (1X PBS supplemented with 2% FBS and 0.5M EDTA). Fluorochrome-labeled extracellular antibodies were incubated for 25 minutes at 4°C. For intracellular staining, cells were stimulated with Cell Stimulation Cocktail (plus protein transport inhibitors) (eBioscience) for 3 hours at 37°C and ICS was performed using the FoxP3transcription factor staining buffer kit (eBioscience). Samples were collected on BD Biosciences flow cytometer, FACS CantoII or Fortessa, and analyzed using FlowJo. All antibodies used are listed in table 2.

For confirmation of *Cftr* excision in LysMcre *Cftr* mice, cells were purified using various methods. Splenic T cells were purified using a CD8a+ Isolation Kit (Miltenyi Biotech). Neutrophils from the bone marrow were isolated using density gradient centrifugation and Histopaque 1077 and 1119 (Sigma). Lastly, bone marrow was cultured for 5 days in RPMI-1640 supplemented with 10% FCS and 30% macrophage colony stimulating factor (MCSF; L929 cells) at 37°C with 5% CO2 to get bone marrow derived macrophages (BMDMs). Cell purity was confirmed on a FACSCanto II.

Western Blot: Cells were lysed in CHAPS buffer (150mM KCl, 50mM HEPES, 0.1% CHAPS) supplemented with protease inhibitor cocktail (Fisher) and Sodium orthovanadate (Sigma).

Lysates were sonicated for 10 minutes (30 seconds on, 30 seconds off) at 40% amplitude (qSONICA) and then cleared. Lysates were separated on SDS-PAGE gels and transferred to nitrocellulose membranes, blocked in 5% skim milk or BSA and probed with primary antibody Anti-CFTR (Thermofisher, CF3) or anti-beta-Actin (Cell Signaling, 13E5), followed by horseradish peroxidase (HRP) conjugated anti-rabbit or anti-mouse antibodies before addition of ECL (Amersham).

DC-T cell Co-Culture: 1 x10^4 steady-state or LPS activated (3 ng/ml) transduced DCs were pulsed with whole Ovalbumin (OVA) protein (1 mg/ml) per well in 96-well plates. 6 hours following stimulation, 1x10^5 sorted $CD4^+$ T cells from OTII, respectively were added (1:10 ratio). On day 3, T cells were examined for proliferation (pre-labeled with e450 Proliferation Dye) and expression of cell-surface activation markers using flow cytometry. Alternatively, T cells were stimulated with phorbol 12-myristate 13-acetate (PMA) (50 ng/ml) and ionomycin (500 ng/ml) for 4 hours, and then cytokine production was examined using flow cytometry.

Antigen Sensitivity Assay: 1 x10^4 steady-state BMDCs were pulsed with whole Ovalbumin (OVA) protein titrations for 6 hrs followed by addition of 25x10^3 sorted $CD4^+$ T cells from OTII mice (1:5 ratio). On day 2, %$CD25^+$ T cells was determined via flow cytometry and normalized to top dose of peptide (10 ug).

Quantification and Statistical Analysis: Statistics were performed on Prism software (GraphPad). Significance was determined using one-way ANOVA or Student T-test. Data was presented as mean showing SEM or SD as indicated. Statistical significance is represented as *$p<0.05$.

3.7 Acknowledgements

The authors thank Dr. Martin Richer, Dr. Ryan Pardy, Rebecca Rabinovitch and the members of the Gruenheid labs for thoughtful discussion and critical analysis of the data. The authors would also like to thank Dr. Bruce Valance for generously providing the bioluminescent strain of *C. rodentium* DBS100. We would also like to thank Dr. Mitchell Drumm for generously providing B6.129-*Cftr*^{tm1Cwr} mice. This work could not have been carried out without the services of Camille Stegen and Julien Leconte at the Cell Vision Flow Cytometry Facility. The authors would like to acknowledge the advanced BioImaging Facility, Histology core Facility at McGill university and the Comparative Medicine & Animal Resources Centre. The work described here was supported by Canadian Institutes of Health Research (CIHR) grant PJT-175166. We also acknowledge salary support for B.C. from CIHR (CGS-D).

3.8 Supplemental Information

Supplemental information inventory

Key Resources Table

- A table containing detailed information about all reagents used in this study

Supplemental figures 1-7

-Further data to support the main text figures and results, the figures are titled as follows:

Figure S1, related to Figure 1

Figure S2, related to Figure 2

Figure S3, related to Figure 3

Figure S4, related to Figure 4

Figure S5, related to Figure 5

Figure S6, related to Figure 6

Key Resources Table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
FITC-CD80 16-10A	eBioscience	Cat# 11-0801-82; RRID: AB_465133
PE-CD80 16-10A	eBioscience	Cat# 12-0801-82 RRID: AB_465752
PeCy7-CD86-GL1	eBioscience	Cat# 25-0862-82 RRID: AB_2573372
PerCP-Cy5.5-CD11c N418	eBioscience	Cat# 45-0114-82 RRID: AB_925727
APC-CD40 IC10	eBioscience	Cat# 17-0401-82 RRID: AB_469386
APC-Cy7-MHC class II M5/114.15.2	eBioscience	Cat# 47-5321-82 RRID: AB_1548783
e450-CD4 RM4-5	eBioscience	Cat# 48-0042-82 RRID: AB_1272194
PerCP-Cy5.5 CD4 RM4-5	eBioscience	Cat# 45-0042-82 RRID: AB_1272194
APC-Cy7 CD8 53.7	eBioscience	Cat# 47-0081-82 RRID: AB_1272185
e450 CD8 53-6.7	eBioscience	Cat# 48-0081-82 RRID: AB_1272198
PE CD25 PC61.5	eBioscience	Cat# 12-0251-83 RRID: AB_465608
PerCP-Cy5.5 CD44 IM7	eBioscience	Cat# 45-0441-82 RRID: AB_925746
Pe-Cy7 CD44 IM7	eBioscience	Cat# 25-0441-82 RRID: AB_469623

REAGENT or RESOURCE	SOURCE	IDENTIFIER
FITC CD69 H1 2f3	eBioscience	Cat# 11-0691-82 RRID: AB_465119
FITC IFNγ XMG1.2	eBioscience	Cat# 11-7311-41 RRID: AB_10718840
APC IFNγ XMG1.2	eBioscience	Cat# 17-7311-82 RRID: AB_469504
e450 Proliferation Dye	eBioscience	Cat# 65-0842-85
PerCP 5.5-CD3 17A2	Biolegend	Cat# 100218 RRID: AB_1595492
FITC-NK1.1 PK136	eBioscience	Cat# 11-5941-85 RRID: AB_465319
FITC-B220 RA3-6B2	eBioscience	Cat# 11-0452-85 RRID: AB_465055
e506 Fixable viability dye	eBioscience	Cat# 65-0866-18
e450 CD11c N418	eBioscience	Cat# 48-0114-80 RRID: AB_1548665
FITC-CD3 17A2	eBioscience	Cat# 11-0032-82 RRID: AB_2572431
APC-CD172a P84	eBioscience	Cat# 17-1721-82 RRID: AB_10733158
Brilliant Violet 650- XCR1 ZET	Biolegend	Cat# 148220 RRID: AB_2566410
Brilliant Violet 711- CD64 X54-5/7.1	Biolegend	Cat# 139311 RRID: AB_2563846
PE/Dazzle 594- F4/80 BM8	Biolegend	Cat# 123146 RRID: AB_2564133
FITC-CD11a M17-4	Thermofisher	Cat# 11-0111-82
Alexa700-CD103 2E7	Thermofisher	Cat#:56-1031-80
FITC-FOXP3 FJK-16s	Thermofisher	Cat#:11-5773-82
Pe-Cyanine7-IL-17A eBio17B7	Thermofisher	Cat#:25-7177-82
APC-eFluor 780 CD45 30-F11	Thermofisher	Cat#:47-0451-82
Fixable viability dye eFluor506	Thermofisher	Cat#:65-0866-18
BV650-RORyt RUO	Biolegend	Cat#:564722
PerCP 5.5- CD11b M1/70	Thermofisher	Cat#: 45-0112-82

REAGENT or RESOURCE	SOURCE	IDENTIFIER
PE- B-catenin 15B8	Thermofisher	Cat#: 12-2567-42
APC-Ly6C HK1.4	Biolegend	Cat#: 128015
PerCP 5.5- Ly6G RB6-8C5	BD	Cat#:552093
E450-NKp46 29A1.4	Biolegend	Cat#:137611
Pe- Siglec-F E50-2440	Biolegend	Cat#: 552126
E450- TCRB H57-597	Thermofisher	Cat#: 48-5961-82
Anti-CFTR, CF3	Thermofisher	Cat# MA1-935 RRID:N/A
anti-beta-Actin	Cell Signaling	Cat# 4970S RRID: AB_2223172
Chemicals, Peptides, and Recombinant Proteins		
Lipopolysaccharide, Escherichia coli, serotype 0111:B4	Sigma-Aldrich	L4391-1MG
Heat-killed Staphylococcus aureus	Invivogen	tlrl-hksa
Heat-killed Escherichia coli serotype 0111:B4	Invivogen	tlrl-hkeb2
Zymosan	Invivogen	tlrl-zyn
Zymosan-Depleted	Invivogen	Tlrl-zyd
House dust mite Dermatophagoides pteronyssinus	CITEQ	N/A
SC79 Akt activator	SelleckChem	S7863
Cell stimulation cocktail (500x)	Thermofisher	00-4970-93
Whole Ovalbumin	Worthington	LS003056
Ovalbumin peptide (SIINFEKL)	Biosynth	FO73537
phorbol 12-myristate 13-acetate (PMA)	Calbiochem	524400
Ionomycin	Sigma-Aldrich	10634
Experimental Models: Organisms/Strains		
Mouse: C57BL/6N	Charles Rivers Laboratories	027
Mouse: BALB/c.129P2-*Cftr*^{tm1Unc}	-	Gift
Mouse: FVB/N.129P2-*Cftr*^{tm1Eur}	-	Gift 002364

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Mouse: B6.129P2-*Cftr*^{tm1Unc}. FVB-Tg(FABPCFTR)1Jaw/J	The Jackson Laboratory	004781
Mouse: B6.129P2-Lyz2$^{tm1(cre)Ifo}$/J	The Jackson Laboratory	003755
Mouse: B6.129S4-*Meox2*$^{tm1(cre)Sor}$/J	The Jackson Laboratory	021504
Mouse: B6.Cg-Tg(Vil1-cre)10000Gum/J	The Jackson Laboratory	Gift
Mouse: B6.Cg-Tg(Itgax-cre)1-1Reiz/J	-	Gift
Mouse: B6.129-*Cftr*^{tm1Cwr}	-	004194
Mouse: OTII transgenic mouse	The Jackson Laboratory	
Oligonucleotides		
Ifng_F: ACTGGCAAAAGGATGGTGAC	IDT	N/A
Ifng_R: ATCCTTTTTCGCCTTGCTGT	IDT	N/A
Il1B_F: CAGGCAGGCAGTATCACTCA	IDT	N/A
Il1B_R: AGGTGCTCATGTCCTCATCC	IDT	N/A
Cxcl1_F: CACCTCAAGAACATCCAGAGC	IDT	N/A
Cxcl1_R: CTTGAGTGTGGCTATGACTTCG	IDT	N/A
tnf_F: CATCTTCTCAAAATTCGAGTGACAA	IDT	N/A
tnf_R: TGGGAGTAGACAAGGTACAACCC	IDT	N/A
Gapdh_F: TGCACCACCAACTGCTTAGC	IDT	N/A
Gapdh_R: GGCATGGACTGTGGTCATGAG	IDT	N/A
Software and Algorithms		
GraphPad Prism8	Graphpad	N/A
FlowJo_V10	FlowJo	N/A

3.8.1 Supplemental Figures

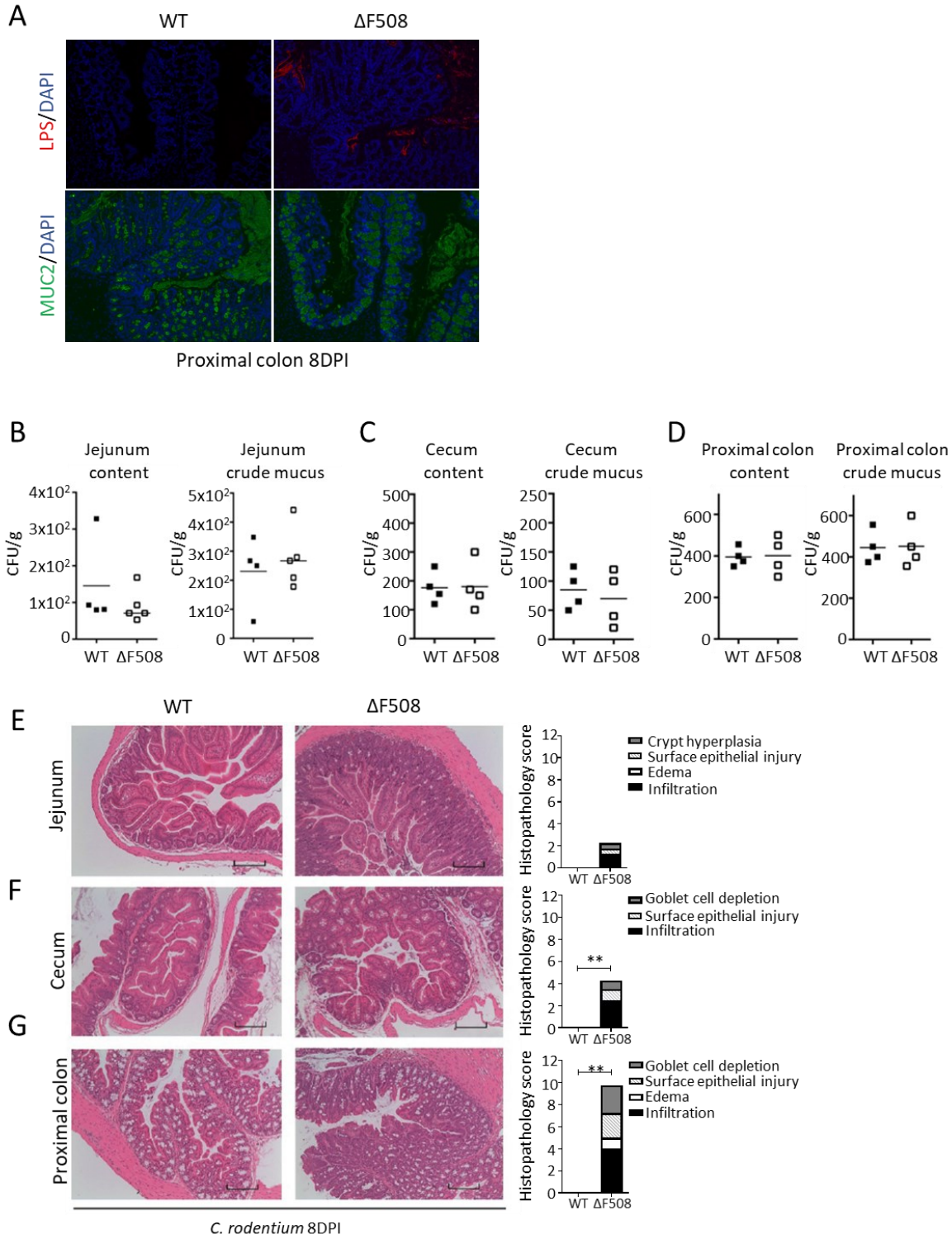

Figure S1: Related to Figure 1: (A) Representative Immunohistochemistry staining images of WT and ΔF508 mouse proximal colon sections stained for LPS (red) , MUC2 (green) and co-stained with DAPI (blue) 8 DPI with *C. rodentium*. Scale bars represent 100 microns. (B-D) Growth of *C. rodentium* in PBS supplemented with diluted (left panels) luminal contents or (right panels) mucus from the (C) jejunum, (D) Cecum and (E) proximal colon of WT and ΔF508 mice (dilution factor 0.01). Data points represent fold-changes compared to growth in non-supplemented PBS from three independent experiments and lines represent the median. (E-G) H and E staining and histopathology scoring of (F) jejunum, (H) cecum and (J) proximal colon tissues of WT (n=4) and ΔF508 (n=4) mice at 8DPI with *C. rodentium* (10X magnification). Scale bars represent 300 microns. Significance was determined by two-tailed unpaired Mann-Whitney test. *$p < 0.05$ **$p< 0.01$

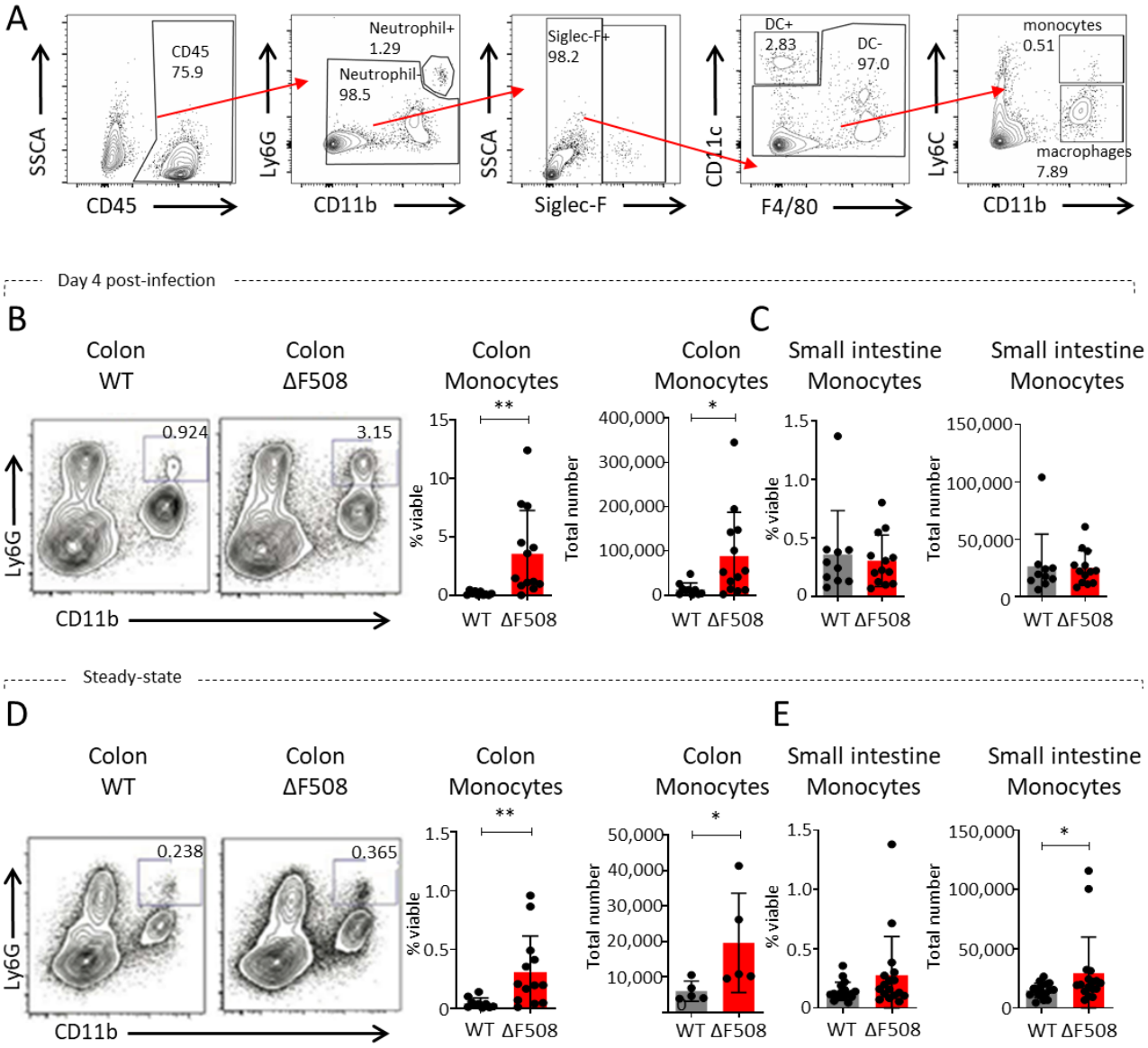

Figure S2: Related to Figure 2: (A) Gating strategy used for identification of CD11b^{+} Ly6G^{+} and CD11b^{+}Ly6C^{hi} monocytes by flow cytometry. Cells were first gated using FSC-H vs SSC-H, followed by single cells using FSC-A vs FSC-H and SSC-A vs Viability Dye. Next the SSC-A vs CD45^{+} cells were gated and Ly6G^{+}CD11b^{+} cells were identified as neutrophils. The non-neutrophils were then gated on Siglec-F- followed by non-dendritic cells. Finally, monocytes were identified by gating on Ly6C^{hi}CD11b^{+} cells as indicated. (B) Representative contour plot, frequency and total number of viable CD11b^{+} Ly6C^{+} monocytes in the colon lamina propria of WT and ΔF508 mice at 4DPI with *C. rodentium* (C) Frequency and total number of viable CD11b^{+}Ly6C^{+} monocytes in the Small intestine of WT (n=10) and ΔF508 (n=13) mice. (D-E) Experiments were performed as in (B-C) but on tissues from WT (n=5) and ΔF508 (n=5) mice at steady-state. Data points represent individual independent experiments and error bars represent SEM. Significance was determined through two-tailed unpaired Mann-Whitney test. *p<0.05 **p<0.01

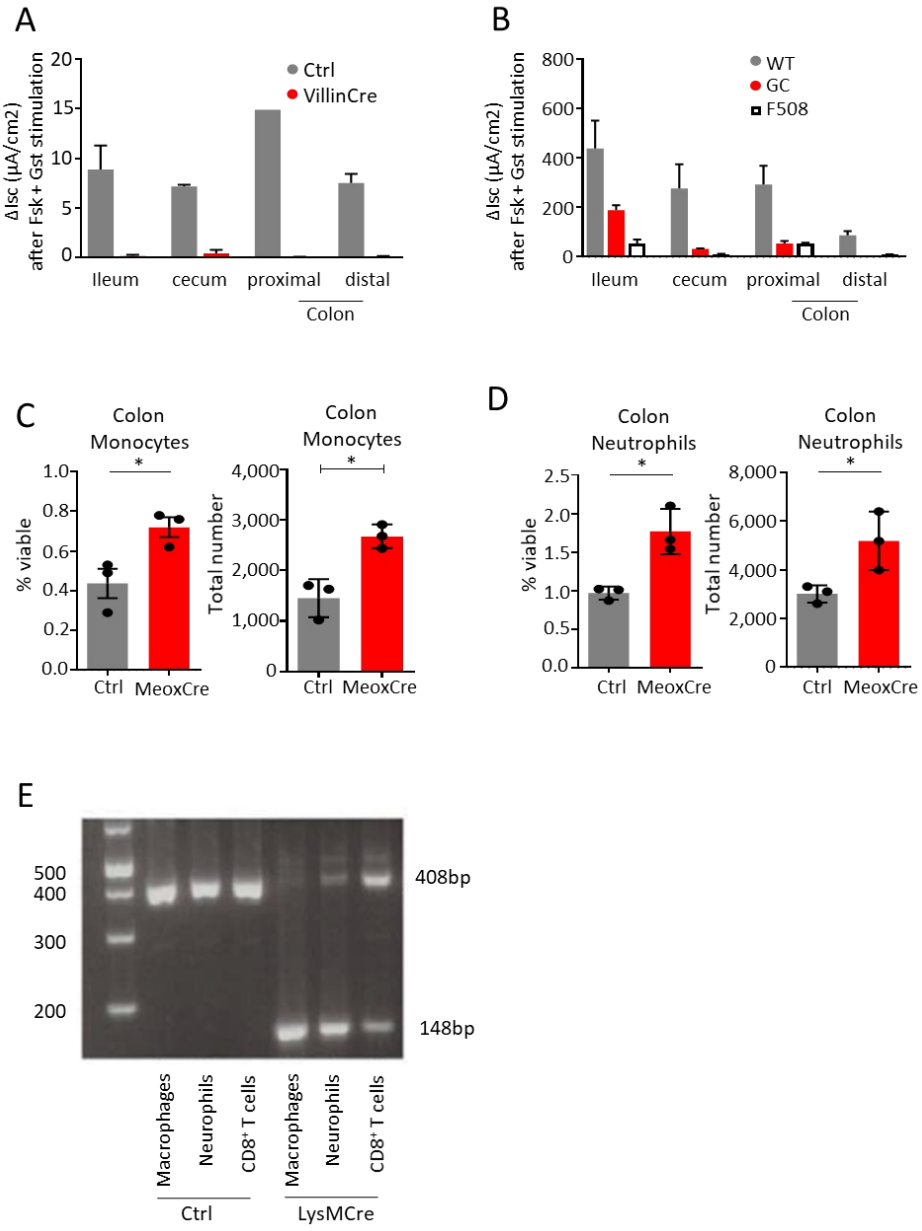

Figure S3: Relates to Figure 3 (A-B) Tissue sections from the Ileum, Cecum, proximal colon, and distal colon were pulsed with forskolin to specifically induce CFTR-mediated chloride ion secretion. Changes in current were then measured to assess CFTR function. (A) Short circuit current response of CFTR in WT (n=4), Gut-corrected mice (n=4) and ΔF508 (n=2) mouse tissues after stimulation with forskolin at steady state. (B) Short circuit current response of CFTR in Control (n=2) and VillinCre (n=2) mouse tissues after stimulation with forskolin at steady state. (C-D) Frequencies and total numbers of (C) viable $CD11b^+$ $Ly6G^+$ neutrophils and (D) $CD11b^+$ $Ly6G^+$ neutrophils in the colon lamina propria of WT and MeoxCre mice at steady state. (E) DNA gel of CFTR expression from macrophages, neutrophils and $CD8^+$ T cells from Control and LysMCre mice. Data shown are representative of at least two independent experiments and error bars represent SEM. Significance was determined by (A-C) one-way ANOVA followed with Sidak's multiple comparison's test or two-tailed unpaired Student's t-test (C-D). *$p < 0.05$ **$p< 0.01$

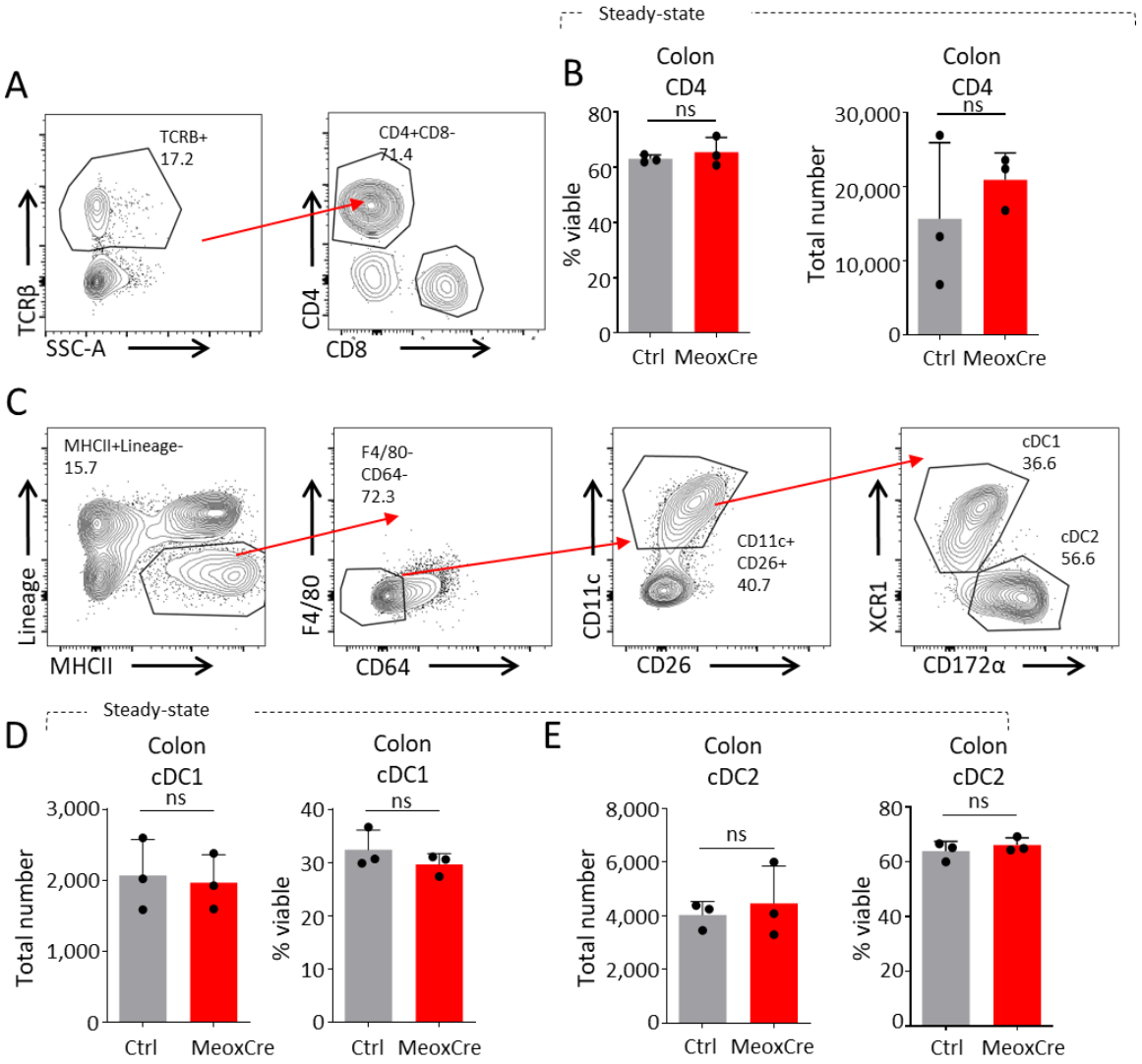

Figure S4: Related to Figure 4 (A) Gating strategy used for gating CD4^{+} T cells from mouse colons. Cells were first gated using FSC-H vs SSC-H, followed by single cells using FSC-A vs FSC-H. Next the TCRβ^{+} cells were gated (see methods for antibody list), followed by CD4^{+} cells. (B) Frequency and total number of viable CD4^{+} T cells in the Colon of WT (n=3) and MeoxCre (n=3) mice. (C) Gating strategy used for gating cDC1s and cDC2s from mouse colons. Cells were first gated using FSC-H vs SSC-H, followed by single cells using FSC-A vs FSC-H. Next the lineage negative, MHCII^{+} cells were gated (see methods for antibody list), followed by CD64^{-} F480^{-} cells and then gated on CD11c ^{hi} CD26^{+} cells. Finally, XCR1 vs CD172a were used to identify cDC1s and cDC2s as indicated. (D-E) Frequency and total number of viable (D) cDC1s and (E) cDC2s T cells in the Colon of WT (n=3) and MeoxCre (n=3) mice. Data shown are best representative of individual independent experiments (n=3) and error bars represent SEM. Significance was determined through two-tailed unpaired Student's t-test. *p<0.05 **p<0.01

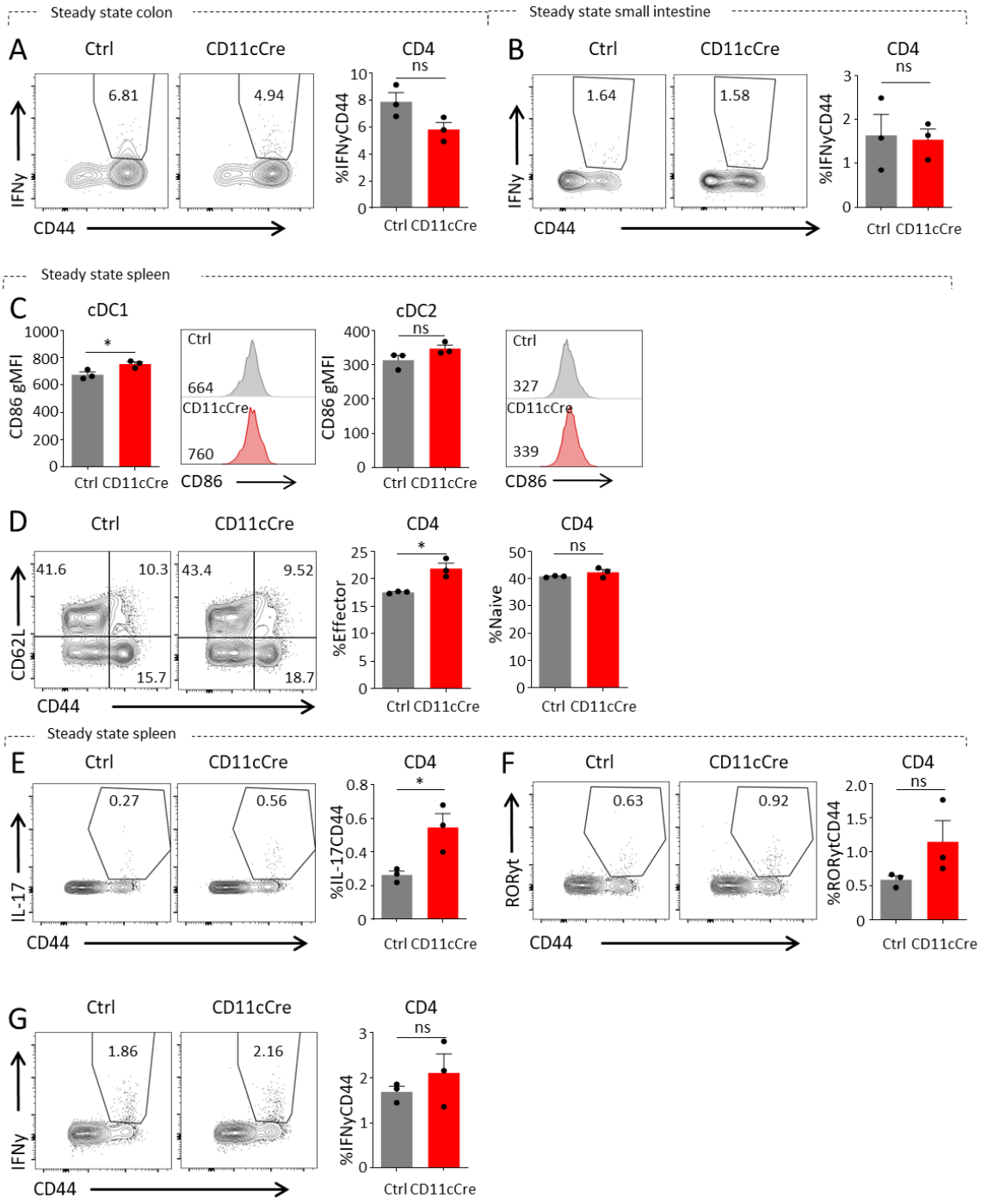

Figure S5: Related to Figure 5: (A-B) Representative contour plots and frequencies of IFNγ^{+}CD44^{+} (A) colonic, (B) small intestinal CD4^{+} T cells from control and CD11cCre mice at steady state. (C-G) Experiments were performed as in Figure 5 but with splenic CD4^{+} T cells from control and CD11cCre mice. Data shown are best representative of individual independent experiments (n=3) and error bars represent SEM. Significance was determined through two-tailed unpaired Student's t-test. *p<0.05 **p<0.01

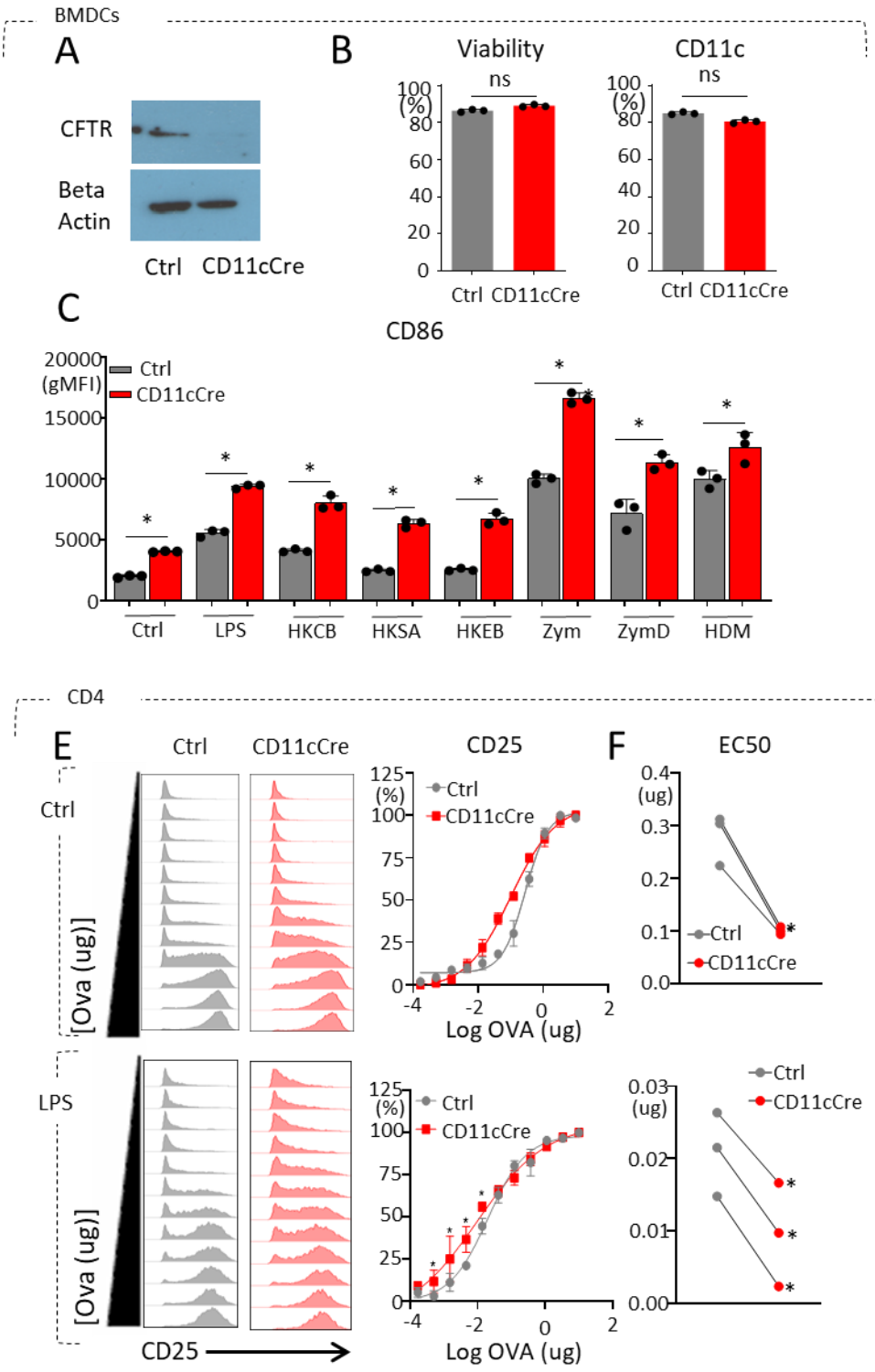
BMDCs
A
CFTR
Beta Actin
Ctrl CD11cCre
B
Viability
CD11c
ns
100 (%) 80 60 40 20 0
Ctrl CD11cCre
C
CD86
20000 (gMFI) 15000 10000 5000 0
Ctrl
CD11cCre
Ctrl LPS HKCB HKSA HKEB Zym ZymD HDM
CD4
E
Ctrl
CD11cCre
Ctrl
[Ova (ug)]
CD25
125 (%) 100 75 50 25 0
-4 -2 0 2
Log OVA (ug)
F
EC50
0.4 (ug) 0.3 0.2 0.1 0.0
LPS
[Ova (ug)]
CD25
0.03 (ug) 0.02 0.01 0.00

Figure S6: Related to Figure 6: (A) Expression of CFTR and beta-Actin in BMDCs from Control and CD11cCre mice by western blot. (B) Percentage of (left panel) viable cells measured through 7-AAD staining and (right panel) %$CD11c^+$ BMDCs generated from control and CD11cCre mice measured by flow cytometry. Data represents the mean percentage of cells that were negative for 7-AAD staining or positive for CD11c staining. Data points represent the mean % positive population and represent one representative of at least three independent experiments. (C) Cell surface expression of CD86 by BMDCs generated from control and CD11cCre mice examined at steady state or after 18 h of LPS treatment (100 ng/mL), 2.5 × 107 cells/mL HKCB (heat-killed Citrobacter rodentium), 2.5 × 107 cells/mL HKSA (heat-killed Staphylococcus aureus), 2.5 × 107 cells/mL HKEB (heat-killed Escherichia coli B), 10 μg/mL Zymosan, 20ug/ml of Zymosan Depleted or 50ug/ml of HDM (house dust mite). Data represents geometric mean fluorescence intensity (gMFI) of best representative of individual independent experiments (n = 3), and error bars represent SEM. (D) Experiments were performed as in Figure 6 but cells were stained for IFNγ via intracellular staining (ICS). (E-F) Percentage of $CD25^+$ $CD4^+$ OTII T cells co-cultured with BMDCs from control or CD11cCre mice (top panels) with or (bottom panels) without LPS stimulation for 6 h pulsed with titrated concentrations of whole OVA. Data were normalized to the proportion of $CD25^+$ cells at saturating OVA concentration (10 μg) and are best representative of three individual experiments. Significance was determined through two-way ANOVA. $*p < 0.05$. (F) EC50 for $CD25^+$ of $CD4^+$ OTII T cells co-cultured with BMDCs from control or CD11cCre mice (top panels) with or (bottom panels) without LPS stimulation for 6 h pulsed. Data in (B) show EC50 values for three individual experiments. Significance achieved for individual experiments is denoted by an asterisk (∗); however, significance was not achieved by combining all experiments due to the variable EC50 in each experiment. Data shown are best representative of individual independent experiments (n=3) and error bars represent SEM. Significance was determined through (B) two-tailed unpaired Student's t-test and (C-J) one-way ANOVA followed with Sidak's multiple comparison's test. *p<0.05 **p<0.01

Chapter 4: Discussion

4.1 Overview

The DC response to stimuli involves a transition from a steady-state phenotype to an activated one, leading to numerous functional changes that promote the differentiation of T-cell lineages. The exact molecular mechanisms that regulate this transition are not well understood. In this thesis, we discovered two novel mechanisms of regulation of DC activation. In Chapter 2, we established the role of miR-9 in promoting DC activation through rapid reduction of the expression of multiple negative regulators. Data presented in Chapter 3 demonstrate the novel role of CFTR in regulating DC activation through modulating anti-inflammatory PI3K-Akt signaling. In summary, this work adds to our understanding of the molecular mechanisms that regulate DC function.

4.2 miR-9 and CFTR are novel regulators of DC activation

As described in Chapter 1, an immense network of molecular mechanisms potently regulate DC activation and function. Despite the complexity of this system of regulation, modulation of single genes has been shown to drastically disrupt DC activation and function [320, 366, 625]. Adding to this body of work, we have shown that modulation of either miR-9 or CFTR has drastic consequences to DC function.

4.2.1 miR-9 promotes DC activation by governing the expression of programs of regulation

In Chapter 2, we showed that increasing the expression of a single microRNA, miR-9, promoted the activation of DCs and greater DC anti-tumour responses in the B16- melanoma

model. Pathway analysis of the putative targets of miR-9 revealed an enrichment for epigenetic regulators, including many factors known to epigenetically silence genes necessary for DC activation [354, 357, 663]. Among these targets was PCGF6, a known negative regulator of DC activation, which mediates demethylation of H3K4Me3 marks on the promoters of key inflammatory genes [366]. We showed that miR-9 targets PCGF6, but reduced expression of PCGF6 did not account for the full phenotype observed in miR-9 expressing DCs. We instead found that miR-9 also reduced the expression of many other epigenetic regulators, which collectively contributes to the greater activation of miR-9 expressing DCs.

miRNAs represent a small percentage of the genes transcribed in mammals, but sixty percent of the genome has been computationally predicted to be regulated by miRNAs [395, 681]. Many reports have observed that single miRNAs regulate programs of expression in a variety of cell contexts [399-401]. Our data suggest that miR-9 promotes DC activation not by inhibiting the expression of a single gene, like the potent suppressor of DC activation PCGF6, but by downregulating the expression of a program of gene silencing in DCs. miR-9 is known to regulate the chromatin landscape in neural progenitors, promoting differentiation into mature neurons by inhibiting epigenetic silencing [434-436]. Since DCs are potently transcriptionally regulated by changes in the epigenetic landscape [340, 368, 369], we speculated that miR-9 also governed the chromatin landscape in DCs. We found that miR-9 expression increased global H3K4me3 marks, which are associated with open chromatin and gene transcription, likely through targeting of PCGF6. Analysis of the expression of other histone marks, particularly marks associated with gene silencing such as H3K27me3 or H3K9me3, can provide further surface level information on the state of chromatin accessibility in miR-9 expressing DCs.

However, analysis by assay for transposase-accessible chromatin using sequencing (ATAC-seq) would provide a more complete and high-resolution analysis of the chromatin landscape in DCs expressing miR-9 or the miR-9 sponge.

Although our work focused on epigenetic regulators, pathway analysis of predicted miR-9 targets indicated that miR-9 may also regulate DC function through a variety of other pathways. In particular, we noted an enrichment of targets that are involved in mRNA stability [728-730]. Pro-inflammatory cytokine mRNA is naturally unstable and is rapidly degraded in steady state DCs through the action of RNA binding proteins (RBPs) and RNA-degrading complexes [374]. For example, the RBP tristetraprolin (TTP) represses DC activation by promoting the decay of CD86 and pro-inflammatory cytokines mRNA by recruiting deadenylation and de-capping complexes [731]. PRR signaling is known to increase stability of activation-induced mRNA by modifying the expression or function of RNA degrading complexes, in order to minimize inflammation in prolonged immune responses [374]. PRR signaling suppresses the function of TTP and promotes increased cytokine mRNA stability in activated DCs [732]. TTP mediates degradation of mRNA in part by recruiting the CCR4-NOT deadenylase complex, which removes the poly(A) tail from the 3' end of target mRNAs to promote their degradation [733]. We found that miR-9 putatively targets CNOT1, CNOT6L and, XRN-1, all of which are involved with CCR4-NOT complex [728-730]. We speculate that miR-9 may inhibit the function of the CCR4-NOT complex early upon stimulation, promoting the secretion of pro-inflammatory cytokines in DCs.

High miR-9 expression has been found to correlate with lesions associated with multiple inflammatory diseases in mice and humans [441-443]. For example, one group found that in a

mouse model of multiple sclerosis, mir-9 expression was significantly upregulated in both the spinal cord and the urine of mice at the peak of disease [443]. Another group studying IBD in humans, found that unaffected colon tissue expressed significantly less miR-9 than adjacent inflamed tissue [441]. Dysregulated DC activation is a significant contributor to MS and IBD pathology [556, 557, 734], so we speculated that high miR-9 expression in inflamed tissue could act as a biomarker for dysregulated DC activation. In agreement with this, our results show that miR-9 expression promoted splenic cDC1 activation and function *ex vivo.* However, in performing complementary experiments inhibiting miR-9 function in cDCs, we found that the miR-9 locked nucleic acid (LNA) and control LNA promoted background activation of splenic cDC subsets. With these limitations to our *ex vivo* studies, we propose to use *in vivo* experiments to study the effects of miR-9 in regulating DC activation and promoting tissue inflammation.

Many groups have shown that miRNA mimics can be delivered systemically in lipid-based nanoparticles to deliver specific miRNAs *in vivo* [735, 736]. However, this form of miRNA delivery would be non-specific and it has been shown to vary in terms of effectiveness [737]. An alternative strategy would be to create fetal liver chimeras via transduction of fetal liver cells with miR-9-overexpressing vectors and reconstitution of lethally irradiated mice to create miR-9-overexpressing mice [738]. Similarly, transduction of fetal liver cells with the miR-9-sponge would enable complementary experiments on reduced miR-9 function to be performed that were impossible with LNA delivery *ex vivo*. Although this method is restricted to hematopoietic cells, unlike miRNA mimic delivery, it would still be difficult to study whether miR-9 promotes inflammation specifically through dysfunctional DC activation as all immune cells would have modulated miR-9 expression.

A more direct strategy for studying miR-9 specifically in tissue cDCs would be to use DC-specific miR-9 knockout mice. However, it is unclear which of the three primary miR-9 transcripts is responsible for the mature miR-9 expression induced by TLR ligation. Analysis of transcription factor binding sites showed that only pri-miR-9-1 contained an NF-kB site, suggesting that pri-miR-9-1 expression could be induced by TLR signaling. However, we found that all three primary miR-9 transcripts were upregulated after cDCs were stimulated with LPS. Thus, it is possible that LPS stimulation could be promoting mature miR-9 expression from all three primary miR-9 transcripts. Therefore, it would likely be necessary to delete all three primary miR-9 transcripts to be certain that mature miR-9 expression is inhibited. Although cumbersome to create, DC-specific knockout mice would allow direct interrogation of the effects of miR-9 expression in cDCs *in vivo* and provide further evidence of the role of miR-9 in promoting inflammatory disorders through dysregulated cDC activation.

4.2.2 CFTR regulates DC activation potentially through PI3K-Akt signaling

Data presented in Chapter 3 establishes a clear role for CFTR in regulating DC activation and function. BMDCs that lacked CFTR expressed significantly higher levels of surface activation markers and preferentially secreted increased Th17-biased cytokines after stimulation with LPS. In accordance with these results, we observed that CD11c-specific loss of CFTR *in vivo* led to the generation of a Th17-biased $CD4^+$ T cell response and induced significant inflammation in the gastrointestinal tract of mice.

DCs are involved in both promoting immune responses to microbes and in preventing harmful inflammatory reactions to non-harmful substances [450-452]. This has been shown to

be particularly crucial in the intestinal tract, where intestinal cDCs are maintained in a tolerogenic state to prevent effector responses against the gut microbiota but can efficiently transition to an activated state upon pathogen encounter [205, 450]. Multiple groups have shown that dysregulated intestinal cDC function plays a role in intestinal inflammatory disorders, including IBD and celiac disease [556, 557]. CF disease is associated with increased inflammation in the gastrointestinal tract that shares many features with IBD and our data provides evidence of the role of dysregulated DC activation in promoting this inflammation. One caveat to our findings is that we utilized CD11c-driven Cre CFTR floxed mice, which does not completely restrict Cre expression to DCs. This promoter will also drive Cre expression in other cell types, such as monocytes, macrophages and B cells [739]. However, our results in LysMCre mice indicated that loss of CFTR in monocytes, neutrophils, and macrophage caused no signs of inflammation at steady state. Further experiments could be done in Zbtb46-driven Cre CFTR floxed mice, which would specifically remove CFTR from cDCs, to be certain that CFTR loss in other cell types is not responsible for our observed phenotypes.

Importantly, we observed that loss of CFTR led to a reduction in PI3K-Akt signaling in BMDCs and that treatment with an Akt activator was able to reverse this phenotype. PI3K-Akt signaling is known to be induced by TLR stimulation, but the exact role of this pathway in DC function is controversial [646, 740, 741]. Multiple groups have shown that PI3K-Akt signalling reduces pro-inflammatory cytokine secretion in BMDCs by acting in a negative feedback loop with TLR signaling [723, 740, 742]. However, other reports have shown that TLR-induced PI3K-Akt signaling plays important roles in cellular processes critical for DC function such as phagocytosis, migration, and metabolism [741, 743-745]. A possible explanation for these

inconsistent findings is that different PI3K isoforms perform specific roles in DC function [746]. For example, specifically inhibiting PI3Kδ increases pro-inflammatory IL-12 secretion in DCs [742], whereas inhibition of PI3Kγ reduced DC activation and migration [744]. Further work has shown that PI3Kδ signaling promotes the expression of IL-10 in DCs but also promotes the internalization of TLR4 post-stimulation, preventing overstimulation of DCs by TLR-agonists [742]. These results suggest that CFTR may be interacting specifically with PI3Kδ signaling, but further work would need to be done to clearly establish this mechanism. Interestingly, PI3Kδ deficiency in mice led to progressively worsening colitis that was dependant on the presence of the microbiome [747]. Human Lymphoma patients on PI3Kδ-specific inhibitors also develop intestinal colitis as a prominent side effect of prolonged use [748, 749]. As discussed in Chapter 1, tolDCs play a critical role in promoting tolerance to the microbiota and dysregulation of tolDC function has been shown to induce colitis in mice [747]. It is possible that dysregulated PI3Kδ signaling in CFTR KO DCs is promoting aberrant tolDC function or inhibiting tolDC differentiation.

It is currently not well understood how CFTR interacts with PI3K-Akt signalling in DCs. One possible mechanism is through sphingosine-1-phosphate (S1P), a potent bioactive sphingolipid that regulates immune cell processes critical for inflammation and immune responses [750]. S1P signals primarily through extracellular surface receptors but can also be imported into the cell to engage in intracellular signaling [750, 751]. Intracellular S1P signaling has been shown to promote anti-inflammatory PI3K-Akt signaling in epithelial cells [752-754]. Furthermore, recent evidence has shown that S1P transport is in part regulated by CFTR [755]. DCs express all five extracellular receptors for S1P, and S1P signaling has been shown to

modulate DC migration and function [607]. BMDCs have also been shown to import S1P, which coupled with S1P export, can be used to regulate intracellular and extracellular S1P levels [756, 757]. Excessive extracellular S1P signaling due to defective S1P import promotes DC activation, pro-inflammatory cytokine secretion, and reduced PI3K-Akt signaling [758-760]. We propose to examine the S1P signaling pathway in BMDCs to determine whether CFTR regulates PI3K-Akt activation through dysfunctional S1P signaling and import.

4.3 cDC1 and cDC2 activation and function are differentially regulated by miR-9 and CFTR

We found evidence of distinct regulatory mechanisms of DC activation in cDC1s and cDC2s throughout the present work. In Chapter 2, we observed that transfection with the miR-9 mimic promoted the activation and function only in splenic cDC1s, with no significant difference measured in splenic cDC2s. Furthermore, we found that transfection of the miR-9 mimic in cDC2s did not decrease the expression of miR-9 targets, as was observed in cDC1s. In Chapter 3, CD11c-specific loss of CFTR promoted splenic cDC1 activation but not splenic cDC2 activation. However, CFTR loss did promote the activation of both cDC1s and cDC2s in the gastrointestinal tract.

One possible explanation for our observations in Chapter 2 is that miR-9 is less functional in cDC2s compared to cDC1s. Multiple long non-coding RNAs (lncRNAs) can acts as natural miRNA sponges and prevent miRNAs from binding to target mRNAs. For example, the lncRNA nuclear paraspeckle assembly transcript 1 (NEAT1) was shown to be highly expressed in cervical cancer cells and was able to directly inhibit miR-9 function thereby promoting survival

and growth [680]. Similarly, DCs stimulated with LPS upregulate the expression of the lncRNA Metastasis Associated Lung Adenocarcinoma Transcript 1 (MALAT1) to inhibit the function of miR-155 and reduce DC activation [761]. We speculate that cDC2s may express more miR-9 inhibiting lncRNA, such as Neat1, than cDC1s.

An alternative explanation is that PRR signaling induces different activation pathways in cDC1s and cDC2s. Our data supports this, as we observed that LPS stimulation only induced miR-9 expression in cDC1s and not in cDC2s. Furthermore, we observed that LPS stimulation led to downregulation of all examined putative miR-9 targets in splenic cDC1s but not all targets were downregulated in splenic cDC2s, including genes known to negatively regulate DC activation. cDC1s and cDC2s are known to express different TLRs, which is thought to relate to the different effector responses generated by these subsets [47]. These results suggest that PAMPs induce different TLR signaling pathways depending on the cDC subsets. For example, LPS stimulation of DCs primarily promotes a Th1 effector response, which is induced primarily by cDC1s, and could explain why LPS stimulation induces miR-9 expression in cDC1s but not cDC2s.

Our data in Chapter 3 showed that CFTR loss promoted cDC2 activation depending on microenvironmental factors. CD11c-specific loss of CFTR promoted the activation of intestinal cDC2s but did not significantly alter the activation of splenic cDC2s. A possible explanation for these findings is the recent discovery that cDC2s are a heterogenous group that can be divided into anti-inflammatory cDC2As expressing T-Bet and pro-inflammatory cDC2Bs expressing RORγt [47]. This differentiation was found to be induced by microenvironmental factors, with cDC2As enriched on mucosal surfaces and cDC2Bs enriched in lymphoid tissue. Antibiotic

treatment reduced the levels of cDC2As in the gastrointestinal tract but not in the spleen, suggesting that microbial signals are likely needed to induce cDC2A [47]. Perhaps the difference in CFTR mediated regulation of the activation of cDC2s in the spleen and intestinal tract is due to differences in regulation of cDC2As and cDC2Bs. It is also possible that if we expressed miR-9 in cDC2s derived from other tissues, such as the intestinal tract, we would observe a greater effect of miR-9 to promote cDC2 activation.

4.4 Therapeutic applications

Since the 1990s, multiple clinical trials have used DCs as cell-based therapeutic vaccines for anti-cancer therapy [762, 763]. These strategies involve using DCs derived from human cancer patients, which are pulsed with tumour antigens and then injected back into the patient to induce anti-tumour immune responses [764]. Unfortunately, DC-based vaccines have been shown to not be very effective in inducing anti-tumour immune responses in all but a small subset of patients [762, 763, 765]. This low efficacy has been attributed to multiple factors, including the choice of tumour antigen, improperly stimulated DCs, and the immunosuppressive effects of the tumour microenvironment (TME) [766]. The TME contains many suppressive cytokines and factors, which recruit immunosuppressive Tregs and induce tolDC differentiation [766]. A variety of strategies has been employed to counteract the suppressive effects of the TME on DCs, including promoting DC activation through addition of different PRR agonists and inhibition of suppressive pathways that regulate DC activation [766-768]. For example, one group observed that the effectiveness of a DC-based breast cancer vaccine was enhanced by promoting miR-155 expression [768]. Mice injected with miR-155-

expressing DCs showed drastic reduction of tumour sizes and increased anti-tumour effector responses [768]. Our work highlights two novel molecular regulators of DC activation, miR-9 and CFTR, that can potentially be utilized to boost DC function in the TME. We have shown that miR-9 downregulates the expression of multiple negative regulators of DC activation, potentially inhibiting multiple suppressive pathways that are promoted by factors in the TME. For example, we have shown that miR-9 expression significantly reduces TGFBR1, the receptor for the suppressive cytokine TGFβ, which is involved in suppressive signaling induced by the TME of certain tumours [769] . Similar to miR-155, our data provide direct evidence that mir-9 expression in BMDCs promotes anti-tumour $CD8^+$ T cell responses against B16 melanoma, highlighting the potential of miR-9 for use in human DC-based vaccines. We have also shown that miR-9 mimics can be easily transfected into sorted cDC1s and can potently promote their activation and function. In Chapter 3, we observed that CFTR regulates DC activation potentially through modulating anti-inflammatory PI3K-Akt signaling. A recent study showed that inhibiting PI3Kδ signaling promoted anti-tumour immunity in a mouse colon tumour model [770]. This study didn't examine the effects of inhibiting PI3K signaling in DCs but we speculate, based on our results, that inhibiting PI3K signaling could promote anti-tumour immune responses by promoting DC activation and function.

4.5 Conclusion

In summary, this work highlights the novel functions of miR-9 and CFTR as regulators of DC activation and function. We established that mir-9 promotes DC activation through negative regulation of the expression of multiple factors known to be involved in suppressing immune

responses. Furthermore, we determined that absence of CFTR promotes dysregulated intestinal cDC activation, possibly through regulation of PI3K-Akt signaling. This work expands our knowledge of the complex processes critical for the transition from the steady state towards activation of DCs and provides further insight into potential new therapeutics for reducing dysregulated DC activation in diseases such as CF.

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