Negative regulation of dendritic cell activation and function by the transcriptional repressor polycomb group factor 6

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Preface

Abstract

Dendritic cells (DCs) are professional antigen presenting cells of the innate immune system. They are finely tuned to sense invading pathogens and danger signals. The initiation of appropriate T cell activation, expansion and effector function relies on the delivery of three signals from DCs: antigen presentation, costimulation and cytokine production. The combination of these three signals determines the type and magnitude of the T cell response. Activation of DCs is accompanied by rapid and global transcriptional reprogramming mediated, in large part, by epigenetic modification downstream of signal transduction pathways. Intrinsic mechanisms exist to restrain DC activation in the absence of proinflammatory stimuli. The polycomb group factor, PCGF6 forms a complex that represses transcription through chromatin modification. In a microarray screen, PCGF6 was found to be downregulated in DCs in response to LPS. We hypothesized that PCGF6 inhibits genes associated with inflammation to maintain DC homeostasis. We characterized the function of PCGF6 by manipulating its expression in bone-marrow derived DCs. We found that PCGF6 downregulation was necessary for optimal DC activation and T cell priming. Through its interaction with the lysine demethylase JARID1c, PCGF6 suppresses key sites related to inflammation by regulating H3K4me3 levels and chromatin accessibility. PCGF6 downregulation made binding sites for the transcription factor EGR1 available for binding. EGR1 binding sites were enriched at genes involved in immune function and lipid metabolism. Finally, we found that PCGF6 in DCs regulated the antigen sensitivity of $CD8^+$ T cells through PDL1. This work highlights the novel function of the epigenetic factor PCGF6 in dynamic regulation of DC activation and function.

Résumé

Les cellules dendritiques (CD) sont des cellules présentatrices d'antigènes, dites 'professionnelles', du système immunitaire inné. Elles sont largement adaptées à percevoir la présence de pathogènes invasifs et à reconnaître des motifs moléculaires associés aux dangers. L'initiation d'une activation appropriée des lymphocytes T, de leurs expansions et de leurs fonctions effectrices dépend de trois signaux de la part des CDs : la présentation d'antigènes, la co-stimulation et la production de cytokines. La combinaison de ces trois signaux détermine le type et la magnitude de la réponse des lymphocytes T. L'activation des CDs est accompagnée d'une rapide reprogrammation transcriptionelle globale déterminée, en grande partie, par des modifications épigénétiques en aval de voies de transduction signalétique. Des mécanismes intrinsèques existent pour restreindre l'activation de CDs en l'absence de stimuli pro-inflammatoire. Le polycomb-group factor, PCGF6 forme un complexe qui réprime la transcription suite à la modification de chromatine. Dans un dépistage à l'aide de biopuces, PCGF6 s'est avéré être régulé négativement dans les CDs en réponse au LPS. Nous avons émis l'hypothèse que PCGF6 inhibe les gènes associés avec l'inflammation pour maintenir l'homéostasie des CDs. De plus, nous avons caractérisé la fonction de PCGF6 en manipulant son expression dans des CDs dérivées de la moelle osseuse. Nous avons donc déterminé que la régulation négative de PCGF6 est nécessaire pour l'activation optimale des CDs et pour l'amorçage des lymphocytes T. Grâce à son interaction avec la déméthylase spécifique de la lysine JARID1c, PCGF6 réprime les sites de transcription clés liés à l'inflammation en régulant les niveaux d'H3K4me3 et l'accessibilité de la chromatine. La régulation négative de PCGF6 permet aux sites de

liaison du facteur de transcription EGR1 d'être disponibles à la liaison. Les sites de liaisons d'EGR1 étaient enrichis au niveau de gènes impliqués dans la fonction immunitaire et le métabolisme lipidique. Finalement, nous avons déterminé chez les CDs que PCGF6 régulait la sensitivité antigénique des lymphocytes $CD8^+$ par l'entremise de PDL1. Ce travail met donc en évidence la fonction novatrice du facteur épigénétique PCGF6 dans la régulation dynamique de l'activation et de la fonction des CDs.

Acknowledgements

There are so many people who have been instrumental in providing their support both technically and emotionally for the completion of this work.

First, I would like to thank my supervisor, Dr. Connie Krawczyk, for her continued guidance and support over the course of my degree. Connie is a brilliant scientist with a contagious excitement for discovery and a keen ability to spark collaboration and discussion among her peers and students. Her multi-disciplinary approach and open-mindedness to new ideas is refreshing and I could not have asked for a better learning environment for my PhD. She has pushed me to constantly strive for self-improvement and through her mentorship I have become a better scientist and a better person.

I would like to thank all of my lab mates who have been amazing colleagues and friends over the years. I feel incredibly fortunate to have worked with these people; together we created this environment of support and friendship that is unparalleled and that I will sorely miss. They have all contributed in various ways to the completion of this thesis. Alborz Borjian who patiently trained me at a time when the only thing I knew about immunology was that it seemed complicated. Hannah Guak who is one of the most

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Research publications

This is a manuscript-based thesis. The work presented in this thesis has been published or will be published in the following publications:

Chapter 1: Literature review

Part of Chapter 1 will be published in an invited review as part of a series for Fronteirs Immunology called "Looking Beyond Pattern Recognition: Perturbations in Cellular Homeostasis and Metabolism as Emerging Regulators of Dendritic Cell Function":

G.M. Boukhaled, H. Guak, M. Corrado, C.M. Krawczyk. Maintaining DC homeostasis through epigenetic modification: beyond development and differentiation. Frontiers Immunology, 2018. *Manuscript in preparation.*

Chapter 2: **G.M. Boukhaled**, B. Cordeiro, G. Deblois, V. Dimitrov, S.D. Bailey, T. Holowka, A. Domi, H. Guak, H.C. Chiu, B. Everts, E.J. Pearce, M. Lupien, J.H. White, and C.M. Krawczyk (2016). The transcriptional repressor Polycomb group factor 6 (PCGF6) negatively regulates dendritic cell activation and promotes quiescence. Cell Reports 16(7), 1829-1837.

Chapter 3: **G.M. Boukhaled**, G. Deblois, S. Bailey, M. Lupien, C.M. Krawczyk. PCGF6 dynamically regulates the availability of Egr1 transcription factor binding sites to fine-tune TLR-mediated maturation. *Manuscript in preparation, to be submitted by end of 2018.*

Chapter 4: **G.M. Boukhaled**, H. Guak, L. Smith, H. Elsaesser, D. Brooks, M. Richer, C.M. Krawczyk. Cooperative and independent control of immunosuppressive programs in DCs by PCGF6 and PDL1. *Manuscript in preparation. To be submitted in fall 2018*

Other research contributions not appearing in this thesis

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

L.K. Smith, **G.M. Boukhaled**, S.A. Condotta, S. Mazouz, J.J. Guthmiller, R. Vijay, N.S. Butler, J. Bruneau, N.H. Shoukry, C.M. Krawczyk and M.J. Richer (2018). Interleukin-10 Directly Inhibits $CD8^+$ T Cell Function by Enhancing N-Glycan Branching to Decrease Antigen Sensitivity. Immunity 48(2), 299-312.

V. Dimitrov, M. Bouttier, **G. Boukhaled**, R. Salehi-Tabar, R. Avramescu, B. Memari, B. Hasaj, G.L. Lukacs, C.M. Krawczyk, and J.H. White (2017). Hormonal vitamin D upregulates tissue-specific PD-L1 and PD-L2 surface glycoprotein expression in human but not mouse. Journal of Biological Chemistry. doi:10.1074/jbc.M117.793885

E.H. Ma, G. Bantug, T. Griss, S. Condotta, R.M. Johnson, B. Samborska, N. Mainolfi, V. Suri, H. Guak, M.L. Balmer, M.J. Verway, T.C. Raissi, H. Tsui, **G. Boukhaled**, S. Henriques da Costa, C. Frezza, C.M. Krawczyk, A. Friedman, M. Manfredi, M. J. Richer, C. Hess, and R.G. Jones (2017). Serine Is an Essential Metabolite for Effector T Cell Expansion. Cell Metabolism 25(2): 482.

Contributions of authors

I performed and analyzed experiments for the majority of the data presented in this thesis. Connie Krawczyk and I conceived and designed experiments for all chapters. I wrote this thesis and I designed the majority of the figures presented. The work presented here was thoughtfully edited by Hannah Guak and Connie Krawczyk. The abstract was translated by Jean-Alexandre Sauvé. The specific contributions of authors for each chapter are outlined below.

Chapter 2

I prepared retrovirus, grew and transduced BMDCs, performed all T cell assays,

flow cytometry and ELISA experiments. I also performed the Seahorse experiment. ChIP-qPCR was performed by Vassil Dimitrov. Genevieve Deblois and I performed the ATAC experiment together. Genevieve Deblois and Swenke Bailey did the bioinformatics analyses of the ATAC-sequencing data. Genevieve Deblois assembled Figure 4A-B and edited the manuscript. The IP was performed by Brendan Cordeiro. Brendan Cordeiro, Hannah Guak, Anisa Domi, Clare Chiu, and I performed the RNA extractions, RT-qPCR experiments. Experiments were designed and interpreted by Connie Krawczyk and I with input from Genevieve Deblois for the sequencing experiment. The manuscript was written by Connie Krawczyk and I.

Chapter 3

I prepared retrovirus, grew and transduced BMDCs. ATAC analyses were performed by Genevieve Deblois and Swenke Bailey. HOMER analysis was performed by Genevieve Deblois. I performed GSEA and assembled Figures 2-4. I adapted Figure 1 and S1 from data and analyses provided by Genevieve Deblois. I performed all T cell assays, BMDC activation and flow cytometry experiments. I wrote the manuscript and it was edited by Connie Krawczyk and Hannah Guak.

Chapter 4

I performed all antigen sensitivity and conjugate assays. I generated fetal liver chimeras. Hannah Guak and I prepared retrovirus, grew and transduced BMDCs. I performed all RNA extractions and RT-qPCR. Hannah Guak performed intracellular cytokine staining of PDL1-overexpressing DCs and provided data presented in Figure 2D-E. Logan Smith and Hannah Guak provided other miscellaneous experimental support. Connie Krawczyk and I designed and interpreted experiments. Hannah Guak

also provided input in terms of data interpretation. I wrote the manuscript and it was edited by Connie Krawczyk and Hannah Guak.

Contributions to original knowledge

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

- Prior to this study, there was no known role for PCGF6 nor its binding partner JARID1c in immune cell function. Therefore, ours is the first to identify a role for both of these proteins in regulating DC homeostasis.
- This study contributes strong evidence that one mechanism by which DCs restrain their activation is through tonic transcriptional repression by chromatin modifying complexes.
- We provide evidence that PCGF6 promotes the expression of IL-10 and PDL1 in response to stimuli such as LPS and type I IFNs. IL-10 is increased at the mRNA level while PDL1 protein is stabilized by suppression of CCND2.
- PCGF6 in DCs regulates naïve T cell antigen sensitivity as well as the stability of interaction between DCs and T cells.
- We found that naïve T cell antigen sensitivity is differentially regulated by cDC1s and cDC2s which to our knowledge has not been previously shown. In addition, we found that only cDC1s increased the T cells antigen sensitivity and stability of their interactions with T cells in response to LPS.
- TLR-mediated maturation is fine-tuned by PCGF6 through the regulation of EGR1 binding site availability.

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List of Abbreviations

3-C – chromatin conformation capture
AIRE – autoimmune regulator
ANOVA – analysis of variance
APC – antigen presenting cell
ATAC-seq – sequencing of assay for transposase accessible chromatin
ATF3 – activating transcription factor 3
BAF – barrier-to-autointegration factor
BCG – bacille Calmette-Guerin
BCR – B cell receptor
BMDC – bone marrow-derived dendritic cell
BRG1 – brahma-related gene-1
c-SMAC – central supramolecular activation cluster
C/EBP – CCAAT/enhancer-binding protein
CBX – chromobox homolog
cDC – conventional DC
CDCM – complete dendritic cell media
CDP – common DC progenitor
CFSE – carboxyfluorescein succinimidyl ester
ChIP – chromatin immunoprecipitation
CIITA – class II transcriptional activator
CLP – common lymphoid progenitor
CMP – common myeloid progenitor

COMPASS – complex of proteins associated with SET1
CTL – cytotoxic T lymphocyte
CTLA-4 – cytotoxic T lymphocyte-associated protein 4
CyTOF – mass cytometry
DAMP – danger associated molecular pattern
DC – dendritic cell
DMEM – Dulbeco's Modified Eagle's medium
DNA – deoxyribonucleic acid
DNMT – DNA methyl transferase
ECAR – extracellular acidification rate
EDTA – Ethylenediaminetetraacetic acid
EED – embryonic ectoderm development protein
EGR – early growth response
EZH2 – enhancer of zeste homolog 2
FACS – fluorescence-activated cell sorting
FCS – fetal calf serum
Flt3L – FMS-like tyrosine kinase 3 ligand
GITRL – glucocorticoid-induced TNFR family related ligand
GM-CSF – granulocyte macrophage colony stimulating factor
GMP – granulocyte-monocyte progenitor
gp33 – glycoprotein 33
GSEA – gene set enrichment analysis
H2AK119Ub – mono-ubiquitinated histone 2A lysine 119
H3K27Ac – acetylated histone 3 lysine 27
H3K27me3 – trimethylated histone 3 lysine 27
H3K36me3 – trimethylated histone 3 lysine 36
H3K4me1 – monomethylated histone 3 lysine 4
H3K4me3 – trimethylated histone 3 lysine 4
H3K9me3 – trimethylated histone 3 lysine 9
HAT – histone acetyl transferase
HBSS – Hank's balanced salt solution
HCV – hepatitis C virus
HDAC – histone deacetylase
HIF1α - hypoxia inducible factor 1 alpha
HIV – human immunodeficiency virus
HLA – human leukocyte antigen
HMT – histon methy transferase
Hox – homeobox
HP1γ - heterochromatin protein 1 gamma
HSC – hematopoietic stem cell
ICAM – intercellular adhesion molecule
IDO – indoleamine 2,3-dioxygenase
IFN – interferon
IFNAR – interferon-alpha/beta receptor
IFNβ – interferon beta
IFNγ – interferon gamma

Ig – immunoglobulin
IL – interleukin
ILC – innate lymphoid cell
iNOS – inducible nitric oxide synthase
IP – immunoprecipitation
iregDC – immunoregulatory DC
IRF – interferon regulatory factor
ISG – interferon signaling genes
JARID – jumonji-arid-domain containing
KDM – lysine demethylase
L3MBTL2 – lethal(3) malignant brain tumour-line protein 2
LAG-3 – lymphocyte activation gene 3
LCMV – lymphocytic choriomeningitis virus
LFA-1 – lymphocyte function associated antigen 1
Lm – *Listeria monocytogenes*
LMP – MSCV-LTRmiR30-PIG
LPS – lipopolysaccharide
M.tb – *Mycobacterium tuberculosis*
MACS – magnetic-activated cell sorting
MAPK – mitogen activated protein kinase
MARCH1 – membrane associated ring CH-type finger 1
MARCH4 – membrane associated ring CH-type finger 4
MARCH9 – membrane associated ring CH-type finger 9
MBLR – MEL-18 BMI-1-like RING protein
MCMV – murine cytomegalovirus
MHCI – major histocompatibility complex class I
MHCII – major histocompatibility complex class II
MMTV-PyMT – mouse mammary tumour virus-polyoma middle T antigen
MSCV – mouse stem cell virus
MSigDb – molecular signatures database
mTOR – mammalian target of rapamycin
NF-κB – nuclear factor kappa-light-chain-enhancer of activated B cells
NK – natural killer
OVA – ovalbumin
PAMP – pathogen associated molecular pattern
PBS – phosphate buffered saline
PCGF – polycomb group factor
PD-1 – programmed cell death 1
pDC – plasmacytoid DC
PDL1 – programmed cell death ligand 1
PDL2 – programmed cell death ligand 2
PGE_2 – prostaglandin E_2
PMA – phorbol 12-myristate 13-acetate
pMHC – peptide-loaded major histocompatibility complex
PRC – polycomb repressive complex
PRR – pattern recognition receptor

qPCR – quantitative polymerase chain reaction
qRT-PCR – quantitative reverse transcription polymerase chain reaction
RACK7 – receptor for activated C-kinase 7
RBBP5 – retinoblastoma binding protein 5
RIP-gp – rat insulin promoter driven LCMV-glycoprotein
RNA – ribonucleic acid
RPMI – Roswell Park Memorial Institute medium
RT – reverse transcription
SET – su(var)3-9, enhancer-of-zeste and trithorax
SHP – Src homology region 2 domain-containing phosphatase
shRNA – small hairpin RNA
SLE – system lupus erythematosus
SMAC – supramolecular activation cluster
SMCX – structural maintenance of chromosomes X
SREBP1 – sterol regulatory element binding protein 1
STAT1 – signal transducer and activator of transcription 1
stimDC – stimulatory DC
SUZ12 – suppressor of zeste 12
TAP – transporter associated with antigen processing
TCA cycle – tricarboxylic acid cycle
TCF1 – T cell factor 1
TCR – T cell receptor
TFBS – transcription factor binding site
T_{fh} – follicular helper T cell
TGFβ – transforming growth factor beta
T_h1 – type 1 helper T cell
T_h17 – type 17 helper T cell
T_h2 – type 2 helper T cell
TIM-3 – T cell immunoglobulin and mucin domain containing-3
TLR – toll-like receptor
TNFα – tumour necrosis factor alpha
T_r1 – type 1 regulatory T cells
T_r2 – type 2 regulatory T cells
T_{reg} – regulatory T cell
TrxG – thrithorax group protein
WDR5 – WD repeat-containing protein 5
ZBTB46 – zinc finger and BTB-domain containing 46
β-ME – beta-mercaptoethanol

Chapter 1: Literature Review

1.1 Overview of the immune system

The immune system is a unique physiological system comprised of a complex combination of cell types. Immune cells orchestrate broad functions throughout the host, including tissue remodeling, wound healing, tumour surveillance, and importantly, protection from invading pathogens. Unlike other physiological systems, the immune system is not bound by specific organs; instead, immune cells circulate through blood and lymphatic vessels and reside in most tissues. Though not restricted to specific organs, there are primary lymphoid organs, the thymus and bone marrow, where immune cells develop, and secondary lymphoid organs, the spleen and lymph nodes, where immune cells can communicate and initiate a response. In mammals, there are two distinct arms of the immune system: innate and adaptive. Virtually every living organism has some form of innate immune system, however, only vertebrates have evolved the more sophisticated adaptive immune system. Both innate and adaptive systems are comprised of many cell types of distinct function (Figure 1).

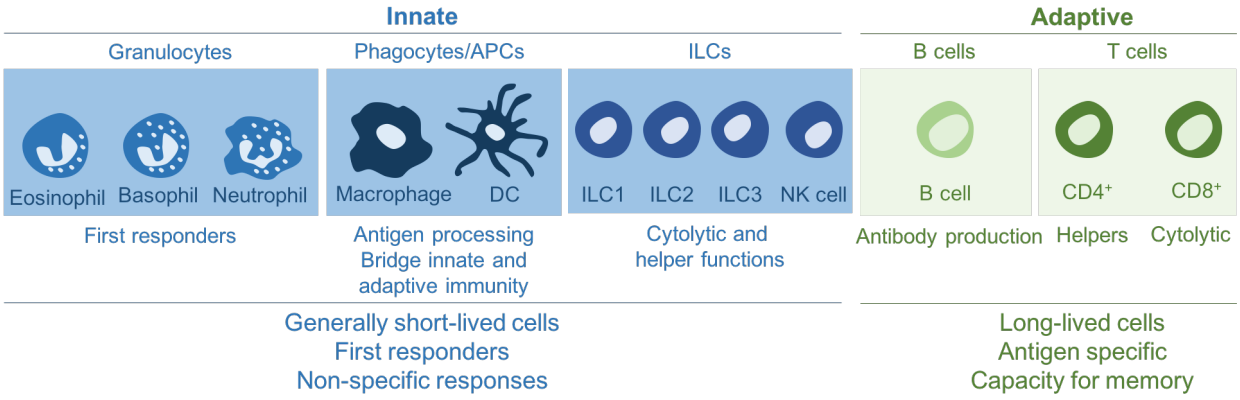

Figure 1. Examples of innate and adaptive immune cells and associated functions

The innate immune system

The innate immune system is the host's first line of defense against pathogens and danger signals. It is comprised of a number of circulating and tissue-resident cells that are all considered "first-responders" of the immune system. These cells include neutrophils, eosinophils, basophils, mast cells, Langerhans cells, innate lymphoid cells (ILCs), macrophages and dendritic cells (DCs).

In the absence of injury or invading pathogens, innate immune cells play roles in many normal physiological processes. As mentioned previously, immune cells, particularly macrophages, are heavily implicated in tissue remodeling (Mantovani et al., 2012). Macrophages, DCs and neutrophils also participate in regulating adipose tissue homeostasis, vascular endothelium integrity and liver function (Gao et al., 2008; Macdougall et al., 2018; Mann, 2011). Granulocytes, ILCs and other innate immune cells promote gut health and protect and maintain mucosal surfaces (Sonnenberg, 2014).

Upon encountering a pathogen or danger signal, each cell type serves a unique purpose both functionally and temporally to resolve the threat. Though the innate immune system is non-specific, the type, timing and action of responding cells depends in part on signals from the pathogen or host. For example, control of a parasitic infection often begins with the recruitment of eosinophils and basophils to the site of infection while neutrophils are the first cells recruited to sites of bacterial or fungal infections. The innate immune system, therefore, in a broad sense, consists of rapidly activating cells that act to quickly control and reduce pathogen burden through proinflammatory cytokine secretion, phagocytosis and direct cell-killing. However, should those efforts fail, antigen

presenting cells (APCs) also recruit cells of the adaptive immune system to ultimately clear the pathogen in a targeted manner.

DCs: Bridging innate and adaptive immunity

Macrophages, B cells and DCs are all able to present antigen with varying degrees of efficiency. Naïve T cells can only become activated by APCs. APCs are cells of the innate immune system that have a unique capacity to phagocytose, process and present antigen on major histocompatibility complex (MHC) molecules. There are two classes of MHC: class I (MHCI) is expressed on every nucleated cell and loads short endogenous peptide sequences on the surface of the cells, and class II (MHCII) expression is restricted to APCs at steady-state, although its expression is inducible on most cells by IFNγ (Truax et al., 2012). MHCII peptide sequences are often longer than those of class I. In humans, MHC complexes are genetically diverse and encoded by HLA genes. The genetic diversity of HLA is crucial, as it determines the compatibility of peptide sequences for loading into the complex.

DCs, are prolific APCs and are therefore highly specialized to present antigen to T cells. The efficiency of antigen presentation depends partly on the phagocytic capacity of the cell and the processing of proteins in the lysosomal compartment (Merad et al., 2013). While macrophages are highly phagocytic cells, their lysosomal compartments are also more acidic than DCs thus lending to destruction of potential peptides that can be loaded onto MHCII complexes. DCs however, are able to process and present a more diverse set of peptide sequences, increasing the probability of encountering antigen-specific T cells.

Brief history of dendritic cells

DCs were discovered in 1973 by Ralph Steinman and Zanvil Cohn (Steinman et al., 1975; Steinman and Cohn, 1973, 1974). The name “dendritic cell” comes from the processes that project from their cell body resembling neuronal dendrites (Steinman and Cohn, 1973). Soon after their discovery, DCs were found to be important for inducing T cell activation and proliferation (Nussenzweig and Steinman, 1980). For several years, the classification of DCs as a distinct immune subset remained controversial as many speculated that they represented a subpopulation of specialized macrophages (Merad et al., 2013). Indeed, even to this day the overlap in function and characterization of DCs and macrophages make it difficult to distinguish between the two cell types (Figure 2).

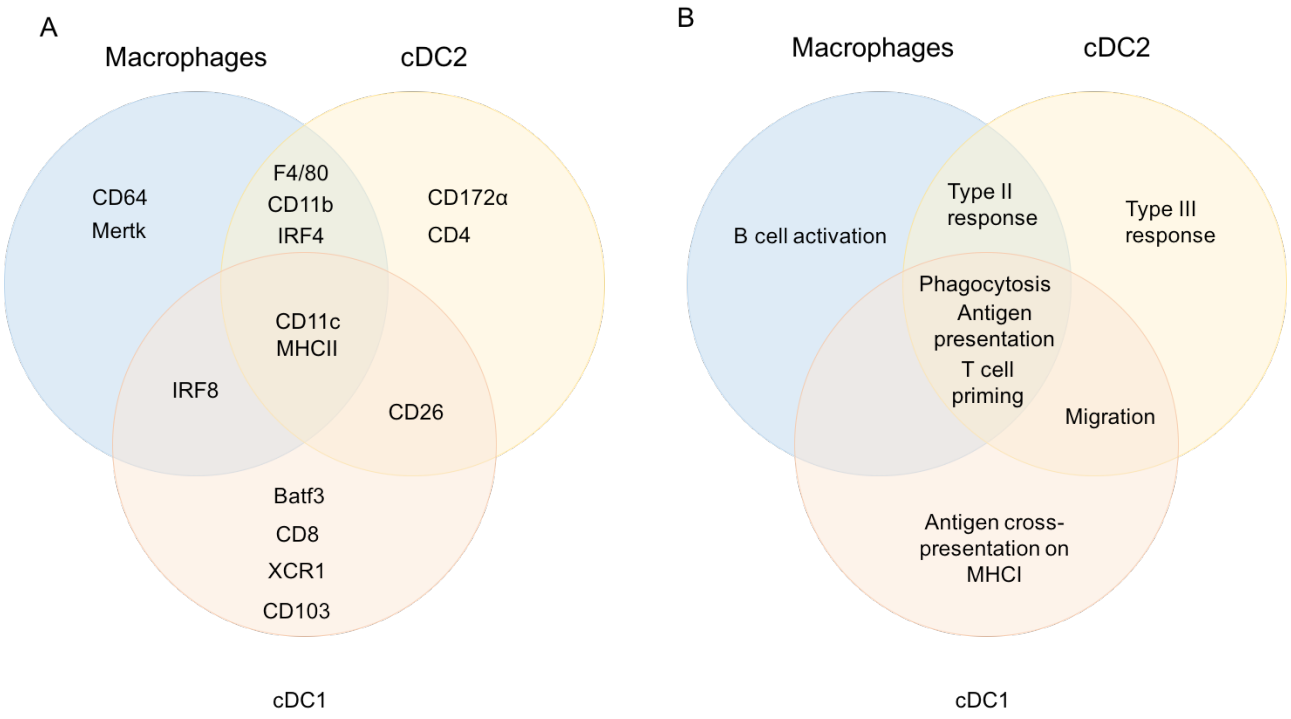

Figure 2. Convergent and divergent function and classification of DCs and macrophages. Venn diagrams representing markers, A, and functions, B, distinguishing cDC1s, cDC2s and macrophages.

Lineage tracing studies have shown unequivocally that DCs and macrophages arise from distinct progenitors (Naik et al., 2013; Naik et al., 2007; Sathe et al., 2014). In addition, the discovery of Flt3L and ZBTB46 further solidify DCs as immune cells that are distinct from macrophages (Meredith et al., 2012; Satpathy et al., 2012; Waskow et al., 2008). Flt3L is a secreted factor that is sufficient to expand conventional DC (cDC) subsets and plasmacytoid DCs (pDCs) (Naik et al., 2007). ZBTB46 is a transcription factor expressed only by cDCs (Meredith et al., 2012; Satpathy et al., 2012). Loss of ZBTB46 does not prevent the development of cDCs per se but it does favour the development of cDC1s (Meredith et al., 2012). Although it is now accepted that DCs are a distinct cell type, until recently the markers used to characterize DCs and macrophages were very similar. In addition to this, a different set of markers was used to identify cDCs depending on the species and organ being studied. However, with tools such as mass cytometry (CyTOF) and single-cell sequencing, classifying DC subsets in an unbiased manner has finally enabled a reliable means of identifying DCs in many tissues and across species (Alcántara-Hernández et al., 2017; Guilliams et al., 2016; Villani et al., 2017). Importantly, this classification also enables the exclusion of macrophages (Figure 2).

DC ontogeny

Hematopoietic stem cells (HSCs) in the bone marrow are multipotent progenitors that give rise to both the common lymphoid progenitor (CLP) and common myeloid progenitor (CMP) (Merad et al., 2013). Historically the CLP was thought to give rise to the adaptive arm of the immune system and the CMP was thought to give rise to the innate arm. However, the discovery of the heterogeneous innate lymphoid population of cells

(ILCs), which include natural killer (NK) cells, has challenged this notion. Further reports of the CLP giving rise to certain types of DCs, for example pDCs, also demonstrates that the CLP does not solely give rise to B cells and T cells as was once thought (Merad et al., 2013). Such studies also emphasize the importance of the innate immune system, given that two distinct progenitors can give rise to innate immune cell development.

The CMP becomes further specialized, giving rise to the granulocyte-monocyte progenitor (GMP), which gives rise to monocytes, granulocytes and the common DC progenitor (CDP) (Fogg et al., 2006). Finally, the CDP gives rise to pre-DCs, which were thought to then seed secondary lymphoid organs and other tissues where they would then differentiate into cDC1s and cDC2s (Liu et al., 2009; Naik et al., 2007; Onai et al., 2007). Therefore, tissue-specific factors would govern the fate of the pre-DC (Figure 3). However, single cell sequencing has revealed that the pre-DCs leaving the bone marrow are already committed to either a cDC1 or cDC2 fate, indicating that the resident organ plays less of a role in the cDC fate determination than previously thought (Schlitzer et al., 2015).

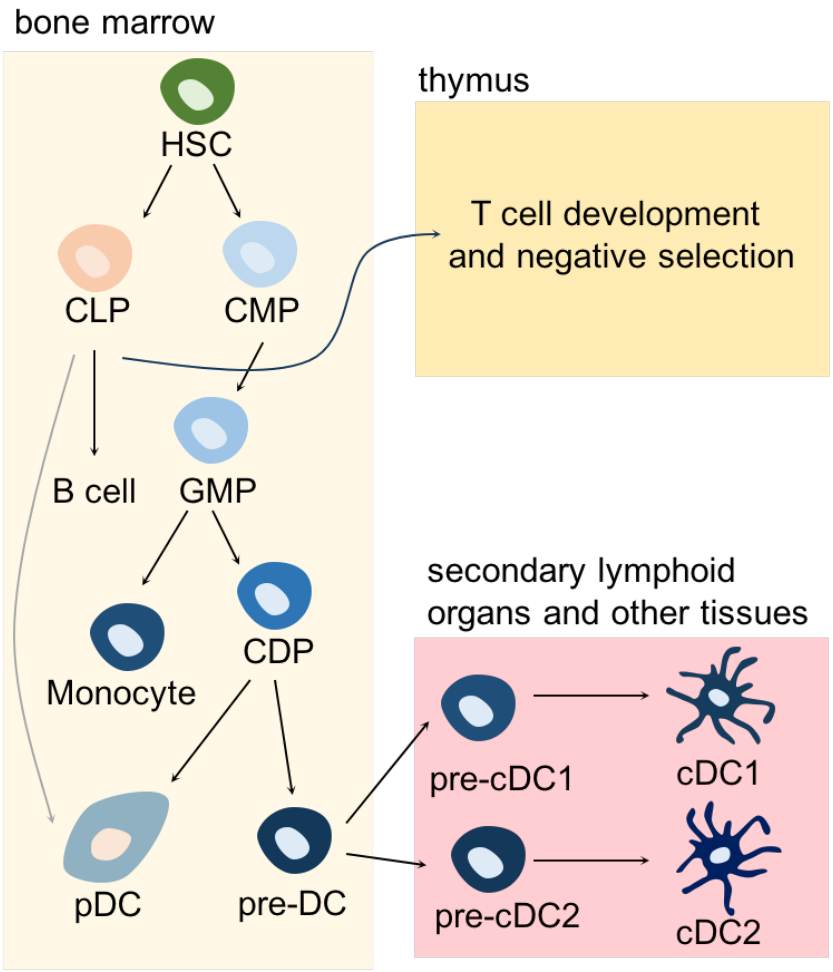

Figure 3. Development of cDCs from bone marrow pre-cursors.

Antigen presentation

Antigen presentation on MHCI and MHCII is the most important process for activating T cell responses. T cells express antigen receptors that bind specifically to peptide sequences called epitopes that are loaded into MHCI or MHCII complexes. Naïve T cells require priming by APCs to initiate their effector or memory function. However, antigen recognition in the absence of sufficient co-stimulation leads to T cell anergy and cell death. The requirement for multiple signals to initiate T cell responses protects the host from spontaneous or aberrant activation. $CD8^+$ T cells are typically activated by

antigen presentation on MHCI while $CD4^+$ T cells are activated by antigen presentation on MHCII. Distinct mechanisms govern antigen presentation on class I and class II.

Antigen presentation on MHCII

MHCII expression is restricted to APCs, with DCs expressing the highest levels at steady state. The importance of presentation on MHCII becomes evident for HIV infection which specifically targets $CD4^+$ T cells. Interestingly, HIV infection triggers the emergence of a $CD8^+$ T cell subset that can be primed by MHCII and contributes to viral control (Ranasinghe et al., 2016). Similarly, CD4 deficiency in mice gives rise to MHCII-restricted $CD8^+$ T cells (Pearce et al., 2004).

Processing and loading of MHCII occurs in the phagosome. TLR stimulation of DCs is accompanied by a brief increase in phagocytosis and antigen processing, however, the later phases of stimulation shut down *de novo* synthesis of MHCII as well as phagocytosis and antigen processing. This is thought to stabilize the peptide:MHCII complexes already exposed on the surface of the cells, which allows for more time for DCs to find and prime antigen-specific $CD4^+$ T cells (Young et al., 2008).

Dynamic MHCII expression is controlled by the class II transcriptional activator (CIITA) and the E3 ubiquitin ligase, MARCH1 (Landmann et al., 2001; Raval et al., 2001). CIITA acts as a recruitment platform for multiple other factors forming a complex known as an enhanceosome. The enhanceosome then triggers initiation and transcription of MHCII. Late activation of DCs triggers downregulation of CIITA and disassembles the enhanceosome which prevents MHCII transcription (Landmann et al., 2001). CIITA expression is regulated by cell-type specific promoters (I, II and IV) (Kitamura et al., 2012). However, transcriptional activation through promoter IV can be achieved in most cells by

IFNγ. Studies have shown that cancer cells can epigenetically silence the CIITA promoter IV to prevent IFNγ-stimulated MHCII expression which facilitates immune evasion (Londhe et al., 2012; Truax et al., 2012).

MARCH1 mediates ubiquitination of MHCII which leads to its internalization and degradation. Interestingly, TLR stimulation also triggers downregulation of MARCH1 which contributes to the stabilization of loaded MHCII at the cell surface. Therefore, the turnover of MHCII is controlled by CIITA and MARCH1 activity. MHCII expression is subject to regulation by environmental factors such as IL-10, which promotes MARCH1 activity and MHCII destabilization (Thibodeau et al., 2008; Tze et al., 2011).

Antigen presentation on MHCI

Antigen presentation on MHCI occurs on all nucleated cells. Short, endogenous peptide sequences are generated by proteasomal degradation of proteins in the cytosol, as opposed to MHCII peptides which are processed in endosomes. The class I peptides are imported into endoplasmic reticulum through the transporter associated with antigen processing (TAP) protein and then loaded onto MHCI. Turnover of MHCI is regulated by MARCH4 and MARCH9 (Neefjes et al., 2011).

Most cells can only load endogenous peptide on MHCI. Therefore, cells have to be infected to present foreign antigen, and this targets them for killing by $CD8^+$ T cells. However, DCs have the unique capacity to acquire exogenous proteins, process and load them on MHCI, termed cross-presentation. Cross-presentation is crucial because it allows DCs to mount a $CD8^+$ T cell response without becoming infected themselves. Similar to presentation on MHCII, cross-presentation is enhanced in response to TLR4 stimulation (Alloatti et al., 2015). The increased capacity for cross-presentation in response to

activation is attributed to lowering of the degradative potential of endosomes (Ackerman et al., 2005; Alloatti et al., 2015). The GTPase, Rab34, participates in enhanced cross-presentation by controlling lysosomal clustering (Alloatti et al., 2015).

cDC1s are considered to be better cross-presenters than cDC2s (den Haan et al., 2000; Schnorrer et al., 2006). The transcription factor IRF8, which is more highly expressed in cDC1s and pDCs, also regulates a set of genes involved in cross-presentation, while cDC2s, which are more dependent on IRF4, dominantly express genes involved in antigen presentation on class II (Vander Lugt et al., 2013). However, IRF8 can also drive expression of class II related genes, therefore it is thought that anatomical localization of cDC1s also restricts their capacity to activate $CD4^+$ T cells (Gerner et al., 2017).

Studies have shown that $CD103^+$ migratory cDC1s are responsible for carrying tumour antigen to the draining lymph node and initiating $CD8^+$ T cell responses (Roberts et al., 2016; Salmon et al., 2016). However, recent studies have challenged the notion that cDC2s do not cross-prime $CD8^+$ T cells. One study identified a subtype of cDC2s in tumours that are capable of efficient cross-presentation (Sheng et al., 2017). Another study has argued that the route of antigen delivery affects the capacity for cross-presentation. Antigen targeted by DEC205 lead to efficient cross-presentation in both cDC1s and cDC2s (Kamphorst et al., 2010). Targeting to different endocytic receptors affects the compartment the antigen is delivered to, which affects antigen export to the cytosol. However, whether this targeting has relevance *in vivo* is still a matter of debate.

Interestingly, gap junction formation between DCs represents an under-appreciated mode of cross-talk between different DC subsets (Matsue et al., 2006;

Mazzini et al., 2014). Gap junction formation has been shown to enhance DC activation and represents a means of rapidly spreading information (Matsue et al., 2006). Furthermore, several lines of evidence have shown that antigen can be shared between DCs, and the DCs that acquire the antigen are not necessarily the ones that prime the resulting T cell response (Allan et al., 2006; Matsue et al., 2006; Wakim and Bevan, 2011). It is possible that antigen acquired efficiently by cDC1s can be shared with cDC2s, bypassing the endocytic receptors.

The adaptive immune system

While the innate immune system is rapidly responding and relatively non-specific, the adaptive immune system is exquisitely specific. Lymphocytes (B cells and T cells) are cells of the adaptive immune system. $CD4^+$ and $CD8^+$ T cells are classified based on the expression of the CD4 or CD8 coreceptor, which determine their interaction with MHC. T cells are uniquely capable of recognizing foreign peptide sequences, or antigens specific to the infecting pathogen, and producing a directed response. B cells produce targeted responses through the recognition of whole, unprocessed antigens. Antigen recognition occurs through antigen receptors on B cells (BCR) and T cells (TCR). Furthermore, antigen is only recognized by the TCR in the context of self. In other words, while BCRs are activated by whole antigen, TCRs on naïve T cells only become activated if their cognate antigen is presented by APCs derived from the host.

Antigen receptor specificity and functional avidity

B cells develop in the bone marrow while T cells develop in the thymus. During lymphocyte development, each cell undergoes a process called VDJ-recombination, where a series of genetic recombinations occur to create a receptor with a unique antigen

specificity. Following VDJ-recombination, T cell precursors undergo a process called selection in the thymus. Thymic epithelial cells express a protein called autoimmune regulator (AIRE) which enables the expression of every self-protein (Bansal et al., 2017). Thymic epithelial cells then test the ability of TCRs to recognize the antigen presented. If lymphocytes elicit too strong a response to a given self-protein, they are deleted (negative selection). However, since TCRs require that antigen be presented in the context of self, the TCR should retain some activity to self-molecules (Mandl et al., 2013). This is ensured by positive selection, whereby a lack of response by T cells promotes their apoptosis. The remaining cells exit the thymus and circulate through the blood and lymphatics and are considered “naïve” until they encounter APCs bearing antigen specifically recognized by the TCR. The capacity to sense low doses of antigen therefore depends on intrinsic factors such as the affinity of the receptor for its cognate antigen determined through VDJ-recombination, and it is refined through negative selection (Slifka and Whitton, 2001).

The BCR also undergoes a process called “somatic hypermutation” where over the course of several divisions, the BCR specificity for its cognate antigen is improved, allowing for a more robust and sensitive response. The TCR does not undergo somatic hypermutation, and its specificity is fixed; however, it does become more sensitive to its cognate antigen (Slifka and Whitton, 2001). Following activation, the antigen sensitivity or functional avidity of T cells can be modulated by inflammatory cytokines over the course of an infection (Richer et al., 2013; Smith et al., 2018). Extrinsic factors serve to fine-tune the TCR signal transduction pathway resulting in an enhanced or dampened response to a given signal. IL-10, for example, decreases the antigen sensitivity of effector T cells during chronic infection by preventing TCR clustering (Smith et al., 2018).

Lymphocyte activation and differentiation

When lymphocytes encounter cognate antigen they undergo a process called "clonal expansion" where the cell with singular antigen specificity proliferates exponentially to give rise to cells with identical antigen specificity. Activated or antigen-experienced lymphocytes that directly participate in the inflammatory process are called effectors. For B cells, that means antibody production resulting in opsonisation and complement-mediated lysis of pathogens expressing epitopes recognized by the antibody. Antibody production can also lead to antibody-dependent cellular cytotoxicity (ADCC) which entails recruitment of innate immune cells to antibody-coated target cells to induce target cell lysis (Gómez Román et al., 2014). $CD8^+$ T cells differentiate into cytotoxic T lymphocytes (CTLs), mediating direct killing of infected cells through perforin and granzyme B production. $CD4^+$ T cells can be further differentiated into several subtypes, driven by the induction of master regulators that control their transcriptional profiles. For example, Type 1 helper T (T_h1) cells are proinflammatory $Tbet^+$ cells that serve as helpers during bacterial and viral infections. $Foxp3^+$ regulatory T cells (T_{reg}), on the other hand, serve to curb the inflammatory response in order to limit toxicity to the host (Luckheeram et al., 2012). B cells and T cells can differentiate into memory cells which enables more rapid responses if the same or a similar pathogen is encountered again.

DCs instruct the type of T cell response that is elicited. Type I immunity is typically involved in the clearance of viruses and other intracellular pathogens while type II immunity involves antibody production and allergic responses. While evidence indicates that a reciprocal negative feedback loop limits one type of immunity in the presence of

the other (T R Mosmann and Coffman, 1989), it is clear that different types of immunity can be elicited to different degrees during an immune response. Furthermore, a number of other $CD4^+$ helper subtypes have been characterized to date and their various functions are still an active area of research (Figure 4).

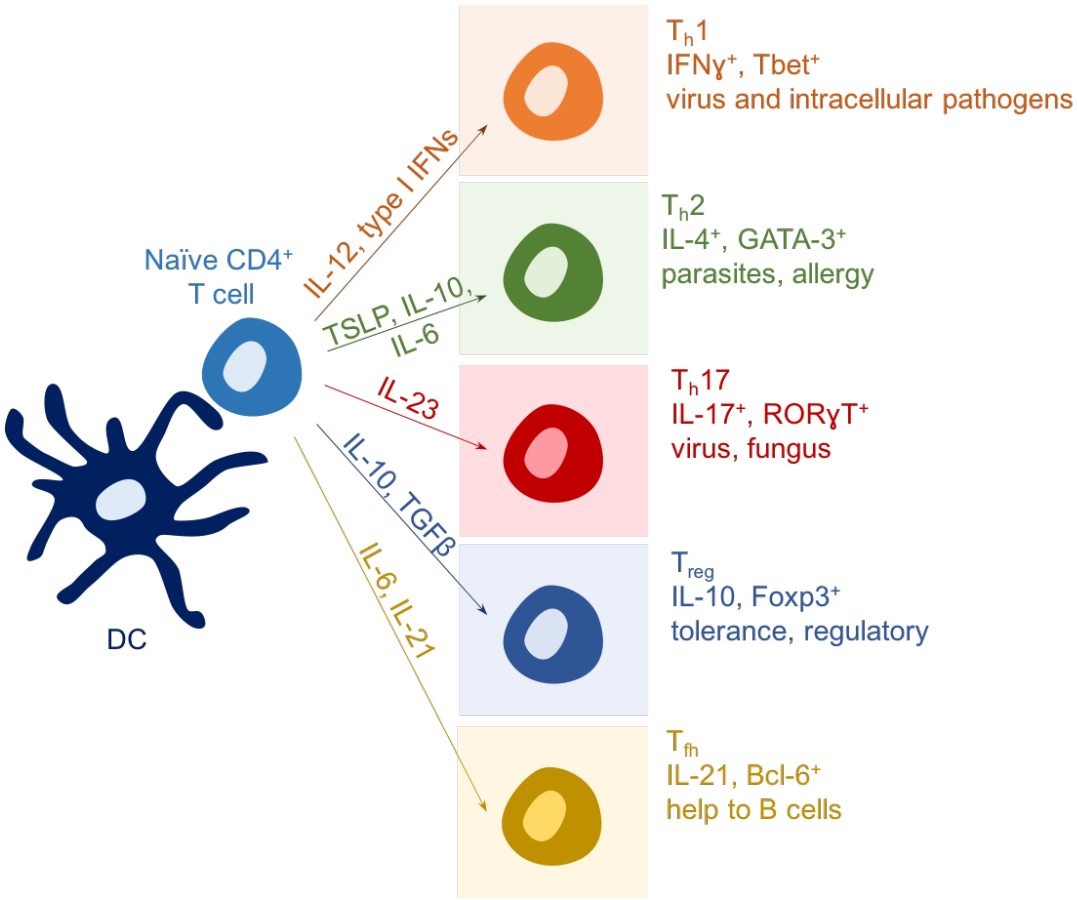

Figure 4. Polarization of CD4+ T cell responses by DCs. Naïve CD4+ T cells receive cytokine signals from DCs which polarize them to a particular fate. The major cytokine, transcription factor, and known function is listed.

Other emerging evidence indicates a previously unappreciated functional subset diversification of $CD8^+$ T cells (Mittrücker et al., 2014). Though controversial, there is growing evidence for $CD8^+$ subsets besides CTLs and memory cells highlighting the complexity of responses that are controlled by DCs.

1.2 Priming T cell responses

Antigen presentation, co-stimulation and cytokine production have long been appreciated as the three fundamental signals required for efficient priming of T cell responses (Figure 5). However, recent studies have demonstrated that other factors contribute to efficient T cell priming (Jain and Pasare, 2017). For example, the stability of interaction between DCs and T cells determines effector and memory function (Henrickson et al., 2013; Mempel et al., 2004; Scholer et al., 2008; Shakhar et al., 2005). Furthermore, a fourth "accumulation" signal has been discovered, required to promote T cell survival after priming (Chang et al., 2017).

Co-stimulation and co-inhibition

Effective priming cannot occur without co-stimulation. Co-stimulation reinforces TCR stimulation as well as DC activation through several mechanisms. The most widely studied co-stimulatory molecules are the members of the immunoglobulin (Ig) superfamily B7: CD80 (B7-1) and CD86 (B7-2). Both are ligands for the CD28 receptor on T cells. One major function of CD28 ligation is to promote and enhance IL-2 production by T cells by stabilizing IL-2 mRNA (Jenkins et al., 1991).

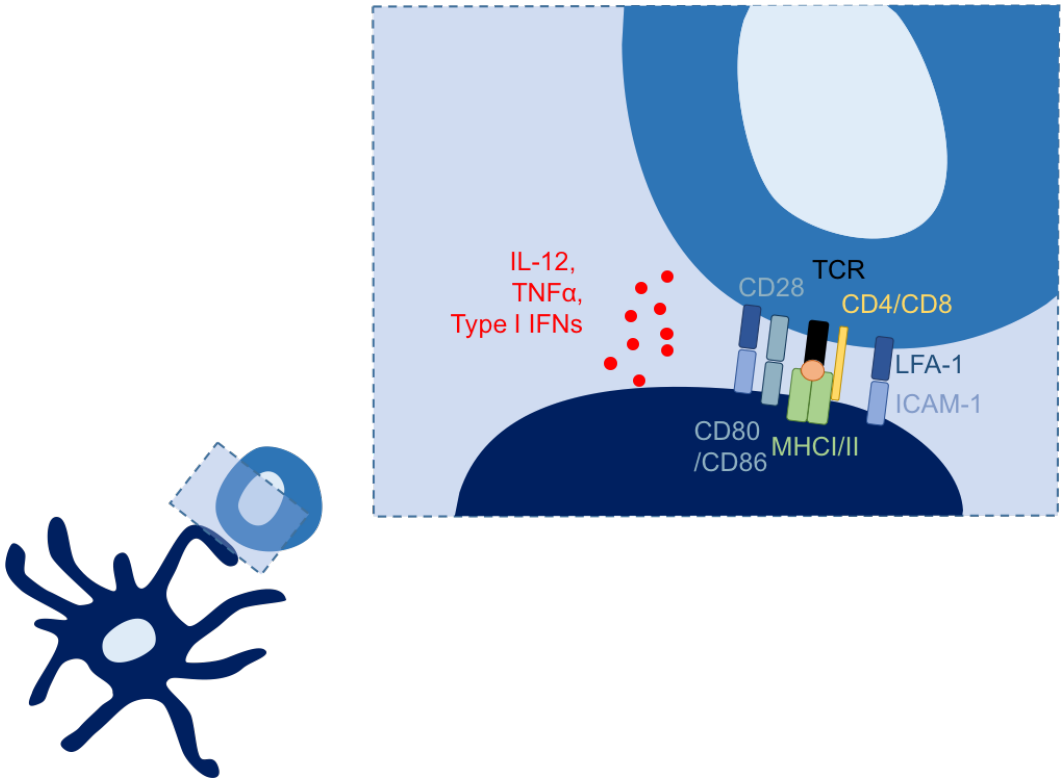

Figure 5. Priming a T cell response. DCs form stable immune synapses in part through ICAM and LFA-1. DCs then present antigen (signal 1), provide costimulation (signal 2) and produce cytokines (signal 3), which activates T cells.

IL-2 is consumed by proliferating T cells and supports their growth and proliferation. Studies in CD28-deficient or CD80- and CD86-deficient mice have informed the mechanisms of co-stimulation. Co-stimulation by CD80 and CD86 affect the phenotype and function of both $CD4^+$ T cells and $CD8^+$ T cells over the course of primary and secondary infections (Dolfi et al., 2011; Eberlein et al., 2012). However, other reports have shown that $CD4^+$ T cells depend more on CD28 signaling than $CD8^+$ T cells (Shahinian et al., 1993). Since $CD8^+$ T cells do not produce as much IL-2 as $CD4^+$ T cells to support their proliferation (Paliard et al., 1988), it is possible that the effects observed on $CD8^+$ T cells are indirect due to insufficient help. Another possibility is that overlap

between different costimulatory molecules could compensate in the absence of CD28. It has also been shown that viral context determines whether co-stimulation through CD28 is important (Welten et al., 2015). CD80/CD86-deficienct mice infected with acute lymphocytic choriomeningitis virus (LCMV)-Armstrong did not exhibit impaired $CD8^+$ T cell activation. However, CD80/CD86 deficiency in the context of other infections such as MCMV and Listeria monocytogenes (Lm) do have a profound effect on the accumulation of antigen-specific $CD8^+$ T cells. Interestingly, fusion of the dominant LCMV MHCI epitope, gp33, into the MCMV-backbone, or over-expression of gp33 by Lm, lead to an impairment in the generation of antigen-specific T cells against gp33 in CD80/CD86 deficient hosts (Welten et al., 2015). This demonstrates that the requirement for co-stimulation is not dictated by the specific epitopes loaded on MHCI but rather by other context-dependent factors.

There is also a growing appreciation for retrograde signaling through CD80 and CD86 (Orabona et al., 2004). The use of soluble CD28 on DCs enhanced the production of IL-6 by splenic DCs through p38-MAPK activity. IL-6 inhibits the immunosuppressive activity of IDO in an autocrine fashion (Orabona et al., 2004). Other co-stimulatory molecules such as CD40 have been long appreciated for their effects on DC function. Upon ligation by CD40L, CD40 activates signaling events in DCs that reinforce their activation and improve their priming capacity.

Members of the B7 family can also be co-inhibitory. CD80 and CD86 can bind to the CTLA-4 receptor on T cells with a higher affinity than CD28 (Pentcheva-Hoang et al., 2004). Expression of CTLA-4 is low on naïve T cells but is upregulated over the course of a T cell response. Unlike CD28, CTLA-4 inhibits T cell activation and curbs the immune

response. Similarly, PD-1 is ligated by either PDL1 (B7-H1) or PDL2 (B7-H2) (Wherry and Kurachi, 2015). Signaling through PD-1 leads to the dephosphorylation of CD28 and the TCR (Hui et al., 2017; Kamphorst et al., 2017). Co-stimulation and co-inhibition therefore dynamically regulate TCR signal strength, contributing to the functional avidity of T cells.

Members of the TNF superfamily of ligands on DCs and receptors on T cells also regulate T cell responses. GITRL for example is induced on DCs in response to type I IFNs and is required for $CD4^+$ T cell accumulation during an active infection (Chang et al., 2017). Since GITRL-signaling is required after priming it is distinct from the canonical signal 2 delivered by co-stimulatory molecules. Therefore, the T cell accumulation signal is now considered signal 4.

Cytokine production

Cytokines convey information about the type of immune response to be elicited. Briefly, cytokines such as type I IFNs and IL-12 promote T_h1 and CTL responses. Meanwhile, IL-4 and TSLP lead to T_h2 responses. Finally, IL-23 promotes T_h17 responses (Kapsenberg, 2003; Luckheeram et al., 2012). Certain cytokines such as IL-6 and IL-10 can contribute to different types of immunity depending on the combinations of other cytokines in the microenvironment. In addition, different types of DCs can be more specialized to produce certain cytokines. pDCs, for example, are major producers of type I IFNs, therefore pDCs play a major role in responses against viral infection (Ito et al., 2006).

Cytokines have very diverse functions, and since they are secreted factors, DCs can also communicate to other cell types including neutrophils and NK cells through

cytokine production (Ferlazzo and Morandi, 2014; Schuster et al., 2013). Though there is some evidence that DCs constitutively express low levels of immunosuppressive factors such as IL-10 (de Saint-Vis et al., 1998), cytokine production is generally elicited by PRR or cytokine receptor signaling. Therefore, activated DCs deliver polarizing signals, depending on the environmental stimulus received (Table 1).

Table 1. Relative cytokine production and T cell polarization by PRRs

PRR	Major cytokines produced	Dominant T cell response	References
TLR4	IL-12 +++ TNFα ++ IL-6 ++ IL-10 + type I IFNs ++	Th1, CTL	(Re and Strominger, 2001)
TLR7/8, TLR9	IL-12 ++ type I IFNs +++ IL-10 +	Th1, CTL	(Gautier et al., 2005)
C-type lectin receptors	IL-1β ++ IL-6 +++ IL-23 ++ IL-10 +++	Th2, Th17	(Wevers et al., 2014)

In addition to polarizing T cell responses, cytokines also feedback in an autocrine or paracrine fashion on DCs to temper or enhance immune responses. For example, type I IFNs drive their own expression in a positive feedback loop where activation of the type I IFN receptor IFNAR activates STAT1 which further drives type I IFN expression (Ma et al., 2015). However, the expression of most proinflammatory cytokines is transient, and resolution of cytokine production occurs through the activation of transcriptional repressors over the course of an inflammatory response, as well as late induction of anti-

inflammatory lipid mediators (Foster et al., 2007; Oishi et al., 2017). For example, IL-10 also suppresses IL-12 and other pro-inflammatory mediators.

Immune synapse

During priming, APCs form relatively stable interactions with T cells. The interaction surface between an APC and a T cell is known as the immune synapse. Different APCs engage distinct interaction behaviours with T cells; B cells form highly stable organized interactions and DCs form more stochastic, disorganized interactions (Gunzer et al., 2004). For both, formation of the immune synapse entails recruitment of adhesion molecules such as intercellular adhesion molecules (ICAMs) (Benvenuti et al., 2004; Scholer et al., 2008). It was originally thought that the immune synapse promotes T cell priming by facilitating the clustering of MHC and co-stimulatory ligands on APCs and TCR and costimulatory receptors on T cells (Dustin et al., 2010). These clusters are called supramolecular activation clusters (SMAC), with the central SMAC or c-SMAC containing the highest concentration of pMHC:TCR clusters. The formation of a synapse also enables local delivery of cytokines and other secretory signals by limiting their diffusion (Dustin et al., 2010). However, the strength of TCR stimulation is inversely correlated with localization within the c-SMAC (Čemerski et al., 2008). Instead, small microclusters of TCRs in the periphery of the c-SMAC (p-SMAC) are responsible for signal transduction, while lateral diffusion into the c-SMAC may be associated with termination of signaling (Varma et al., 2006). In addition, unlike B cells, DCs do not form organized SMACs; therefore, the effect of immune synapse formation on T cell priming is controversial (Benvenuti et al., 2004; Gunzer et al., 2004; Shakhar et al., 2005).

Antigen dependent DC:T cell interactions

Still, stable interactions between DCs and T cells do contribute to T cell activation, differentiation and effector function. T cells transit through lymph nodes, pausing briefly on DCs. Once an antigen-specific T cell contacts a DC bearing its cognate antigen, it stops for longer; TCR signaling also delivers a signal for T cells to decrease their motility. Three phases of interaction between DCs and T cells occur over the first 12 hours of priming and these involve increasingly stable interactions (Mempel et al., 2004). The final phase results in contacts that last greater than 30 minutes. Finally, there is a gradual regain of T cell motility where T cells then migrate from lymph nodes (Mempel et al., 2004). One report has indicated that PD-1 blockade facilitates the recovery of T cell motility (Honda et al., 2014), while another report indicates that PD-1 ligation prevents stable interactions of DCs and T cells by impeding the TCR-mediated stop signal (Fife et al., 2009). To reconcile these two conflicting studies, it is possible that PD-1 plays different roles during distinct phases of priming. One group has shown that ICAM-1 on DCs is not required for early interactions of DCs and T cells, however it is required for more stable interactions (Scholer et al., 2008). Interestingly, loss of ICAM-1 did not impair effector function of $CD8^+$ T cells, however it did limit memory formation, suggesting that longer contacts between DCs and T cells are important to initiate T cell memory. This is supported by other studies that have demonstrated DCs bearing low doses of antigen or low affinity antigen spend more time in the first phase of more transient DC:T cell interactions, resulting in adequate effector function but impaired memory formation (Henrickson et al., 2008; Henrickson et al., 2013; Ozga et al., 2016).

Long interactions of DCs and T cells favour $CD4^+$ T follicular helper (T_{fh}) cell differentiation which is essential to promote antibody production by B cells (Benson et al., 2015). During chronic infection, *de novo* $CD4^+$ T cells are restricted to a T_{fh} cell fate, at the expense of T_h1 and this has been shown to contribute to T cell dysfunction (Osokine et al., 2014; Snell et al., 2016). The T_{fh} restriction is attributed to priming by APCs exposed to chronic type I IFNs (Snell et al., 2016). Furthermore, recent work in chronic infection has shown that the $CD8^+$ T cells reinvigorated following PD-1 blockade are T cell factor 1 (TCF1)-expressing $CD8^+$ cells (Im et al., 2016). The gene profiles of these cells resemble T_{fh} cells as well as $CD8^+$ memory precursor cells. Blockade of PD-1 leads to rapid proliferation of these cells as well as egress from lymph nodes (Im et al., 2016). It is possible that prolonged interactions between DCs and T cells can lead to T cell dysfunction by skewing their differentiation as well as by preventing them from regaining their motility.

Antigen independent DC: T cell interactions

In the absence of cognate antigen, T cells transiting through lymph nodes interact with DCs. Self-peptide MHC interactions have a demonstrated role in maintaining peripheral tolerance. Interestingly, the absence of $CD11c^+$ cells limits the foreign antigen sensitivity of $CD4^+$ T cells, indicating that self-peptide loaded MHCII interacting with TCR serves a purpose of tuning the antigen sensitivity of T cells (Stefanová et al., 2002). Isolating T cells from the host for as little as 30 minutes is sufficient to cause diffusion of TCRs on the surface of the cells which slowed the kinetics of T cell activation *in vitro*. DCs interact non-specifically with T cells in order to maintain TCR clusters, which enables more robust activation in response to foreign antigen (Stefanová et al., 2002). In addition,

the T cells with the strongest affinity for foreign antigen are also the most self-reactive (Mandl et al., 2013). Low-level tonic TCR stimulation delivered by self-peptide MHC may also be important for naïve T cell survival in the absence of foreign antigen (Revy et al., 2001). However, the antigen sensitivity of T cells is dynamically modulated by environmental factors (Richer et al., 2013; Slifka and Whitton, 2001; Smith et al., 2018). Lowering the threshold for T cell activation is beneficial in the context of infection or tumour clearance because it enables the detection and elimination of target cells bearing only low levels of antigen. However, bystander T cells also experience increased antigen sensitivity in response to inflammatory conditions (Richer et al., 2013), which could be a mechanism by which autoreactive T cell responses and autoimmunity occur.

Understanding the dynamics of DCs interacting with T cells, and how these dynamics alter T cell function could lend important insight into how T cell dysfunction or hyperactivation occur in chronic infection and autoimmunity, respectively.

1.3 Regulation of the immune system in health and disease

The innate and adaptive immune systems have developed a number of checks and balances to ensure that inappropriate or aberrant responses do not occur. One example is negative selection, as discussed, which limits self-reactivity of lymphocytes. Furthermore, antigen presentation (signal 1) alone is not sufficient to trigger a response from a naïve T cell. T cells require two more signals to both reinforce their activation (costimulation, signal 2) and trigger their effector function (usually, cytokine production, signal 3). Signal 1 in the absence of signals 2 and 3 triggers abortive deletion of the T cells or renders them anergic. The collection of strategies employed by immune cells to prevent or suppress self-reactivity as well as reactivity towards commensal and normal

flora is termed tolerance. Central tolerance encompasses processes that occur during the development of lymphocytes (positive and negative selection), while peripheral tolerance encompasses strategies employed outside of the thymus to maintain tolerance (Xing and Hogquist, 2012). DCs play a crucial role in both central and peripheral tolerance. They constantly sample the environment through pinocytosis and present self-antigens to T cells in lymphoid organs. The advantage of these brief interactions are two-fold: they prevent self-reactive responses through mechanisms discussed above, and they maintain TCR clusters such that when T cells do encounter DCs bearing their cognate antigen they can respond in a robust manner (Stefanová et al., 2002).

The onset of most, if not all autoimmune disease is associated with a break in tolerance, resulting in an inflammatory response and destruction of self-tissues. In many cases, the cause of the break in tolerance remains elusive and is an active area of research. It is likely that a combination of genetic and environmental factors contributes to the onset of autoimmunity. Several studies suggest that certain infections can trigger genetic vulnerabilities to autoimmunity (Ercolini and Miller, 2009). Since DCs play such a pivotal role in maintaining peripheral tolerance, it comes as no surprise that they are often implicated in autoimmune disease progression. In type I diabetes, for example, many patients diagnosed carry a specific MHC haplotype that makes them more susceptible (Davies et al., 1994). The haplotypes of MHC determine the diversity and specificity of peptide sequences that can be loaded on the surface of DCs. Limitations in peptide diversity could allow low threshold self-reactive T cells to escape negative selection in the thymus. In addition to antigen processing, metabolic alterations in DCs can trigger the onset of autoimmunity. For example, cholesterol accumulation in DCs leads to systemic

lupus erythematosus (SLE)-like autoimmunity in mice (Ito et al., 2016; Westerterp et al., 2017).

Tolerance takes on another meaning during prolonged and active immune responses. Over time, immune cells responding to prolonged inflammation accumulate "tolerogenic" markers that actively counter-balance the proinflammatory immune response. These markers are often induced by the same factors that induce inflammation (ie. Type I IFNs, NF-κB etc) and the purpose is to curb the immune response and limit host tissue damage. A cell is considered tolerized or exhausted when it fails to respond appropriately to secondary signaling (Butcher et al., 2018). Tolerance during an immune response differs from self-tolerance in that there are sufficient pathogen and danger signals to elicit a response but the cells are rendered unresponsive through inhibitory molecules and regulatory cells that actively prevent immunity. For example, T cells become exhausted through the upregulation of receptors such as PD-1, LAG-3, TIM-3 and CTLA-4 which suppress TCR signaling in different ways (Wherry and Kurachi, 2015). PD-1 binds one of its ligands PDL1 or PDL2 which leads to the activation of phosphatases (SHP-1 and SHP-2) that dephosphorylate key signaling molecules in the TCR and CD28 signaling cascades (Dong et al., 1999; Freeman et al., 2000; Ishida et al., 1992; Latchman et al., 2001). Recent evidence demonstrates that PD-1 activation directly interferes with CD28 costimulation required for the reinforcement of TCR stimulation (Hui et al., 2017; Kamphorst et al., 2017). CTLA-4 also interferes with co-stimulation by capturing CD80 and CD86 through transendocytosis, leading to degradation of the costimulatory markers within T cells (Qureshi et al., 2011). IL-10 and TGFβ are examples of anti-inflammatory cytokines that are produced by many immune cells and even some epithelial cells, and

serve to inhibit immune responses through a variety of mechanisms. In addition to the T_{reg} cells mentioned earlier that circulate at steady state, $CD4^+$ T cells can differentiate into various regulatory subsets (T_r1, T_r2).

Should the immune system become suddenly overwhelmed by pathogen burden, often a dysfunctional immune response due to tolerization and exhaustion of the immune cells results. Sepsis, for example, is caused by a systemic bacterial infection that leads to a cytokine storm and an overwhelming and life-threatening immune response (Wysocka et al., 2001). Patients that survive septic shock often go through a refractory period of immunodeficiency caused by tolerized immune cells unable to respond to secondary threats.

Chronic infection and cancer are other characteristic examples where abundance of antigen and over-production of type I IFNs cause dysfunctional T cell responses that prevent the complete clearance of the virus or tumour cells (Cunningham et al., 2016). A complex combination of factors contributes to the persistence of viruses and tumour cells. For example, the chronic LCMV strain clone 13 persists in murine hosts due to a mutation that increases the affinity for alpha-dystroglycan, a receptor expressed on DCs that clone 13 uses to gain entry into the cells (Sullivan et al., 2011). Infected DCs are then impaired in their capacity to mount an efficient immune response. Clone 13 is also able to replicate faster within infected host cells, allowing it to accumulate faster than the immune system is able to clear it. Other chronic viruses like HCV and HIV use similar strategies to persist in the host, often co-opting the host's own defense mechanism (Tsubouchi et al., 2004; Wu and KewalRamani, 2006).

Tumour cells originate from the host themselves, and so intrinsically avoid detection. However, they acquire and accumulate mutations as they grow that allow them to be recognized and cleared by the immune system. They also harness mechanisms to actively evade immune detection, again by co-opting processes that protect the host from tissue damage. Immune evasion is one of the hallmarks of cancer and has become a prominent area of research for developing therapeutics. Anti-PDL1 therapy, for example, is a promising treatment for a number of different cancer types, including melanoma (Topalian et al., 2016). However, the success rate is variable and PDL1 status in the tumour does not always correlate with success of the therapy. Anti-PDL1 therapy can also target and enhance the function of immune cells to promote tumour clearance. New strategies that involve first inducing PDL1 expression on immune cells in order to sensitize non-responders to anti-PDL1 treatment have proven successful in mice (Salmon et al., 2016; Spitzer et al., 2017; Zhang et al., 2017).

It is also clear that the process of immune cell exhaustion evolved to protect the host from the dangers of a hyperactive immune system. Unrestrained, activated immune cells can cause damage to tissues through prolonged inflammation. Evidence in the literature support the notion that immune cell exhaustion is an adaptation, rather than a dysfunctional response, to meet the demands of prolonged stimulation (Speiser et al., 2014). For example, PD-1 expressing T cells at the onset of an immune response are the cells producing the most IFNγ, TNFα and Granzyme B—indicators of a polyfunctional effector response (Barber et al., 2006). However, prolonged antigen exposure and inflammation leads to an accumulation of inhibitory receptors including PD-1 (Chen, 2004). In the case of chronic viral infection, a low level of viral control remains, although

it is insufficient to clear the infection (Jin et al., 1999; Schmitz et al., 1999; Speiser et al., 2014). Finally, T cell exhaustion is reversible, since blockade of PD-1 can reinvigorate T cells, allowing them to regain their effector function (Barber et al., 2006).

Like T cells, DCs also undergo adaptation to chronic stimulation. Although short-lived, DCs are sensitive to their environment and can respond to highly inflammatory signals by engaging a counter-regulatory program that promotes immune suppression through the production of anti-inflammatory cytokines and the induction of regulatory T cell responses (Figure 6). How DCs decide whether to promote or suppress an immune response is still an active area of research. However, evidence shows that immunoregulatory DCs (iregDC) and stimulatory DCs (stimDC) coexist during chronic infection and cancer (Cunningham et al., 2016). These cells are thought to arise from distinct lineages; iregDCs are monocyte-derived while stimDCs are cDC2s. iregDCs are potently inhibitory, not just by contributing to the inflammatory microenvironment, but also by directly impairing the priming of $CD4^+$ T cells (Cunningham et al., 2016; Snell et al., 2016). Chronic infection also impacts *de novo* differentiation of DCs from the bone marrow by skewing their development at the level of HSCs (Sevilla et al., 2004). Understanding the factors that regulate DC function and differential responses on a molecular level is necessary to develop effective treatments for chronic disease.

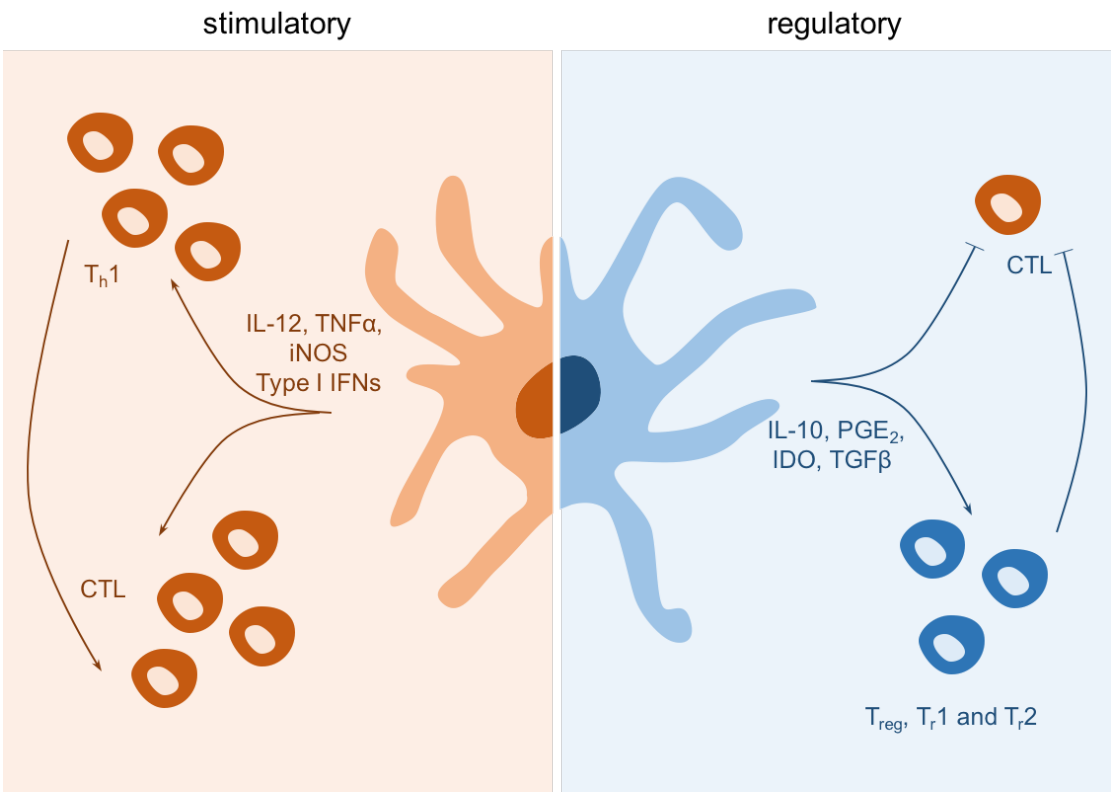

Figure 6. Dual responses of DCs to stimuli. Proinflammatory mediators promote Th1 differentiation and prime CTLs (left panel). Anti-inflammatory mediators promote regulatory T cell activation and inhibit CTLs (right panel).

In terms of cancer and tumourigenesis, many treatment strategies now consider ways to enhance DC function. For example, one study using the murine model for spontaneous breast cancer, MMTV-PyMT, demonstrates that while PD-1 therapy is ineffective in this model, anti-tumour antibody treatment combined with IFNγ and CD40L aimed at boosting DC activation was highly effective in inducing tumour regression (Spitzer et al., 2017). Another study demonstrates that expanding the $CD103^+$ migratory subset of cDC1s through Flt3L administration causes regression of B16 melanoma tumours in mice (Salmon et al., 2016). Flt3L is most effective as a therapy in combination with poly(I:C), which acts as an adjuvant to activate the expanded DCs. Interestingly, both of these studies demonstrate that post-treatment, PDL1 is upregulated on immune and/or

tumour cells. The upregulation of PDL1 in those contexts protects tumours and confers resistance to the therapy, however it also sensitizes the cells to anti-PDL1 therapy (Salmon et al., 2016; Spitzer et al., 2017). Therefore, in both of these studies, combining anti-PDL1 therapy with a therapy serving to increase DC function results in highly effective tumour regression. This highlights the importance of understanding dynamics of DC activation and its effect on systemic immunity.

1.4 Molecular regulation of dendritic cell function

On the molecular level, orchestrating an efficient immune response requires massive transcriptional reprogramming in response to environmental cues. Context becomes essential to instruct the type of immune response. For DCs and other innate immune cells, context is communicated by engagement of pattern-recognition receptors (PRRs), which recognize distinct pathogen-associated or damage-associated molecular patterns (PAMPs or DAMPs), and through cytokine receptors. PRRs and cytokine receptors often engage distinct but converging signaling pathways. However, specificity in a response can be achieved through the combination of signals received. The activation of dynamic transcription factors accompanies signal transduction through PRRs, dictating the gene signatures that are expressed or suppressed during an immune response (Belz and Nutt, 2012; Bornstein et al., 2014; Garber et al., 2012). Regulation of gene expression by dynamic factors also depends on the accessibility of transcription factor binding sites. The chromatin landscape therefore plays an important role in regulating innate immune cell function. A hierarchical transcription factor network allows for maintenance of lineage specification, priming of lineage-specific genes and dynamic regulation of those genes in response to environmental cues (Garber et al., 2012).

Transcriptional networks and epigenetics orchestrate DC function

It is well-established that development and differentiation of immune cell subsets relies on epigenetic modification and transcription of cell-type specific gene signatures. A growing body of evidence demonstrates that even after development, DCs and other immune cells require epigenetic modification for even short-term integration of signals from their environment which in turn has an impact on transcription factor binding and gene profiles (Bornstein et al., 2014; De Santa et al., 2009; Foster et al., 2007; Huang et al., 2012). The chromatin landscape can be conserved during mitosis therefore it is considered a form of inheritance. However, for most DC subsets, epigenetic modification serves a purpose beyond inheritance, since once differentiated, DCs do not divide. Instead, dynamic regulation of the chromatin landscape serves as a means to commit context-specific information in order to convey it readily after migrating from local tissues. Dynamic chromatin remodeling is intimately related to coordination of transcription factor networks, since transcription factors are limited by the availability of binding sites in the genome.

Factors such as PU.1 and C/EBP that specify lineage, are stably and ubiquitously bound to enhancer elements in the genome. Other factors such as ATF3 and JUNB, act at promoters and enhancers to prime genes for transcription and set the steady-state expression levels. Finally, the induction and suppression of genes in response to stimulation relies on dynamic transcription factors, which are guided by the priming factors (Garber et al., 2012).

Pioneer factors

PU.1 and C/EBP (both C/EBPα and C/EBPβ) are the major pioneering factors

(Guerriero et al., 2000; Laiosa et al., 2006). During hematopoiesis, PU.1 and C/EBP are the first to localize to lineage-specific enhancers of developing HSCs (Lin et al., 2015). Their binding is associated with the recruitment of chromatin modifying enzymes that regulate chromatin architecture and establish the initial chromatin landscape. It has long been appreciated that the dose of PU.1 correlates with lineage specification; the highest expression of PU.1 is associated with myeloid cell lineage (Carotta et al., 2010; Guerriero et al., 2000). Interestingly, PU.1 over-expression in T cell progenitors is sufficient to drive re-commitment to myeloid DCs and C/EBPα recommits to a macrophage cell fate (Laiosa et al., 2006). Since PU.1 enables the recruitment of other transcription factors, one mechanism by which it specifies lineage is by scavenging transcription factors from sites that specify other lineages leading to their suppression (Hosokawa et al., 2018).

Priming factors

Priming factors determine the cell-specific gene levels and prime genes activated in response to environmental stimuli. JUNB, ATF3 and IRF4 are examples of DC-specific priming factors (Garber et al., 2012). IRF4 is necessary for cDC2 development and is therefore likely a priming factor for cDC2s (Kovats et al., 2016; Vander Lugt et al., 2013). One possible mechanism by which factors prime genes for transcription is by serving as a docking site or maintaining the availability of a binding site (Mullen et al., 2011). Studies have shown that environmental stimuli activate transcription factors that then replace priming factors of the same family (Garber et al., 2012; Mullen et al., 2011).

Dynamic factors

Dynamic factors are regulated by cues in their environment. These factors include, EGR1, EGR2, NF-κB and STAT family proteins (Garber et al., 2012; Mullen et al., 2011).

Dynamic factors are enriched in the promoters of cell-type specific response genes and initiate transcription. Dynamic factors are mobilized downstream of signaling pathways in order to convey context, by dictating specific transcriptional programs. For example, LPS stimulation of DCs leads to a signaling cascade downstream of the Toll-like receptor (TLR), TLR4 that results in NF-κB activation and translocation into the nucleus. NF-κB activates transcription of thousands of LPS-response genes necessary to orchestrate inflammation (Vander Lugt et al., 2017). Similarly, type I IFNs stimulate STAT1 activation through their receptor, IFNAR which leads to the activation of interferon signaling genes (ISGs) that include anti-viral response genes (Ivashkiv and Donlin, 2013).

The transcription factor networks and dynamics have been exhaustively studied; however, less is known about the specific factors that modify the chromatin architecture, their interaction with priming and dynamic factors, and their effect on DC function.

Histone modification

The enzymatic addition or removal of chemical groups to histone tails regulates chromatin accessibility and transcription in a number of ways. Histone modification influences heterochromatin formation, the binding and recruitment of transcription factors, as well as the shaping of regulatory regions in the genome (Bannister and Kouzarides, 2011). There are many types of modifications; the most commonly studied are methylation, acetylation and ubiquitination (Table 2). Our work and the work of others suggest that DCs, monocytes and macrophages dynamically regulate their epigenome in response to environmental stimuli (De Santa et al., 2007; Foster et al., 2007; Saeed et al., 2014). Methylation and demethylation rely on the metabolic activity of the cell. For example, methyl groups are converted from methionine in the cytosol through one-carbon

metabolism for methyltransferases, and the TCA-cycle intermediate α-ketoglutarate can serve as a substrate for demethylases (Arts et al., 2016b). Therefore, substrate availability may contribute to dynamic changes of histone methylation status, since activation of DCs is also accompanied by metabolic reprogramming (Krawczyk et al., 2010). The link between metabolism and histone modification has been largely explored in cancer cells, however, understanding whether the metabolic changes of DCs contribute to their transcriptional reprogramming remains an area of interest.

Table 2. Commonly studied histone modifications and their associated functions

Histone modification	Common genomic localization	Transcriptional activity of associated genes
H3K4me3	promoters	active
H3K4me1	enhancers	active/poised
H3K27me3	promoters/enhancers/ heterochromatin	repressed
H3K36me3	Intergenic/heterochromatin	repressed
H3K9me3	heterochromatin	repressed
H2AK119Ub	heterochromatin	repressed
H3K27Ac	promoters/enhancers	active

Enhancers are most commonly associated with mono-methylated lysine 4 (H3K4me1) (Bannister and Kouzarides, 2011). Meanwhile, promoters are associated with H3K4me3. The tri-methyl group is added co-transcriptionally, and therefore H3K4me3 can serve as a marker for transcriptionally active loci (Xiao et al., 2003). However, most histone modifications do not alone indicate the activity or accessibility of their nearest genes. Instead, it is a combination and balance of modifications that dictate the resulting

transcriptional profile; this is known as the histone code. One exception is acetylation or tri-methylation of H3K27, which act as switches to turn a gene on or off, respectively. For example, an enhancer is considered "poised" if it carries H3K4me1, it is considered active in combination with H3K27ac and repressed in combination with H3K27me3. While much work has been done to interpret and understand the histone code, continued discoveries of new modifications along with consideration of direct modification of DNA, DNA:RNA interactions, as well as transcription factor binding, highlight the complexity of genomic regulation through epigenetic modifications.

Relatively new strategies aimed at assessing open regions of chromatin have been developed, such as sequencing the assay for transposase accessible chromatin (ATAC-seq) (Buenrostro et al., 2013). ATAC-seq coupled with other sequencing techniques can give a very thorough picture of chromatin states.

DNA methylation

DNA can also be directly modified, most commonly by methylation. DNA methylation serves a very important role in self-recognition for mammalian cells (Teitell and Richardson, 2003). Methylation of CpG islands is a eukaryotic process therefore bacteria and other invading pathogens can sometimes be recognized through unmethylated CpG islands. CpG stimulates the intracellular receptor TLR9, which results in a very strong inflammatory response involving IL-12 and type I IFN production.

Besides self-recognition, DNA methylation has been long appreciated for its role in transcriptional repression and formation of heterochromatin (Riggs, 1975). Methyl groups are deposited onto cytosine residues by DNA methyl transferases (DNMT family of enzymes). DNA methylation is readily maintained during cell division due to interaction

of DNMT1 with the DNA replication machinery (Probst et al., 2009) Removal of DNA methylation can be facilitated by ten eleven translocation (TET) hydroxylases. Recent work has revealed that DNA de-methylation or hypo-methylation can contribute to inflammation. For example, one study has demonstrated that bacterial infection of human DCs leads to rapid DNA demethylation that occurs most frequently at enhancers (Pacis et al., 2015). Loss of DNA methylation was associated with enhancer activation and recruitment of dynamic transcription factors.

Interestingly, a study in cancer cells demonstrates that drug-resistant, triple-negative breast cancer cells acquire modified one-carbon metabolism, which converts methionine to other metabolites such as methyl groups for various cellular processes (Deblois et al., 2018). Importantly, this altered metabolism leads to hypomethylation of DNA which is compensated for by EZH2-mediated H3K27me3. This altered epigenetic signature allows drug-resistant cells to be targeted by EZH2 inhibitors, which exposes the unmethylated DNA and activates the anti-viral response of the cell. Whether DNA demethylation in response to bacterial infections is also the result of metabolic adaptation of DCs in response to infection could provide an interesting intersection between dynamic chromatin remodelling and metabolic reprogramming.

Factors that regulate epigenetic modification

To effect a single chromatin modification requires the action of many factors: “readers” that scan chromatin for specific binding sites, “writers” that catalyse the addition of a chemical group, and “erasers” that trigger removal of a chemical group. There are several families of “writers” and “erasers” depending on their catalytic activity (Table 3). Some writers and erasers also contain reading domains (Torres and Fujimori, 2015).

Furthermore, complexes of multiple factors often act together to mediate chromatin modifications. There are two broad families of chromatin modifying complexes; trithorax (TrxG) family complexes are responsible generally for transcriptional activation through chromatin modification and polycomb repressive complexes (PRC) are responsible for transcriptional repression (Schuettengruber et al., 2007).

Table 3. Enzymes mediating epigenetic modification

Enzyme family	Examples	Catalytic activity	Reviewed in
HDAC	HDAC1-11 SIRT1-7	histone deacetylase	(Seto and Yoshida, 2014)
HAT	HAT1 p300 CBP hGCN5 TAF1	histone acetyl transferase	(Lee and Workman, 2007)
HMT	MLL1-5 SET domain-containing EZH1 EZH2	histone methyl transferase	(Dillon et al., 2005)
KDM	JARID2 KDM5C (JARID1C) JARID1D (KDM5D) KDM2B	lysine demethylase	(Shi, 2007)
DNMT	DNMT1 DNMT3	DNA methyl transferase	(Lyko, 2017)
TET	TET1-3	DNA de-methylase	(Kohli and Zhang, 2013)
RING	RING1A RING1B	E3 ubiquitin ligase	(Di Croce and Helin, 2013)

Trithorax complexes

TrxG complex members were first identified in *Drosophila melanogaster* for their role in opposing PRC-mediated transcriptional repression (Ingham, 1983). They do so by catalyzing H3K27 demethylation and H3K4 trimethylation. There are two classes of TrxG

complexes, SWI/SNF complexes and COMPASS, both of which have been shown to counteract PRC activity (Schuettengruber et al., 2017). Core COMPASS members include WDR5, RBBP5 and DPY30. These complexes are SET-domain containing, and therefore confer histone methyl transferase activity. The SWI/SNF complex contains BRG1, which mediates ATPase-dependent chromatin modification, as well as a number of BAF proteins that have various DNA-binding and histone-binding activities (Schuettengruber et al., 2017).

Polycomb repressive complexes

PRC was first identified in *Drosophila melanogaster* for its role in suppressing *Hox* gene family members (Lewis, 1978). *Hox* genes determine the fate of embryonic segments during development. Failure to appropriately regulate *Hox* genes leads to developmental abnormalities that lead to the nomenclature “polycomb” (Lewis, 1978). Therefore, the PRC was described early on for its role in development and specifying cell fate. More PRC family members were identified over time that form unique complexes (Gao et al., 2012). There are two classes of PRCs, PRC1 and PRC2 (Figure 7). PRC2 has been widely studied in cancer cell lines since some members of PRC2 can be over-expressed in certain cancers. PRC2 is composed of several core complex members including EZH1/2, EED and SUZ12. PRC2 catalyses the repressive H3K27me3 mark. It is thought to reinforce transcriptional repression by mediating spreading of H3K27me3 around the initial mark (Lee et al., 2018; Oksuz et al., 2018). EED is a reader of H3K27me3, and enhances the methyl transferase activity of EZH2. Therefore, the functional association of EZH2 and EED along with other associated factors such as JARID2, promotes propagation of H3K27me3 and gene repression (Oksuz et al., 2018).

EZH2 also binds to nascent mRNA of active genes which inhibits its activity (Kaneko et al., 2014). These two mechanisms of feedback regulation serve to reinforce and amplify the repression or activation of specific genes.

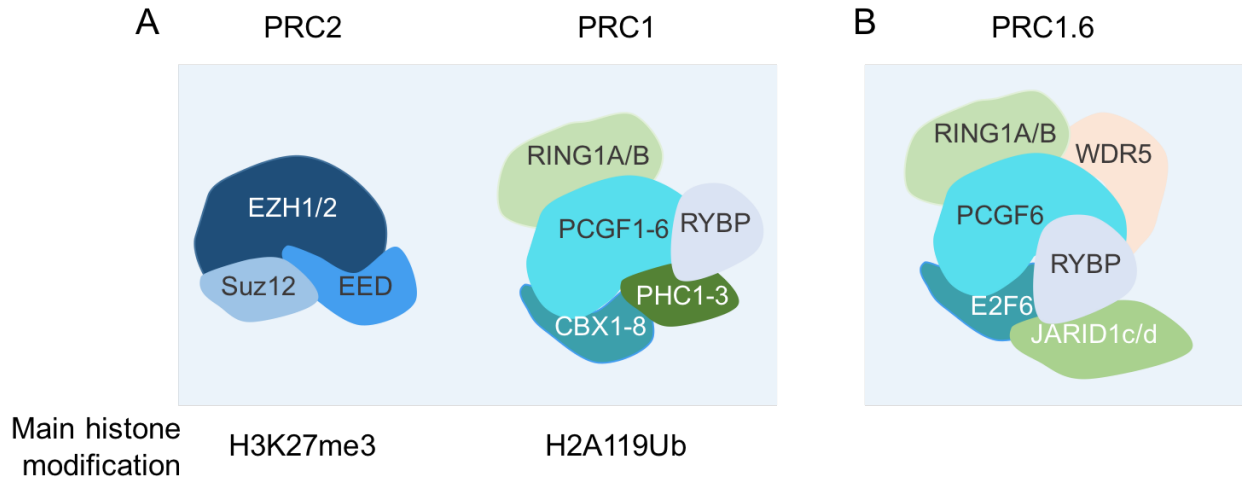

Figure 7. Components of PRC complexes. A. Core PRC1 and PRC2 complex members. B. Subunits of PRC1.6.

PRC1 represents a more diverse set of complexes that are functionally distinct. The core PRC1 complex is composed of one of six polycomb group factors (PCGF1-6), RING1A/B which is an E3 ligase that catalyses monoubiquitination of H2A119, and one of eight CBX proteins that recognize H3K27 methylation (Gao et al., 2012). The canonical role of PRC1 complexes is to recruit PRC2 which then mediates spreading of the repressive signal over the genomic region. However, there is a growing body of evidence for distinct functional roles, and it is now appreciated that PRC1 complexes carry out numerous different histone modifications to repress transcription across the genome (Gao et al., 2012; Schuettengruber et al., 2017). They are also capable of forming much larger complexes of proteins, involving other factors that are not always associated with PRCs (Trimarchi et al., 2001; Trojer et al., 2011).

Transcriptional repression through PRC1.6

PCGF6 was first identified as MEL-18, BMI-1-like RING protein, or MBLR for its similarity to MEL-18 and BMI-1 in protein domain and function (Figure 8) (Akasaka et al., 2002). It forms a complex with RING1B and the atypical E2F transcription factor, E2F6 (Akasaka et al., 2002; Ogawa et al., 2002). E2F6 represses transcription by recruiting chromatin modifiers including HP1γ, that mediates H3K9me3 (Ogawa et al., 2002). A recent study found that E2F6 recruits the PCGF6-containing repressive complex (PRC1.6) to its target sites (Stielow et al., 2018). Interestingly, PCGF6 genomic localization is balanced by both E2F6 and the protein L3MBTL2, each of which retain a distinct set of genomic targets.

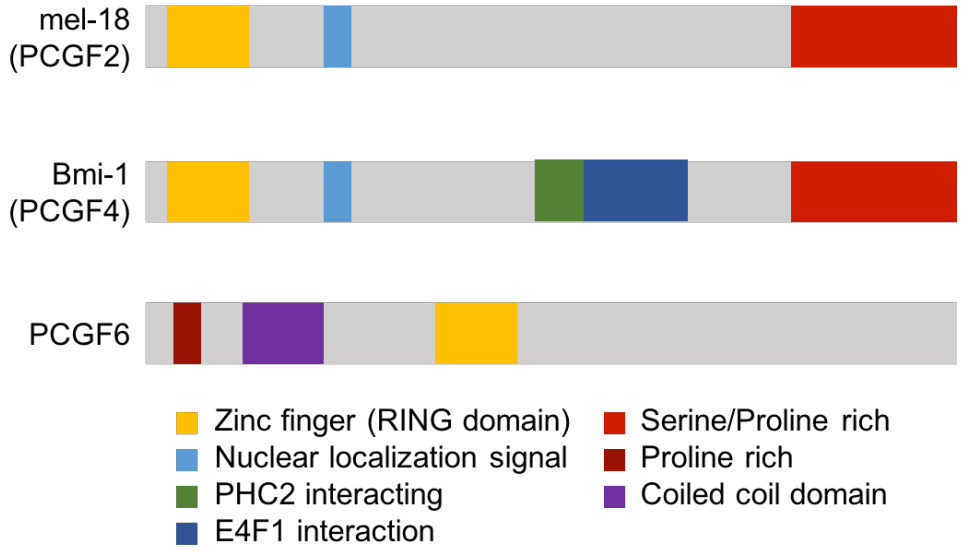

Figure 8. Unique and shared domains in PCGF proteins

PCGF6 is most highly expressed in the reproductive organs of male mice, where it is involved in spermatogenesis (Endoh et al., 2017; Sun et al., 2015; Zdzieblo et al., 2014). It has also been shown to maintain stem cell identity (Yang et al., 2016; Zhao et al., 2017b). Its genomic localization resembles TrxG complexes rather than polycomb

group complexes; one study has proposed that PRC1.6 might also contribute to transcriptional activation (Yang et al., 2016). Indeed, PCGF6 has been found in complex with WDR5, a core COMPASS member (Gao et al., 2012), however, whether this binding hinders its activity or promotes it is unclear. Despite theories that PRC1.6 may achieve both activation or repression, many studies have confirmed a repressive role (Akasaka et al., 2002; Endoh et al., 2017; Stielow et al., 2018; Trojer et al., 2011; Zhang et al., 2017).

In addition to E2F6, PCGF6 has been found in complex with Jumonji-ARID domain containing 1D (JARID1D) and JARID1C (SMCX, KDM5C) (Lee et al., 2007). JARID1D and JARID1C are members of a family of histone lysine demethylases. JARID1C is an X-linked and JARID1D is a Y-linked repressor of transcription through H3K4me3 demethylase activity. An early study established the association of JARID1D and PCGF6, demonstrating that PCGF6 was required for the enzymatic activity of JARID1D (Lee et al., 2007). JARID1D catalyses the demethylation of H3K4me3 near transcription start sites and this impedes transcriptional initiation. Less is known about the association of PCGF6 and JARID1C; they have been identified in complex through mass spectrophotometry studies (Qin et al., 2012), however the functional consequence of their association is unexplored.

Studies that have identified JARID1C near sites of active promoters and enhancers have raised the question of whether JARID1C can also participate in transcriptional activation or if it serves to limit the activity (Outchkourov et al., 2013). Similar to PCGF6, JARID1C has a widespread genomic localization. One study reports that JARID1C promotes enhancer activity but silences promoters (Outchkourov et al., 2013). However,

JARID1C has also been shown to associate with RACK7 at many active enhancers (Shen et al., 2016). This interaction was shown to limit enhancer activity, and loss of RACK7 in cancer cells promotes the expression of genes involved in tumourigenesis. Therefore, it is possible that the specific function of JARID1C depends on the other members of the complex it forms, or that it's role is to fine-tune the activity of promoters or enhancers rather than to strictly activate or suppress them.

Enhancer dynamics and DC function

Recently, there has been a growing interest in and appreciation for the dynamic regulation of enhancer elements. With the advent of new tools such as chromatin conformation capture (a technique used to characterize chromatin looping and long-range interactions; 3-C) and better bioinformatics prediction software, studying enhancers and their target genes has become more feasible and effective (Shlyueva et al., 2014). However, reliably identifying enhancers and their associated genes is still an area of ongoing work. What has become clear is that dynamic regulation of enhancers is an important part of integrating environmental cues. Though previous work has shown that accumulation of epigenetic modifications and specific transcription factors on enhancers serves mainly to specify lineage, there is a growing appreciation of their continued role, for example, during an inflammatory response.

A subclass of enhancers, "super-enhancers", are thought to be extremely stable and are required to specify cell identity (Hnisz et al., 2013). Super-enhancers regulate large clusters of genes, and are often associated with a relatively high number of transcription factors. AIRE, the transcription factor required for negative selection in the thymus, associates with super-enhancers in thymic stromal cells (Bansal et al., 2017).

The super-enhancer loops to interact with their target promoters and this looping likely facilitates the interaction of AIRE with transcription start sites.

Super-enhancers can control other enhancer elements as well. Interestingly, TLR4 stimulation of macrophages lead to dynamic changes in enhancer RNA profiles mediated by super-enhancer elements (Hah et al., 2015). This illustrates an interaction between stable and dynamic elements which ultimately determines lineage-specific, but functionally diverse responses.

Lineage-determining factors such as PU.1 and C/EBP are broadly bound to enhancer elements genome-wide and facilitate lineage specification (Garber et al., 2012). This can occur, for example, through mutual negative feedback inhibition which leads to activation or silencing of cell-type specific enhancers. For example, C/EBPβ specifies monocyte-derived DC lineage and inhibits IRF8, which specifies pDC cell fate (Bornstein et al., 2014). During hematopoiesis, IRF8 reinforces a pDC cell fate by suppressing C/EBPβ and by activating pDC specific enhancers. Similarly, monocyte-derived DCs depend on C/EBPβ to regulate enhancers, and suppress IRF8. This mutual inhibition can ensure cell fate and appropriate enhancer activity regardless of the environment, since the presence of one factor ensures the suppression of the other.

Trained immunity and tolerance

While the epigenetic modifications that specify lineage are highly stable, the environment shapes the functional responses of DCs. DCs can be conditioned by their environment to either increase their sensitivity to stimuli (trained immunity) or to decrease their sensitivity (tolerance). Global epigenetic remodelling underlies both of these processes. β-glucan treatment of human monocytes conditions them to become more

stimulatory compared to untreated monocytes in response to LPS (Garcia-Valtanen et al., 2017; Quintin et al., 2012). Underlying this are widespread changes in multiple histone modifications including H3K4me3 and H3K27Ac. Pre-treatment of DCs with LPS leads to tolerance, where DCs become refractory to stimulation due to epigenetic silencing of inflammatory genes. In some ways, trained immunity and tolerance can be thought of as a form of short term memory that instructs DCs and other innate immune cells on how to respond to subsequent threats.

Trained Immunity

Much of the work studying trained immunity has been focused on monocytes. Training by β-glucan leads to genome-wide changes in H3K4me3 and H3K27Ac profiles which primes proinflammatory genes (Saeed et al., 2014). Therefore, subsequent stimulation leads to a more rapid and robust proinflammatory response. The *Mycobacterium tuberculosis* (M.tb) vaccine, BCG also results in training of monocytes to enhance their protection against M.tb (Arts et al., 2016a; Kaufmann et al., 2018). Training occurs at the level of HSCs as well, leading to unique epigenetic and metabolic signatures in macrophages arising from BCG-trained monocytes (Kaufmann et al., 2018). Glycolysis induced through mTOR and HIF1α is required for BCG and β-glucan training, and blocking these pathways abrogates the effects of training (Arts et al., 2016a; Cheng et al., 2014). In addition to this, fumarate from the TCA cycle, fuelled by glutaminolysis, is directly required to provide substrates that mediate H3K4me3 and H3K27Ac during training (Arts et al., 2016b).

The idea that training also occurs in hematopoietic progenitors is relatively novel. However, one recent study demonstrates that β-glucan treatment promotes myelopoeisis

and favours epigenetic and metabolic alterations that enhance the response of newly differentiated myeloid cells to further stimulation (Mitroulis et al., 2018). Developing innate immune cells are therefore conditioned by their environment to not only modify their differentiation but also the type and magnitude of the response that they generate. This demonstrates an important overlap between immune cell development and dynamic regulation of immune cell function, which are dependent on chromatin modification and metabolic reprogramming. Developing immune cells receive feedback from the periphery to instruct the needs of the host.

The benefits of trained immunity are clear; it confers protection against pathogen reinfection and can even protect against other pathogens as well. However, there is also evidence that trained immunity contributes to certain autoimmune diseases by sensitizing innate immune cells to harmless stimuli (Arts et al., 2018). For example, monocytes from SLE patients demonstrate modified histone acetylation profiles suggestive of a hyperactive state (Zhang et al., 2009). Therefore, strategies to re-train or prevent training of innate immune cells through metabolic or epigenetic intervention could be a viable strategy to manage symptoms of certain autoimmune diseases.

Tolerance

As opposed to trained immunity, tolerance represents a state of immune unresponsiveness. As mentioned previously, tolerance can refer to the process of recognizing self and harmless stimuli or it can refer to a refractory period following proinflammatory stimulation whereby the immune system fails to recognize subsequent threats. With respect to the latter, monocytes, DCs, and macrophages adopt a chromatin landscape that predominantly favours immune suppression. Through the upregulation of

suppressive factors such as IL-10, PDL1, IDO and TGFβ, along with concomitant silencing of IL-12 and other pro-inflammatory mediators, DCs potently suppress immune responses directly or through the activation of T_{reg} cells (Berg et al., 1995; Fallarino et al., 2015; Wysocka et al., 2001; Yoon et al., 2017). A common model to study the mechanisms of tolerance is the "LPS tolerance" model. Simply, an overnight treatment of DCs or macrophages with LPS is sufficient to program a tolerogenic response (Fallarino et al., 2015; Foster et al., 2007). Restimulation favours the production of IL-10 over IL-12 and also induces other anti-inflammatory mediators discussed above. A study in macrophages demonstrates a class of genes that are activated with stimulation and become silenced upon re-stimulation (Foster et al., 2007). A separate class of genes become primed and more rapidly increase in expression upon re-stimulation. Silencing of tolerized genes occurs through a decrease in H3K4me3 at the promoters and a failure to induce H3K27Ac (Foster et al., 2007). It is thought that the factors induced by the other class of genes contribute to the silencing. Similarly, a study of human monocyte-derived DCs tolerized by TGFβ demonstrates a global redistribution of H3K4me3 and H3K27me3 (Huang et al., 2012). This remodeling contributes to the suppression of proinflammatory cytokines, MHCII, and co-stimulatory molecules.

Interestingly, LPS induces cross-tolerance; that is, secondary challenge with other TLR ligands is also impaired by LPS stimulation (de Vos et al., 2009). This is likely due to the stability of epigenetic silencing; the transcription factors downstream of TLR signaling cascades are not able to access sites to induce transcription.

1.5 Active Maintenance of DC homeostasis

At steady state, DCs constantly balance two states: a state of maintaining

quiescence and restraining an immune response and a state of readiness to react and mount a potently proinflammatory response. Maintaining this delicate homeostasis requires tight control of the cellular processes that are modulated with stimulation. Evidence indicates that homeostatic maturation occurs in cDC1s through spontaneous NF-κB activation (Ardouin et al., 2016; Baratin et al., 2015). Interestingly, the rate of homeostatic maturation of DCs is higher in the thymus, and this was found to be important for central tolerance (Ardouin et al., 2016). The purpose of homeostatic maturation in the periphery is unclear. However, it is clear that increasing the frequency of homeostatic maturation promotes auto-inflammatory disease and autoimmunity (Kool et al., 2011).

Mature cDC1s arise from immature cDC1s, rather than arising from distinct progenitors (Ardouin et al., 2016). The transcriptional profile of spontaneously activated cDC1s resembles that of TLR4-matured cDC1s. Since PRR-signaling is not required to elicit a proinflammatory transcriptional program, it suggests that DCs actively restrain maturation by preventing transcriptional reprogramming. When those restraints fail, DCs become spontaneously matured.

The dual role of NF-κB at steady state and following activation also highlights that maintenance of DC homeostasis is an active process. As mentioned, spontaneous NF-κB activity contributes to homeostatic maturation of DCs (Baratin et al., 2015). In addition, at steady state, NF-κB restrains DC activation and prevents DCs from inducing self-reactive CTL responses (Dissanayake et al., 2011). This was shown using the RIP-gp model, which expresses the LCMV glycoprotein under the rat insulin promoter, restricting its expression to the pancreatic β-islets. In this model, infection with LCMV induces an immune response against LCMV and the β-islets of the pancreas which then induces

diabetes, measurable by increased blood sugar. Activated DCs bearing the specific epitopes from the LCMV glycoprotein also induce diabetes in this model. Interestingly, deficiency of NF-κB in DCs leads to the spontaneous induction of diabetes, in the absence of stimulation (Dissanayake et al., 2011). Through genome foot-printing studies, it has been shown that the genomic loci bound by NF-κB are different in steady state and activated DCs suggesting that NF-κB finds new targets when DCs become activated (Smith et al., 2011). Therefore, the dynamics of transcription factor binding contribute to the maintenance of steady state by DCs.

Furthermore, ZBTB46, was recently identified as a transcription factor restricted to cDCs (Meredith et al., 2012; Satpathy et al., 2012). ZBTB46 belongs to a zinc-finger DNA-binding family of transcription factors. ZBTB46 deficiency in mice promotes a cDC1 cell fate and also promotes an activated, proinflammatory state. This study found that downregulation of ZBTB46 accompanies TLR-maturation and was necessary to permit activation (Meredith et al., 2012). Interestingly, a number of studies have demonstrated that lineage-specific factors that contribute to the differentiation of DCs also are downregulated in response to maturation signals (Ardouin et al., 2016; Meredith et al., 2012; Seguin-Estevez et al., 2014). Once committed, the lineage of DCs is highly stable (Bornstein et al., 2014; Schlitzer et al., 2015), therefore sustained expression of lineage-specifying factors may serve to restrain the full maturation of DCs until the appropriate signals are received.

The role of epigenetic modification in maintaining DC homeostasis is a more recent area of interest. Factors such as PU.1 and IRF4 that regulate the differentiation and dynamics of DC activation and function can also recruit histone modifying enzymes and

complexes to affect the chromatin landscape of DCs (Garber et al., 2012; Seguin-Estevez et al., 2014; Vander Lugt et al., 2017). While many studies have shown that histone modification occurs during DC maturation, the factors that mediate these changes remain largely unidentified. It is likely that factors maintain a repressive state at sites important for inflammation, and a first step for initiating an inflammatory response would be overcoming that suppression (Figure 9).

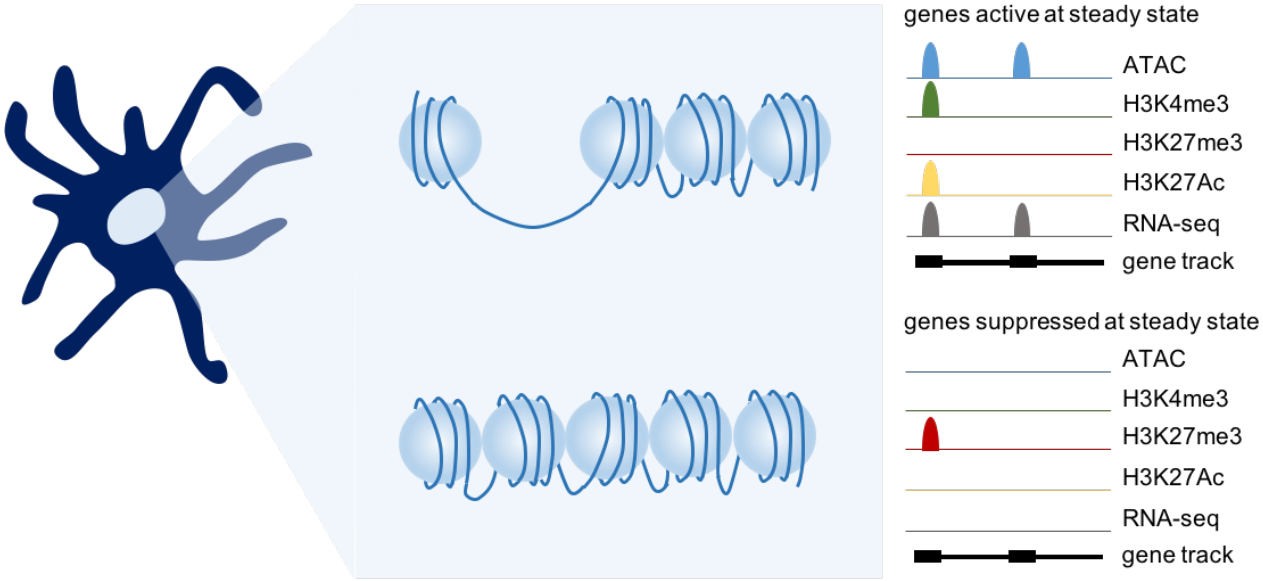

Figure 9. Proposed model for maintenance of steady state through histone modification. Genes that are active at steady state would be accessible and constitutively expressed and would bear chromatin modifications associated with transcriptional activation. Meanwhile, genes that are induced upon stimulation are maintained in a repressed state.

1.6 Rationale

DCs play a central role in orchestrating T cell responses. At steady state, DCs participate in maintaining central and peripheral tolerance. DC activation promotes the clearance of infections and tumour cells by priming T cell responses.

It is clear that the chromatin landscape plays a major role in dictating dynamic responses of DCs (Belz and Nutt, 2012; Bornstein et al., 2014; Garber et al., 2012). Furthermore, DC homeostasis is maintained by factors that actively restrain proinflammatory processes at steady state (Dissanayake et al., 2011; Kool et al., 2011; Meredith et al., 2012). Identifying the specific factors that mediate and maintain epigenetic modifications in DCs could lend important insight into the mechanisms by which DCs regulate their homeostasis, differentially respond to stimuli and prime T cell responses.

We identified PCGF6 in a microarray screen that compared unstimulated DCs to LPS-stimulated DCs (Kane et al., 2004). PCGF6 was downregulated in response to LPS. Given its role as a transcriptional repressor, we hypothesized that PCGF6 maintains DC quiescence through chromatin modification.

Preface to Chapter 2

The goal of this study was to characterize the function of PCGF6 in DCs. PCGF6 has a known role as a mediator of transcriptional repression. In DCs, it was downregulated in response to LPS in a microarray screen. Therefore, we predicted that PCGF6 was involved in maintaining DC quiescence.

Chapter 2: The transcriptional repressor Polycomb group factor 6, PCGF6, negatively regulates dendritic cell activation and promotes quiescence

Adapted from the published work:

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The transcriptional repressor Polycomb group factor 6, PCGF6, negatively regulates dendritic cell activation and promotes quiescence

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2.1 Short summary

Pro-inflammatory signals provided by the microenvironment are critical to activate Dendritic Cells (DCs), components of the innate immune system that shape both innate and adaptive immunity. However, to prevent inappropriate immune activation, mechanisms must be in place to restrain DC activation to ensure DCs are activated only once sufficient stimuli have been received. Here we report that DC activation and immunogenicity are regulated by the transcriptional repressor polycomb group factor 6 (PCGF6). Pcgf6 is rapidly downregulated upon stimulation and this downregulation is necessary to permit full DC activation. Silencing PCGF6 expression enhanced both spontaneous and stimulated DC activation. We show that PCGF6 associates with the H3K4me3 demethylase JARID1c, and together they negatively regulate H3K4me3 levels in DCs. Our results identify two key regulators – PCGF6 and JARID1c – that temper DC activation, and implicate active transcriptional silencing via histone demethylation as a previously unappreciated mechanism for regulating DC activation and quiescence.

2.2 Introduction

Dendritic cells (DCs) play an essential role in host defense by recognizing invading pathogens, initiating inflammation, and stimulating the activation and differentiation of T cells, central mediators of the adaptive immune response. Key to the capacity of DCs to fulfill these functions is their ability to transition from a resting or quiescent state to an active state. A major pathway for DC activation is provided by pattern recognition receptors (PRRs) that recognize molecular patterns from microbes termed PAMPS/MAMPS (pathogen–associated molecular patterns/microbe-associated molecular patterns) and self-molecules termed DAMPS (damage-associated molecular patterns) (Hammer and Ma, 2013; Macagno et al., 2007; Mogensen, 2009).

Despite their diversity, all PRRs initiate signaling pathways that lead to coordinated changes in cellular biology necessary for the effector function of DCs, most of which are underpinned by broad changes in gene expression (Hammer and Ma, 2013; Johnson and Ohashi, 2013). DCs that have undergone activation enhance their antigen-presentation capacity, increase the expression of costimulatory molecules such as CD80 and CD86 and produce many cytokines, chemokines and lipid mediators that shape the inflammatory microenvironment (Sallusto et al., 1995). While the pathways that promote transcriptional activation downstream of inflammatory and PRR signals are well described (Mäkelä et al., 2009; Mogensen, 2009; Yoshimoto et al., 1997), less is known about negative regulators that counter activation signals.

One way of reinforcing transcriptional states in immune cells is through reversible, gene-specific, post-translational modifications of histones (Wen et al., 2008b).

Methylation of lysine 4 of histone 3 (H3K4) marks transcriptionally active and accessible genes (Dong et al., 2012; Muramoto et al., 2010). In DCs and macrophages, H3K4me3 has been reported to be important for IL-12p40 production following TLR activation (Foster et al., 2007; Wen et al., 2008a; Yu et al., 2014). Mechanistically, H3K4me3 can facilitate transcription by promoting the initiation and elongation of transcripts of H3K4me3 marked genes (Benayoun et al., 2014; Lauberth et al., 2013; Ruthenburg et al., 2007).

Polycomb group factor 6 (PCGF6) (also known as MBLR for Mel18 and Bmi1-like RING finger protein) is a member of the Polycomb group (PcG) family of transcriptional repressors (Akasaka et al., 2002; Lee et al., 2007; Sun et al., 2015; Yang et al., 2016; Zdzieblo et al., 2014). PCGF6 has been identified in polycomb repressive complexes (PRC1) that have H3K9 methyltransferase and those that have H3K4 demethylase activity (Gao et al., 2012; Lee et al., 2007). PCGF6 has been reported to have a role in embryonic stem cell differentiation and male germ cell development (Sun et al., 2015; Yang et al., 2014; Zdzieblo et al., 2014), however a function for PCGF6 in the biological context of DCs or any immune cell has not been reported.

Here we show that PCFG6 is a negative regulator of DC activation and function, and is integral to maintaining DC quiescence. Downregulation of PCGF6 expression accompanies, and is necessary for, DC activation by pro-inflammatory stimuli. We demonstrate that H3K4me3 levels in DCs are regulated both by PCGF6 and the histone demethylase JARID1c, and that both proteins maintain a quiescent state. These findings identify two important negative regulators of DC activation and function, and demonstrate that maintaining DC quiescence is an active process involving transcriptional silencing mechanisms.

2.3 Results

Pcgf6 is expressed in quiescent DCs and downregulated following activation

Pcgf6 was identified as being down-regulated by LPS, a strong proinflammatory stimulant, in a previously performed microarray-based comparison of gene expression in quiescent versus activated DCs (Kane et al., 2004). Since Polycomb proteins are broadly known to regulate cellular gene expression programs in cells, we hypothesized that PCGF6 may repress gene expression at the steady state and its downregulation following DC activation may favor increased transcription of the many genes that are coordinately expressed during DC activation. *Pcgf6* expression was characterized in DCs and was found to be rapidly downregulated as early as 2h post activation with LPS, coinciding with the induction of the proinflammatory cytokine *Il12b* (p40 subunit) and was maintained for up to 36h (Figure 1A). Importantly, *Pcgf6* was downregulated to similar extents in splenic DCs and GM-CSF-derived DCs (Figure 1B).

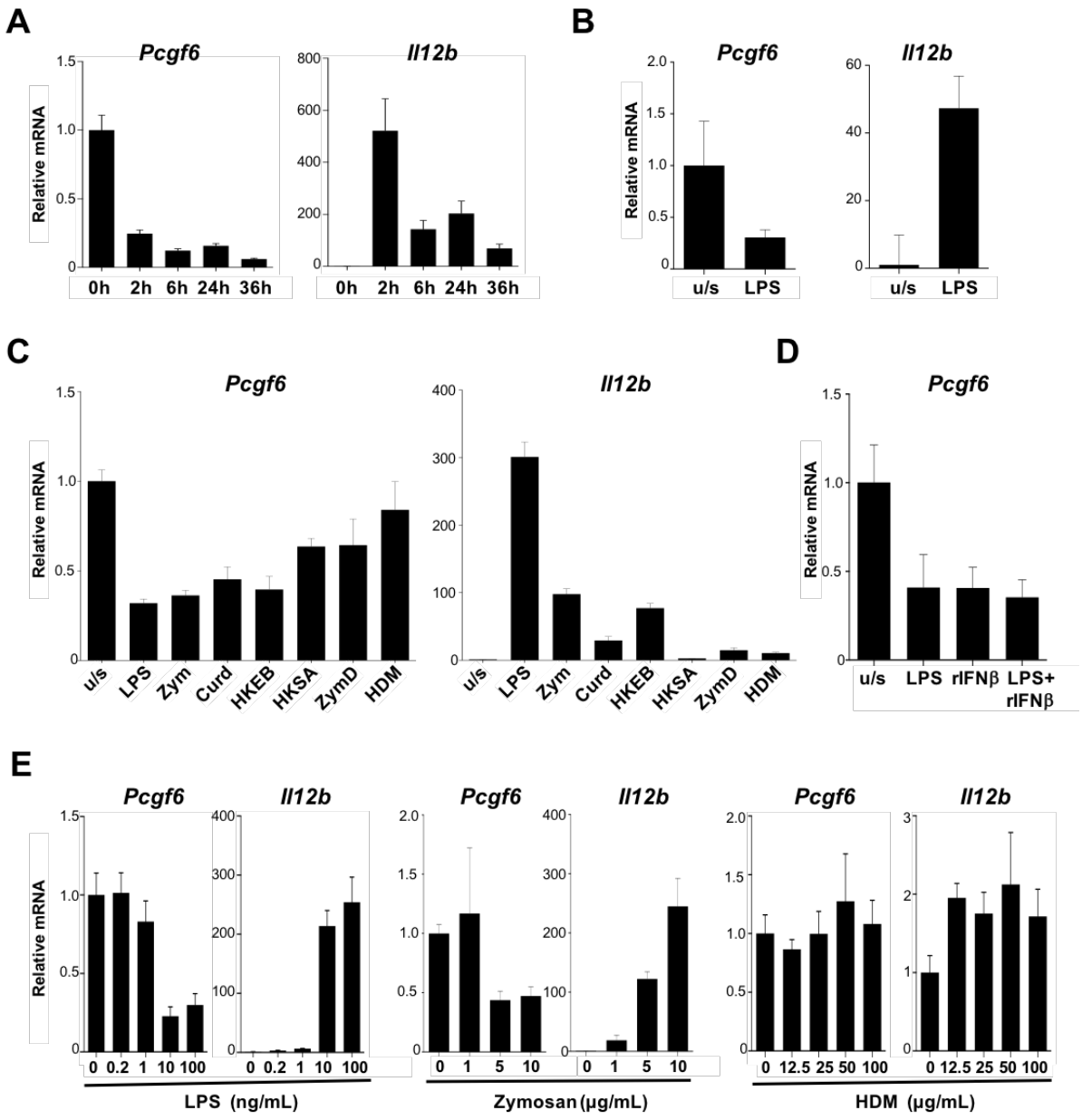

Figure 1. *Pcgf6* expression inversely correlates with activation phenotype. A-E. Gene expression of *Pcgf6* and *Il12b* in DCs stimulated with the indicated activators determined by qRT-PCR normalized to *Hprt* A. DCs stimulated with 10 ng/mL LPS for the indicated times. B. Splenic DCs sorted from mouse spleens activated *ex vivo* with 10 ng/mL LPS for 4h. C. DCs stimulated with the indicated activators for 2h. D. DCs stimulated with the rIFNβ (1000U/mL) and/or LPS for 18h. E. DCs activated with indicated doses of stimuli for 18h. Data is shown for one representative experiment of at least 2-5 independent experiments. LPS (lipopolysaccharide, 10 ng/mL); Zym (Zymosan, 10 μg/mL); Curd (Curdlan, 50 μg/mL); HKEB (heat-killed *Escherichia coli* B); HKSA (heat-killed Staphylococcus aureus); ZymD (Zymosan-depleted, 10 μg/mL); HDM (house dust mite, 50 μg/mL). See also Figure S1.

To determine whether *Pcgf6* downregulation was stimulant-dependent the expression of *Pcgf6* following exposure of DCs to a variety of stimulants was determined. *Pcgf6* expression inversely correlated with the extent of DC activation as measured by expression of CD80, CD86 and MHCII and induction of the inflammatory cytokine *Il12b* (Figures 1C, 1D, and S1). DCs activated by strong pro-inflammatory stimuli such as LPS, Zymosan, Curdlan and IFNβ reduced the expression of *Pcgf6* by 50%-90%, whereas weaker stimuli such as HDM and ZymD induced downregulation of *Pcgf6* expression to a lesser extent (Figure 1C). DCs were stimulated with titrated doses of select activators to determine whether the failure to downregulate *Pcgf6* was due to the dose, and not the nature of the stimulus. Decreases in *Pcgf6* expression again correlated with the extent of DC activation (Figure 1E). These findings demonstrate that the transcriptional repressor *Pcgf6* is downregulated following DC stimulation, and directly correlates with the activation status of DCs.

PCGF6 maintains DC quiescence by negatively regulating DC activation

To test the hypothesis that the downregulation of PCGF6 permits, or facilitates, DC activation we used retroviral-mediated gene transfer to force continued expression of PCGF6 in DCs during activation (Figure S2A) (Krawczyk et al., 2008). Forced expression of PCGF6 resulted in decreased expression of CD80, CD86 and MHCII at steady state and following LPS-stimulation, which could not be rescued by increasing doses of stimuli (Figures S2B, S2C). DCs constitutively expressing PCGF6 also produced lower levels of proinflammatory cytokines, including IL-6, TNF-α, and IL-12p40 (Figure 2A). Decreased production of IL-12p40 and IL-12p70 and expression of *Il12b* were also detected by ELISA and qRT-PCR, respectively (Figure 2B). This defect was not rescued by increasing

the dose of LPS stimulation (Figure S2D). DCs derived from FLT3L-treated cultures overexpressing PCGF6 also produced less IL-12p40 following LPS activation (Figure S2E). Concomitant with DC activation is an increase in glycolysis to support the bio-energetic requirements of DC activation (Everts et al., 2014; Krawczyk et al., 2010). Consistent with a role for PCGF6 in suppressing DC activation, we found that DCs constitutively expressing PCGF6 had lower extracellular acidification rate (ECAR) (surrogate of glycolysis) following LPS stimulation (Figure 2C).

Interestingly, DCs that constitutively expressed PCGF6 produced more of the anti-inflammatory IL-10 both at rest and following LPS stimulation (Figure 2D, S2F). IL-10 antagonizes DC activation by suppressing the upregulation of activation markers and cytokines upon maturation (Corinti et al., 2001). However, neutralizing the IL-10R had no effect on PCGF6-mediated suppression of activation (Figure 2E, S2G). Furthermore, rIL-10 could not prevent Pcgf6 downregulation (Figure 2F). These results suggest that PCGF6 and IL-10 operate in independent pathways to suppress DC activation.

To address the functional role of PCGF6 in DCs, the ability of transduced DCs to stimulate OVA-specific $CD4^+$ and $CD8^+$ TCR transgenic T cells (OT-II and OT-I) was examined. DCs with constitutive expression of PCGF6 were less efficient at inducing CD4+ T cell activation, measured by CD44 and CD25 expression, proliferation and Th1 differentiation (measured by IFN-γ) (Figure 2G-I). Likewise, DCs constitutively expressing PCGF6 were poor stimulators of $CD8^+$ T cell activation, proliferation and IFN-γ production in $CD8^+$ T cells (Figure 2J-L).

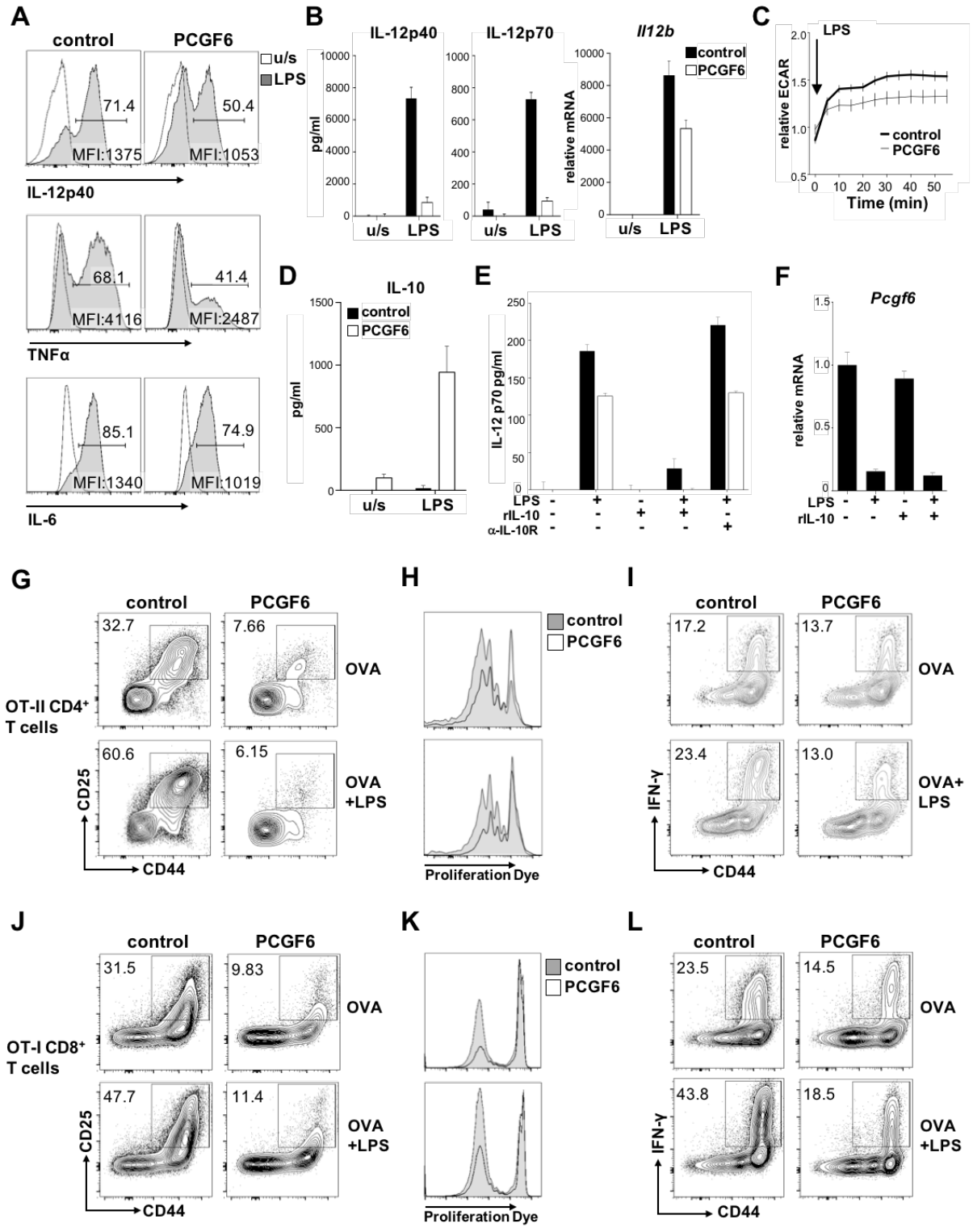
A
control
PCGF6
u/s
LPS
71.4
MFI:1375
50.4
MFI:1053
IL-12p40
68.1
MFI:4116
41.4
MFI:2487
TNFα
85.1
MFI:1340
74.9
MFI:1019
IL-6
B
IL-12p40
IL-12p70
Il12b
control
PCGF6
pg/ml
relative mRNA
u/s
LPS
C
LPS
relative ECAR
control
PCGF6
Time (min)
D
IL-10
control
PCGF6
pg/ml
u/s
LPS
E
IL-12 p70 pg/ml
LPS
rIL-10
α-IL-10R
F
Pcgf6
relative mRNA
LPS
rIL-10
G
control
PCGF6
32.7
7.66
OVA
OT-II CD4+
T cells
60.6
6.15
OVA
+LPS
CD25
CD44
H
control
PCGF6
Proliferation Dye
I
control
PCGF6
17.2
13.7
OVA
23.4
13.0
OVA+
LPS
IFN-γ
CD44
J
control
PCGF6
31.5
9.83
OVA
OT-I CD8+
T cells
47.7
11.4
OVA
+LPS
CD25
CD44
K
control
PCGF6
Proliferation Dye
L
control
PCGF6
23.5
14.5
OVA
43.8
18.5
OVA
+LPS
IFN-γ
CD44

Figure 2. PCGF6 negatively regulates DC activation. A. DCs transduced with MSCV-based retrovirus (control) or virus expressing *Pcgf6* cDNA (PCGF6) were stimulated for 6h and stained intracellularly for the indicated cytokines. Plots show cells that are transduced; gated on hCD8 reporter. Frequency of gated events and the geoMFI of the gated cells (bottom right) are indicated. B. Secreted IL-12p40 and IL-12p70 and expression of *Il12b* following 18h stimulation with LPS. C. Real time ECAR (mpH/min) of transduced DCs during LPS stimulation (100 ng/ml) measured by a Seahorse bioanalyzer. Error represents SEM. D. IL-10 secretion and expression levels were determined as in B. E. IL-12p70 production by transduced DCs stimulated with LPS +/- rIL10 (20 ng/ml) or anti-CD210 (10 μg/ml) for 18h. F. *Pcgf6* and *Il12b* expression in DCs stimulated with LPS +/- rIL-10. G-L. Sorted transduced DCs were pulsed with ovalbumin protein +/- LPS for 6h. Following stimulation, DCs were co-cultured with $CD4^+$ OTII T cells or $CD8^+$ OTI T cells that were labeled or not with proliferation dye. G,H,J,K. 3-4 days after co-culture, extent of T cell activation and proliferation were measured by CD25 and CD44 expression and proliferation dye dilution, respectively. H shows $CD4^+CD44^+$ cells, K shows $CD8^+$ cells. I,L. 4-5 days after co-culture, T cells were stimulated for 4h with PMA and ionomycin and stained intracellularly for IFN-γ. Unless otherwise indicated, error bars represent error calculated by standard deviation of 2-3 replicates. Data are representative of at least 3 independent experiments. See also Figure S2.

In complementary experiments, PCGF6 expression was silenced by expressing a small hairpin RNA (shRNA) targeting PCGF6 (sh*Pcgf6*) (Figure S3A). DCs with reduced PCGF6 expression (sh*Pcgf6*) displayed increased levels of CD86 and MHCII in the resting state and following stimulation with LPS over a range of concentrations (Figure 3A and S3B). Likewise, silencing PCGF6 resulted in increased levels of IL-12p40 production both at rest and following activation (Figure 3B,C). A second hairpin targeting *Pcgf6* yielded similar results (Figure S3C-E). DCs with reduced PCGF6 expression derived from FLT3L-treated cultures displayed a similar phenotype to GM-CSF-derived DCs (Figure S3F). Both resting and LPS-activated DCs with reduced PCGF6 expression induced greater antigen-specific CD4+ and CD8+ T cell activation, as determined by CD44 and CD25 expression, proliferation and IFN-γ production (Figures 3D-I). These results indicate that PCGF6 expression is necessary for the maintenance of the resting state of DCs, and that decreasing PCGF6 expression, even in resting DCs, is sufficient to stimulate stronger T cell responses.

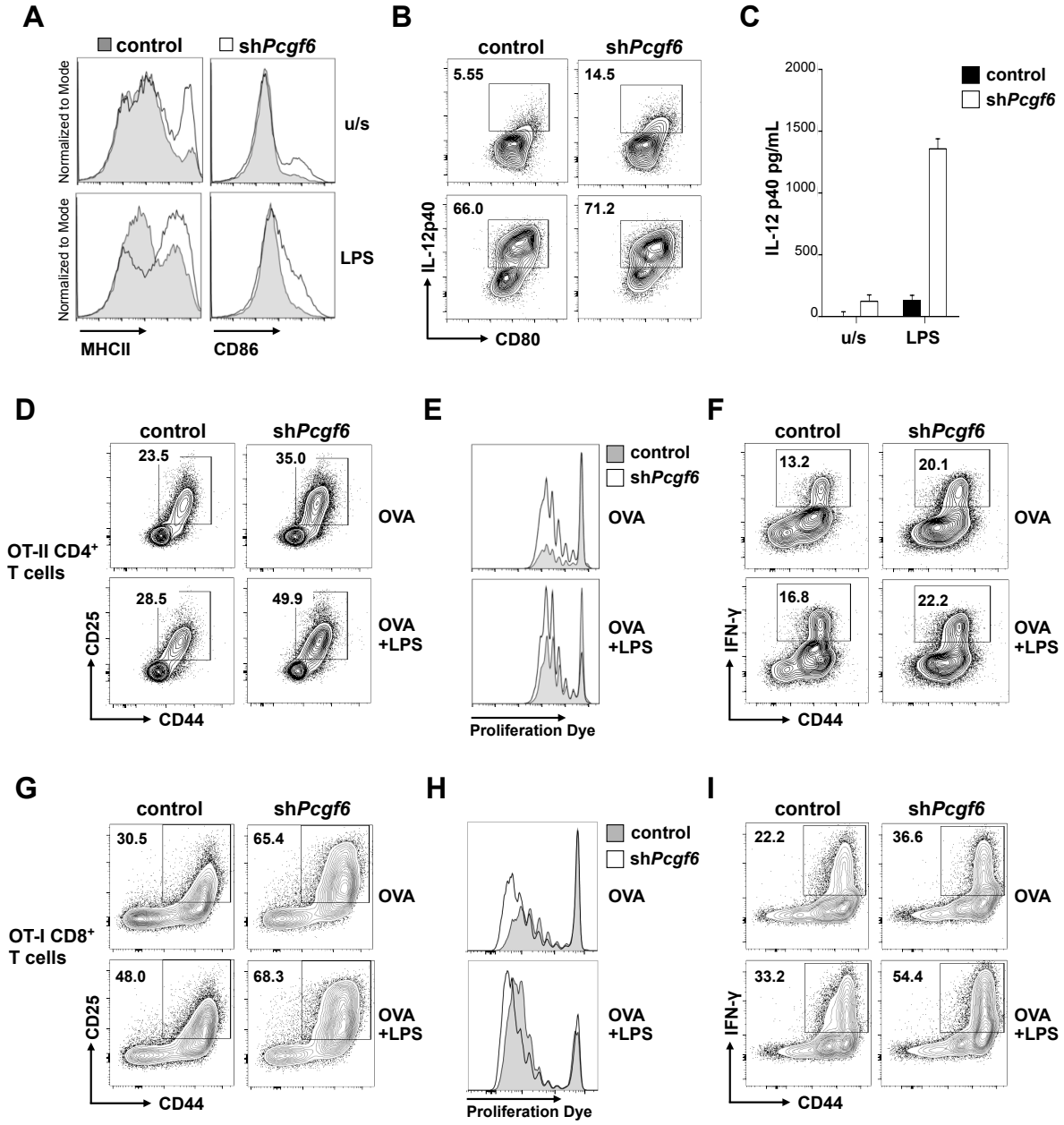

Figure 3. PCGF6 regulates both the resting and activated states of DCs. A,B. MHCII, CD86 and IL-12p40 expression in DCs transduced with an shRNA targeting PCGF6 (sh*Pcgf6*) or empty vector (control) at rest and following stimulation with LPS for 6h. Plots show transduced (hCD8$^+$) cells. C. Secreted levels of IL-12p40 following 18h of LPS stimulation. D-I. CD4$^+$ and CD8$^+$ T cell co-culture experiments were performed as in Figure 2. Data is representative of 3-5 independent experiments. See also Figure S3.

Gene-specific regulation of chromatin accessibility and H3K4me3 levels by PCGF6

Polycomb proteins are broadly known to regulate gene expression programs in cells, often through the regulation of histone modifications that influence chromatin structure and/or transcription (Schuettengruber et al., 2007). To examine changes in chromatin accessibility in the presence or absence of PCGF6, we used Assay for Transposase-Accessible Chromatin (ATAC) to examine specific genes important for DC activation. ATAC libraries were generated from DCs transduced with either *Pcgf6* cDNA or shPcgf6. The accessibility of the promoters of *Ciita*, *H2-Ab1* (MHCII, IAb), *Il12a*, *Il12b* and *Actb* was examined using high-throughput sequencing data (Figure 4A, B) and validated by qRT-PCR on an independent set of samples (Figure S4). We observed decreased ATAC-qPCR enrichment relative to input at the promoters of *Ciita* and *Il12a* in DCs overexpressing PCGF6 (Figure S4), corresponding to reduced accessibility at these loci. An overall trend to decreased signal intensity by ATAC-seq and relative enrichment by ATAC-qPCR was observed in PCGF6-overexpressing cells at the promoters of *Ciita*, *H2-Ab1*, *Il12a*, and *Il12b* (Figure 4A and S4). Conversely, there was a trend towards increased signal intensity by ATAC-seq and relative enrichment by ATAC-qPCR observed at these promoters upon depletion of PCGF6 (Figure 4B).

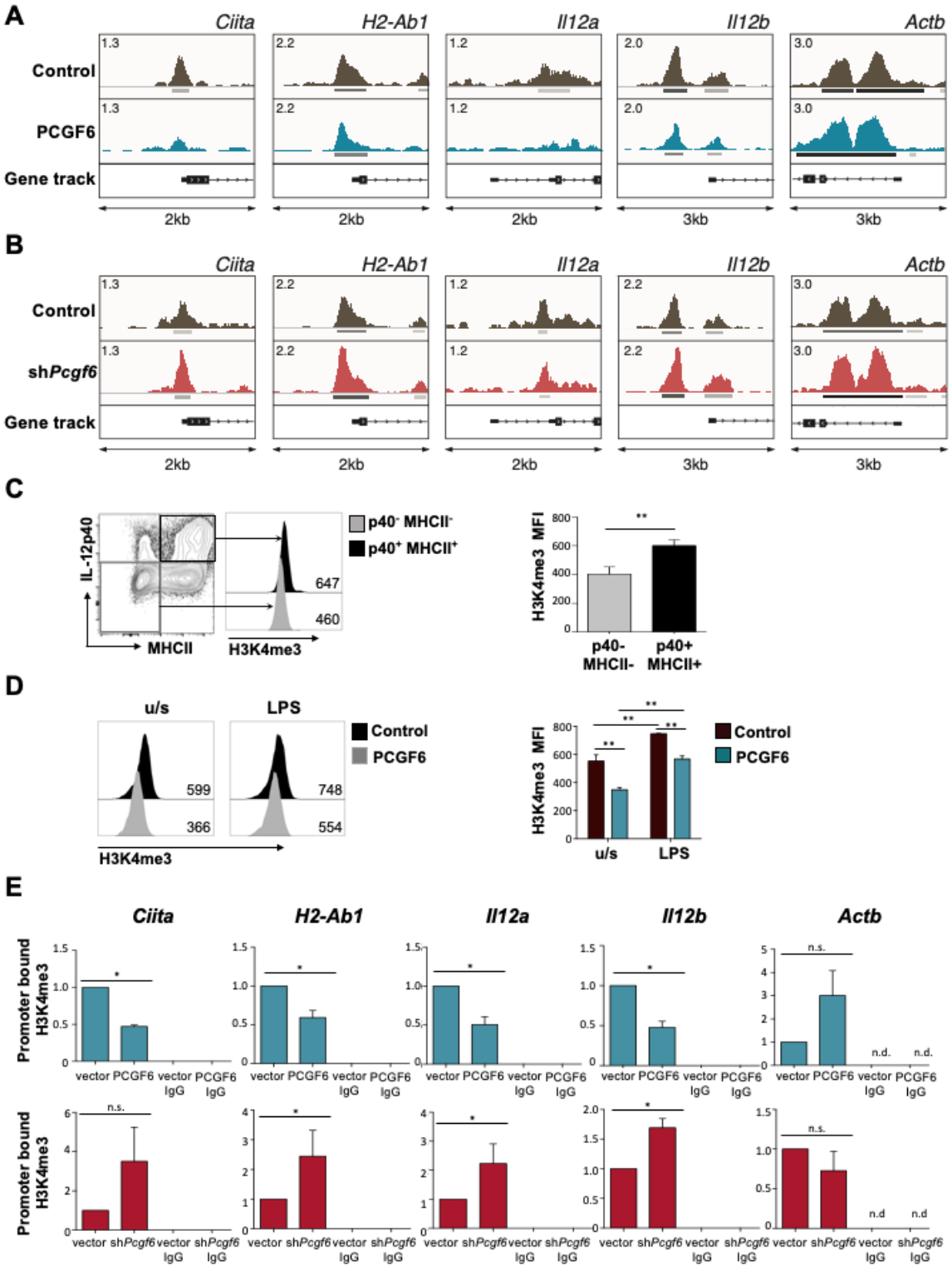
A
Ciita
H2-Ab1
Il12a
Il12b
Actb
Control
PCGF6
Gene track
2kb
3kb
B
shPcgf6
C
IL-12p40
MHCII
H3K4me3
p40- MHCII-
p40+ MHCII+
647
460
H3K4me3 MFI
p40-
MHCII-
p40+
MHCII+
D
u/s
LPS
599
366
748
554
Control
PCGF6
E
Promoter bound H3K4me3
vector
PCGF6
IgG
shPcgf6
n.s.
n.d.

Figure 4. PCGF6 suppresses H3K4me3 levels in resting and activated DCs. A,B. ATAC-seq of transduced resting DCs. Screenshot from a genome browser of peaks generated by ATAC-seq observed in proximity to the indicated genes. Peaks are a pileup of sequence alignments over the specified regions resulting from the ATAC reaction. The number in the top left of each panel represents the scale of the signal intensity. Called peaks are indicated by the shaded rectangles, where darkness of the rectangle represents relative intensity of the signal. C. DCs activated with LPS for 6h cells stained intracellularly for MHCII, IL-12p40 (left) and H3K4me3 levels (right) and analyzed by flow cytometry. Inset values are the geoMFI of the adjacent histogram. D. H3K4me3 levels determined by flow cytometry in resting and LPS-stimulated.DCs GeoMFI is quantified on the right panel. C, D. Error bars represent standard deviation of triplicate samples. E. ChIP of H3K4me3 in transduced DCs followed by qPCR of the promoters of indicated genes. ChIPs were performed on three biological replicates. Error bars indicate the standard error of the mean of pooled qPCR values from 3 independent ChIP experiments. * $p<0.05$, ** $p<0.005$ See also Figure S4.

Since PCGF6 has been found in complexes with H3K4 demethylase activity (Lee et al., 2007), we examined whether PCGF6 could regulate H3K4me3 levels in DCs. We first examined whether changes in *Pcgf6* expression correlated with changes in H3K4 methylation in DCs using flow cytometry. We specifically gated on LPS-stimulated cells that were either IL-12p40^{hi}MHCII^{hi} or IL-12p40^{low}MHCII^{low}, and compared the fluorescence intensity (Figure 4C). Consistent with its role as an activating epigenetic signature, we observed a positive correlation between the extent of H3K4me3 and the degree of cellular activation. Ectopic PCGF6 expression resulted in a decrease in H3K4me3 in both resting and LPS-stimulated DCs (Figure 4D), indicating that PCGF6 promotes H3K4 demethylation in DCs.

We examined H3K4me3 levels at the promoters of *Ciita*, *H2-Ab1*, *Il12a*, and *Il12b* using α-H3K4me3 ChIP-qPCR (Figure 4E). We found a significant decrease in the enrichment of H3K4me3 at these promoters upon ectopic expression of PCGF6 and an increase in H3K4me3 enrichment with Pcgf6 knockdown (Figure 4E). Levels of H3K4me3 were not significantly changed at the *Actb* promoter.

Maintenance of DC quiescence by PCGF6 is dependent on JARID1c

PCGF6 was shown to be associated with and promote the histone demethylase activity of JARID1d, a histone demethylase that acts on H3K4me2 and H3K4me3 (Lee et al., 2007). Our studies were performed with DCs from female mice that do not express the Y-linked JARID1d, therefore we focused our attention on the X-linked homolog, JARID1c, another H3K4 demethylase (Outchkourov et al., 2013). PCGF6 and JARID1c were found to interact in DCs by co-immunoprecipitation (Figure 5A). Unlike PCGF6, *Jarid1c* expression increased slightly (approximately 2-fold) in response to LPS stimulation (Figure S5A). DCs expressing JARID1c-specific shRNA (sh*Jarid1c*) had increased levels of H3K4me3 (Figure 5B and S5B), consistent with the role of JARID1c as a histone demethylase in DCs.

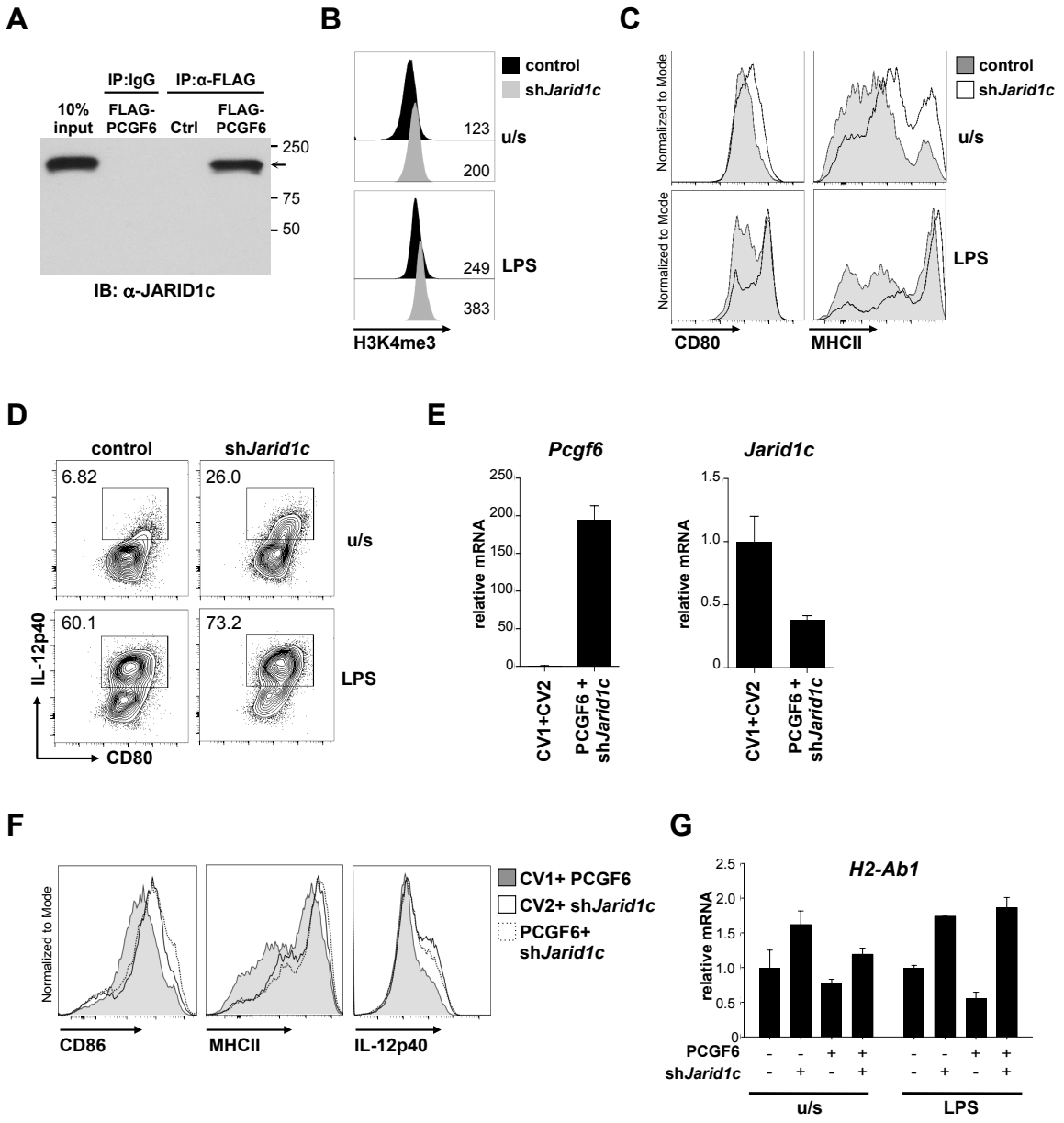

Figure 5. PCGF6 negatively regulates DC activation in part through JARID1c. A. Immunoprecipitation (IP) performed on lysates from transduced DCs immunoblotted with anti-JARID1c. B. DCs transduced with a control vector or sh*Jarid1c* were activated with LPS for 6h. H3K4me3 levels were measured by flow cytometry. C,D. Transduced DCs treated as in B were assayed for surface marker expression and intracellular cytokine production (gated on $hCD8^+$ cells). E-G. DCs were transduced on day 1 and on day 2 of culture with either a relevant control vector (CV) or one expressing sh*Jarid1c* or PCGF6. E. Expression of *Pcgf6* and *Jarid1c* in double-transduced DCs measured by qRT-PCR and normalized to *Hprt*. F. Expression of MHCII, CD86 and intracellular IL-12p40 on human $CD8^+$ cells. G. Expression of *H2-Ab1* mRNA in transduced DCs. Data is shown from one representative of three different experiments. See also Figure S4.

Similar to cells expressing *Pcgf6* shRNAs, expressing sh*Jarid1c* in DCs led to increased CD80, MHCII and Il-12p40 levels both at rest and in response to LPS stimulation (Figure 5C, D). To examine whether JARID1c activity was needed for PCGF6-mediated suppression we co-expressed PCGF6 and sh*Jarid1c* (Figure 5E). In the absence of JARID1c, PCGF6 was only partially able to suppress the expression of costimulatory molecules and IL-12p40 production (Figure 5F). Furthermore, PCGF6 could not suppress *H2-Ab1* and *Ciita* mRNA levels to the same extent in the absence of JARID1c (Figures 5G, S5C). Together, these data demonstrate that PCGF6 and JARID1c work in concert to negatively regulate the expression of key genes involved in DC activation, and that JARID1c is required in part for PCGF6-mediated suppression.

2.4 Discussion

Controlled activation of DCs is necessary to prevent the inappropriate induction of inflammation and activation of T cell responses, resulting in immunopathology and/or autoimmunity. Thus, it is important that the induction of the proinflammatory phenotype be restrained until sufficient, and appropriate, stimuli have been received. The maintenance of DC quiescence and the transition to activation is a dynamic process that requires the coordinated induction of many genes necessary for their effector function. Here, we have identified two transcriptional repressors, PCGF6 and JARID1c, which cooperate to suppress the expression of genes that are associated with DC activation. Our work contributes to emerging evidence that maintaining the resting state in DCs is an active process involving negative regulators of transcription (Huang et al., 2012; Johnson and Ohashi, 2013). Our data also reveals that expression of PCGF6 must be

downregulated to permit full activation of genes necessary for the acquisition of effector function of DCs in response to proinflammatory stimuli.

DCs are poised to respond rapidly to their environment and one mechanism to facilitate rapid responses could be transcriptional readiness wherein the locus is primed and awaiting the arrival of activated transcription factors. Based on the ATAC experiments performed, the promoters of genes important for DC activation and function are relatively "open" at rest, in line with the need to have them immediately transcribed following PRR signaling. We found that expression levels of PCGF6 affected the "openness" of these promoters. While there are many chromatin modifications that can affect chromatin structure, we focused on H3K4me3 given the association of H3K4me3 with transcriptional activation and the published report that PCFG6 can interact with the H3K4 demethylase JARID1d (Lee et al., 2007). We found that H3K4me3 levels are increased in highly activated cells ($MHCII^{hi}p40^{hi}$) compared to immature cells and that PCGF6 expression decreased the global signal of H3K4me3. We specifically analyzed promoters of genes important for DC activation and found that PCGF6 expression decreases the level of H3K4me3 at these promoters. Mechanistically, we demonstrate that PCGF6 can associate with JARID1c and that the suppressive activity of PCGF6 in DCs is, in part, dependent on JARID1c. PCGF6 can also promote the transcription of genes such as IL-10, however the net effect of PCGF6-mediated regulation of gene expression is to dampen DC activation and promote a more quiescent state.

Active maintenance of the quiescent state is important for the fine-tuning of DC responses to a range of stimuli that have the potential to activate DCs. Here, we provide evidence that the extent to which these activating signals translate into transcriptional

activation is tempered by negative regulators of transcription such as PCGF6 and JARID1c. PCGF6 and JARID1c antagonize mRNA expression by regulating H3K4me3 levels and in doing so promote a quiescent phenotype. In the absence of JARID1c and PCGF6, DCs are more active at rest, and at least for PCGF6, stimulate enhanced T cell responses. Therefore, maintenance of the quiescent state via transcriptional repression in DCs has important consequences for the induction of both innate and adaptive immune responses.

Our findings demonstrate that PCGF6 and JARID1c regulate DC activation and function by actively suppressing H3K4me3 levels. These data support emerging evidence that constitutive inhibitory mechanisms are in place to regulate DC quiescence and temper activation. In the case of PCGF6, the expression of these factors must be downregulated to permit DC activation and subsequent triggering of adaptive immunity.

2.5 Contributions

Conceptualization, C.M.K., G.M.B.; Methodology, C.M.K., G.M.B., G.D., V.D.; Validation, G.M.B., B.C., A.D., H.G.; Formal Analysis, S.D.B., G.D.; Investigation, C.M.K., G.M.B., B.C., G.D., V.D., T.H., A.D., H.G., H.C.C.; Resources, J.H.W., M.L.; Data Curation, G.D.; Writing- original draft, C.M.K., G.M.B.; Writing – Review and editing, C.M.K., G.M.B., J.H.W., M.L.; Visualization, C.M.K., G.M.B.; Supervision, C.M.K., E.J.P., M.L., J.H.W. ; Project administration, C.M.K., G.M.B.; Funding acquisition, C.M.K

2.6 Experimental Procedures

Mice and reagents

C57BL/6 (Charles River Laboratories). OTII transgenic (Jackson 004194) and OTI (Jackson 003831) mice were maintained under specific pathogen-free conditions at McGill University. All procedures were carried out in accordance with guidelines of the Canadian Council on Animal Care, as approved by the Animal Care Committee of McGill University. DC activators: LPS (*Escherichia Coli* serotype 0111:B4) (Sigma-Aldrich); HDM (low endotoxin, Greer laboratories); Zymosan and Zymosan-depleted of TLR ligands (Invivogen); Cytokines: GM-CSF, IL-10, IFN-β (Peprotech). $CD4^+$ and $CD8^+$ T cell negative selection kit (STEMCELL Technologies); Pan-DC enrichment kit and PE positive selection kit (Miltenyi Biotec). Antibodies listed in Table S1. ELISA Ready-set-go kits: IL-12/23 p40, IL-12 p70 and IL-10 (eBioscience). Media reagents: RPMI (Corning, ThermoFisher). Iscove's Modified Dulbecco's Media, FCS, L-glutamine and penicillin/streptomycin (Wisent), Non-essential amino acids (Gibco, Invitrogen).

BMDC Culture and transduction

Bone marrow cells were cultured and stimulated as described (Krawczyk et al., 2008). For intracellular staining BMDCs were incubated with brefeldin for 2hrs before the end of stimulation then fixed and permeabilized (eBioscience kit). Flt3L-derived BMDCs, were generated as above using media containing 20% conditioned supernatants from a B16 cell line expressing FLT3L in place of GM-CSF. BMDC cultures were transduced as described using retrovirus produced in 293Ts transfected with either MSCV or LMP-based (Krawczyk et al., 2008; Paddison et al., 2004) retroviral vectors using Lipofectamine 2000.

Gene expression

RNA was extracted (Trizol) and cDNA was generated to perform SYBR-based qRT-PCR (For primers, see Table S2). Relative fold change was calculated using the ΔΔCq method. The standard deviation of triplicate samples was combined with the standard deviation of the reference gene by error propagation. The range of fold change was then calculated by the formula: $2^{(-\Delta\Delta Cq +/- s)}$ and the standard deviation of the range in fold change represents the error bars indicated in the qPCR bar graphs.

Splenic DC preparation and ex vivo activation

Spleens from C57BL/6 mice were treated with collagenase and DNase for 20 min at 37°C, homogenized through a 70um filter and red blood cells were lysed with ammonium chloride solution (150mM NH4Cl, 10mM Tris, pH7). DCs were isolated from total splenocytes by MACS pan-DC enrichment according to the manufacturer's protocol.

Seahorse assay

Seahorse Extracellular Flux Analyzer was used to measure ECAR (mpH/min) following LPS stimulation. Briefly, sorted DCs were plated in 6 replicates in seahorse media freshly supplemented with 10% FCS, 2 mM L-Glutamine, 25 mM Glucose and 300 nM NaOH (pH adjusted to 7.4). LPS was injected directly by the Seahorse (final concentration 100 ng/mL) measurements of extracellular acidification rate (ECAR) were recorded every 5 min.

DC-T cell co-culture

$2x10^4$ sorted DCs were stimulated or not with LPS (10 ng/ml) and whole OVA protein (2 µg/ml) per well in 96-well plates. Six hours following stimulation, $2x10^5$ sorted CD4+ T cells from OTII mice were added (1:10 ratio). On day 3 or 4, T-cells were examined for proliferation (pre-labeled with CFSE or e450 Proliferation Dye) and expression of cell surface activation markers. To detect cytokine production by intracellular staining, cells were stimulated for 4h with PMA (50 ng/ml) and ionomycin (500 ng/ml). For $CD8^+$ co-cultures $1x10^4$ sorted DCs were stimulated or not with LPS (0.5 ng/ml) and whole OVA protein (1 µg/mL) in 96-well plates and co-cultured and analyzed as described above.

H3K4me3 staining and Chromatin Immunoprecipitation

To detect intracellular H3K4me3 cells were fixed with 4% PFA for 20 min at room temperature, washed and permeabilized with 100% methanol at 4°C for at least 30 min. Cells were then washed and stained at 4°C. ChIP was performed essentially as described in (Memari et al., 2015) with modifications detailed in Supplemental Methods. Briefly, cells were cross-linked and lysed to obtain nuclei. Nuclei were lysed with sonication and chromatin was used for overnight IP at 4^oC. IP'ed DNA was used as a template for qPCR specific to the promoter region of indicated genes. Statistical significance was determined by an unpaired t-test.

ATAC-seq

ATAC-seq libraries from transduced DCs were sorted by FACS for high hCD8, $CD11c^+$ cells and were prepared as previously described (Buenrostro et al., 2013) using

5x104 cells per sample and 50ng DNA per input. Library preparation was achieved with DNA Library Preparation Kit (Illumina) and fragments were size-selected using Caliper LabChIP system (PerkinElmer). Reads were aligned to the Mouse genome (mm10) using BWA (Li and Durbin, 2009). Duplicate reads were marked and removed using Picard (https://github.com/broadinstitute/picard). For comparisons the larger dataset was downsampled to match the size of smaller dataset using Picard (https://github.com/broadinstitute/picard). Peaks were called using MACS2.0 (https://github.com/taoliu/MACS/) (Zhang et al., 2008) For ATAC-qPCR, ATAC libraries were prepared from 3 independent biological replicates as described above and used in real-time qPCR using the same primers listed for ChIP-qPCR. The fold enrichment relative to input was normalized to a negative control region.

Co-Immunoprecipitation

Cell lysates from transduced DCs were prepared using RIPA lysis buffer (NaCl 150uM; EDTA 5mM; 1M pH 5 Tris 50mM; NP-40 10%; 10% Sodium deoxycholate 0.5% and 10% SDS 0.1%) with protease inhibitor cocktail tablets (Roche). The lysates (1 mg) were cleared then incubated with beads pre-coated with either anti-FLAG M2 or normal mouse IgG for 2h. The beads were washed and the proteins were eluted by boiling and processed for western blot using anti-JARID1c. 10% input (100 μg) was loaded as a control.

Statistical Analysis

Statistical analysis was performed using Prism 6.0b software and/or Microsoft Excel. Statistical significance was determined by unpaired t-tests and indicated by the

following: n.s., not significant; * $p<0.05$; ** $p<0.05$. Error bars represent either standard deviation or standard error of the mean as described in the figure legends.

2.7 Accession Numbers

The accession number for ATAC-sequencing data is GSE83640.

2.8 Acknowledgements

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2.10 Supplemental Information

Supplemental Information Inventory

Supplemental Experimental Procedures

-Detailed description of H3K4me3 ChIP protocol
-sequences of hairpins from the RNAi codex

Table S1. List of Antibodies

- A comprehensive list of the antibodies used throughout the study

Table S2. List of primers used for qPCR

-A table containing the forward and reverse primer sequences used throughout the study for qPCR

Table S3. List of primers used for ATAC and ChIP qPCR

-A table containing the forward and reverse primer sequences used for ATAC and ChIP-qPCR

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 4.

Figure S5, related to Figure 5.

Supplemental Experimental Procedures

Chromatin Immunoprecipitation (ChIP)

ChIP was performed essentially as described in (Memari et al. 2015) with the following modifications. Cells ($4x10^6$ – $10x10^6$) were collected and cross-linked with fresh formaldehyde (252549-500ML, Sigma-Aldrich) added directly in the culture medium to final concentration of 1%. Cells were left for 10 min at room temperature (RT) with occasional shaking. Crosslinking was quenched with 125 mM glycine (G7126-5KG, Sigma-Aldrich) for 5 min at RT. Cells were pelleted by centrifugation at 4^{o}C for 5 min at 500 rcf, followed by washing with ice-cold PBS. The pellet was re-suspended in 10 ml cell lysis buffer (10 mM TRIS pH 7.5, 10 mM NaCl, 0.2% NP-40, protease inhibitor cocktail) containing protease inhibitor cocktail (PIC; 04 906 837 001, Roche) and incubated on ice for 10 min with occasional swirling. Nuclei were pelleted and washed in 10 ml micrococcal nuclease (MNase) buffer (buffer B; 7007BC, NEB), then re-suspended in 1ml of MNase buffer. 0.4 µl of MNase (M0247S, NEB) per 10 **x** 10^6 cells was added and incubated with rotation at 37^{o}C for 30 min. 100 µl of 0.5M EDTA (EDT001.1, BioShop) was added to stop the reaction, and samples were centrifuged for 2 min at 10,000 rcf. Nuclei were re-suspended in 500 µl lysis buffer and incubated for 30 min on ice with occasional mixing. Samples were sonicated for 10 sec at 30% using Vibra Cell sonicator (Sonics). Cell debris was pelleted by centrifugation at 10,000 rcf for 10 min at 4^{o}C and supernatant was transferred to a new tube. 50 µl of chromatin, 420 µl dilution buffer and 5 µg of anti H3K4me3 antibody (Abcam, ab8580) or anti-GFP antibody (ab290, Abcam) were incubated overnight at 4^{o}C with rotation. 20 µl of Dynal magnetic beads (161-4013, Bio-Rad) were added and incubated for 2 h at 4^{o}C with rotation. The beads were then washed

3 time for 5 min with buffer 1, once with buffers 2, 3, and TE, and were re-suspended in 130 µl of extraction buffer containing 5 µl of RNase (AM2286, Ambion). After incubation at 37^{o}C while shaking at 900 rpm, 5 µl of proteinase K was added (25530-049, Ambion) and samples were incubated for another 30 min at 37^{o}C while shaking at 900 rpm. Overnight decrosslinking was performed at 65^{o}C while shaking at 650 rpm. Magnetic beads were precipitated and DNA was purified using PCR/Gel purification kit (FAGCK001-1, FavorGen). The resulting DNA was diluted 1 in 15 and used in quantitative real-time PCR (qPCR) with primers as indicated and SsoFast EvaGreen supermix (1725200, Bio-Rad) using LightCycler 96 (Roche). The qPCR protocol is as follows: pre-incubation for 10 min at 95^{o}C, followed by 45 2-step amplification cycles consisting of 95^{o}C (15s) and 60^{o}C (15s). The quality of each primer pairs was assessed by inspecting its melting curve.

Knockdown of Pcgf6 and Jarid1c

PCGF6 and JARID1c hairpins were obtained from the RNAi codex.

PCGF6:

HP_109991,TGCTGTTGACAGTGAGCGCCCAGCTTGTCAGGTAGATATATAGTGAAG
CCACAGATGTATATATCTACCTGACAAGCTGGATGCCTACTGCCTCGGA

HP_252718,

TGCTGTTGACAGTGAGCGCCCAGACTCAGCCTCTTTATAATAGTGAAGCCACAGAT
GTATTATAAAGAGGCTGAGTCTGGTTGCCTACTGCCTCGGA.

JARID1c: HP_32183,

TGCTGTTGACAGTGAGCGCGCCCAGTTTATTGAGTCATATTAGTGAAGCCACAGAT

GTAATATGACTCAATAAACTGGGCATGCCTACTGCCTCGGA)

2.11.3 Supplemental Tables

Table S1. List of antibodies

Antigen	**Clone**	**Fluorophore(s)**
Cell Surface Markers		
CD80	16-10A1	FITC
MHCII	M5.114.15.2	PE,PE-Cy7
CD86	GL1	PE-Cy7
CD40	HM40-3	APC
CD11c	N418	PerCP-Cy5.5
hCD8	SK1	APC-e780, PE
CD4	RM4-5	PerCP-Cy5.5
CD44	IM7	PE-Cy7
CD25	PC61.5	PE
Cytokines		
IL-12/23 p40	C17.8	PE, APC
IL-6	MP5-20F3	eFluor-450
TNFα	MP6-XT22	PE-Cy7,APC
IFNy	XMG1.2	FITC
Epigenetic marks		
H3K4me3	mAbcam1012	AlexaFluor-647
Western blot and IP antibodies		
JARID1c	D29B9 Cell Signaling	-
FLAG M2	M2 Sigma	-
Functional		
α-CD210	1B1.3a	-

Table S2. List of primers used for qPCR

Target	Forward Primer	Reverse Primer
Pcgf6	GGA GAA GCA ACT ATC GGG CA	CCA GTA AGT GAT CCC CAC AGA
Il12b	CTG GAG CAC TCC CCA TTC CT	CGC CTT TGC ATT GGA CTT CG
IL-10	TGA ATT CCC TGG GTG AGA AG	TGG CCT TGT AGA CAC CTT GG
Jarid1c	AGA AGG AGC TGG GGT TGT AC	CCA CAC ACG CAG ATA GAA GC
Ciita	ACA CCT GGA CCT GGA CTC AC	GCT CTT GGC TCC TTT GTC AC
H2-Ab1	CGG CTT GAA CAG CCC AAT GT	CGC ACT TTG ATC TTG GCT GG
HPRT	AGG ACC TCT CGA AGT GTT GG	GGC TTT GTA TTT GGC TTT TCC

Table S3. List of primers used for ATAC and ChIP qPCR

Target	Forward Primer	Reverse Primer
Il12a	ACCTGGATGGCAGGAACTAC	CTTGCCCAGGAGGTTACAAT
Il12b	TTCCCCCAGAATGTTTTGACA (Wen et al 2008)	TGATGGAAACCCAAAGTAGAAACTG (Wen et al 2008)
H2-Ab1	TGGGATTTCAGATCACTCCA	ACAGGTAATGGCAGTCACCA
Ciita	CCTTTGAGTCAAGGCAACAA	GGATGCTCTGATCAATGTGG
Actb	TAGGCGTAAAGTTGGCTGTG	TCGCTCTCTCGTGGCTAGTA

2.11.4 Supplemental Figures

Figure S1

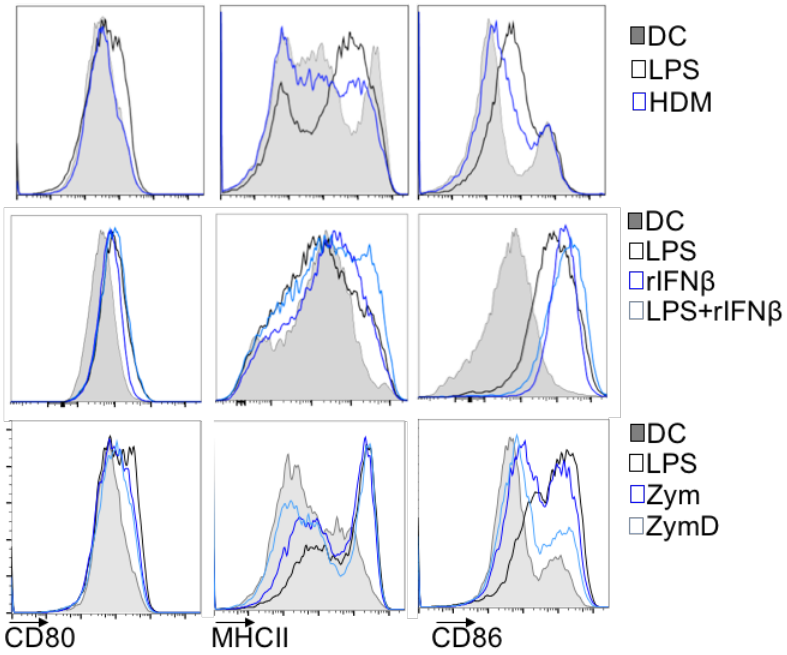

Figure S1, related to Figure 1. DCs were stimulated with the indicated activators for 18h. CD80, MHCII and CD86 surface expression was measured by flow cytometry.

Figure S2

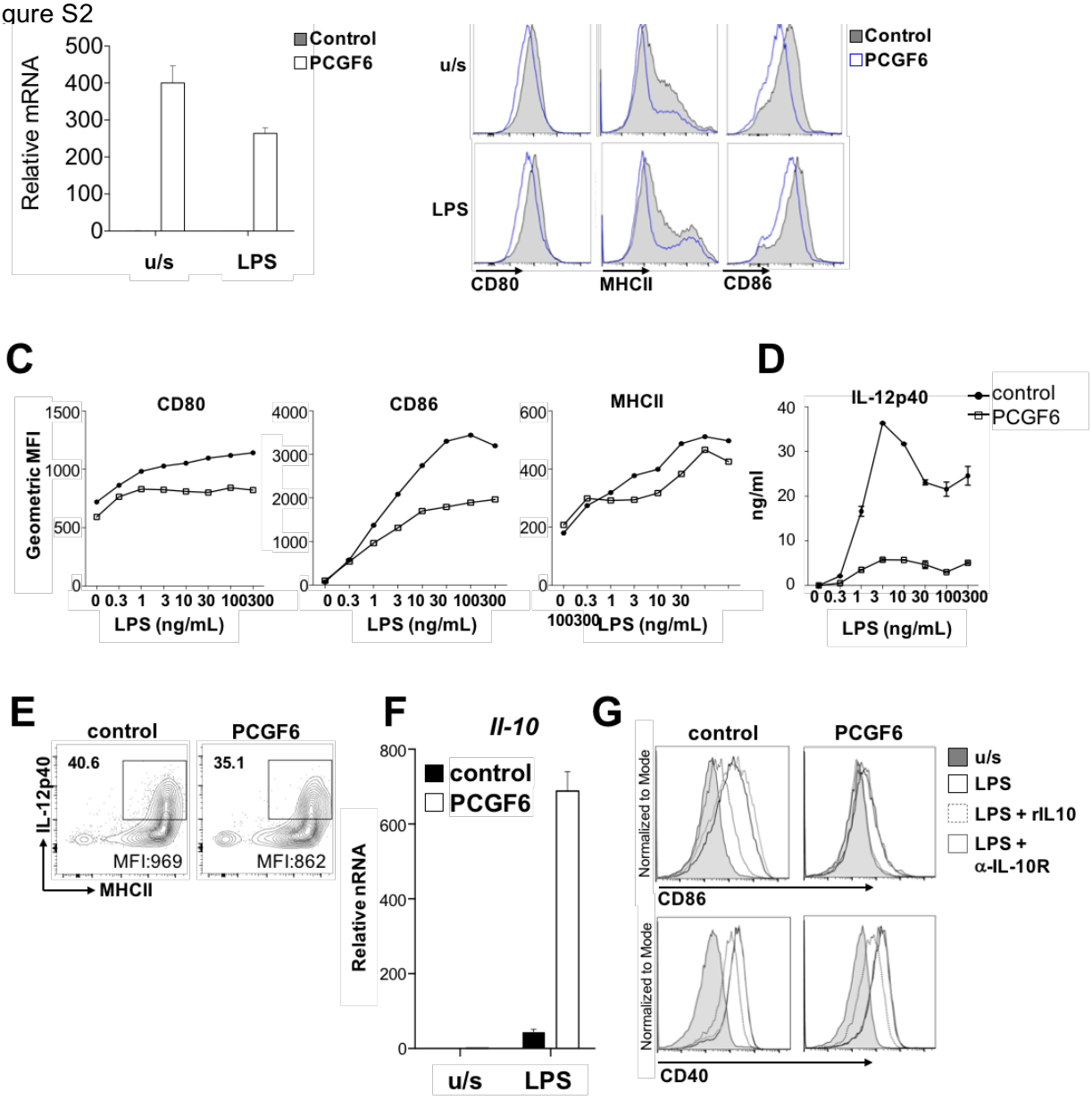

Figure S2, related to Figure 2. A. Transduced DCs were stimulated with LPS for 6h and expression of *Pcgf6* was determined by qPCR. B. DCs were activated as in A and CD80, MHCII and CD86 surface expression was measured by flow cytometry. (u/s: unstimulated). C. Transduced DCs activated for 18h with the indicated amounts of LPS were stained and geometric MFI was measured by flow cytometry for CD80,CD86 and MHCII. D. DCs were activated as in C, and IL-12p40 secretion was measured by ELISA. E. Transduced DCs generated by Flt3L-treated bone marrow were activated with LPS for 6h and IL12p40 production was measured by flow cytometry. F. *Il-10* expression in transduced DCs activated for 4h with LPS. G. Expression of CD40 and CD86 on transduced DCs treated with the indicated stimuli for 18h.

Figure S3

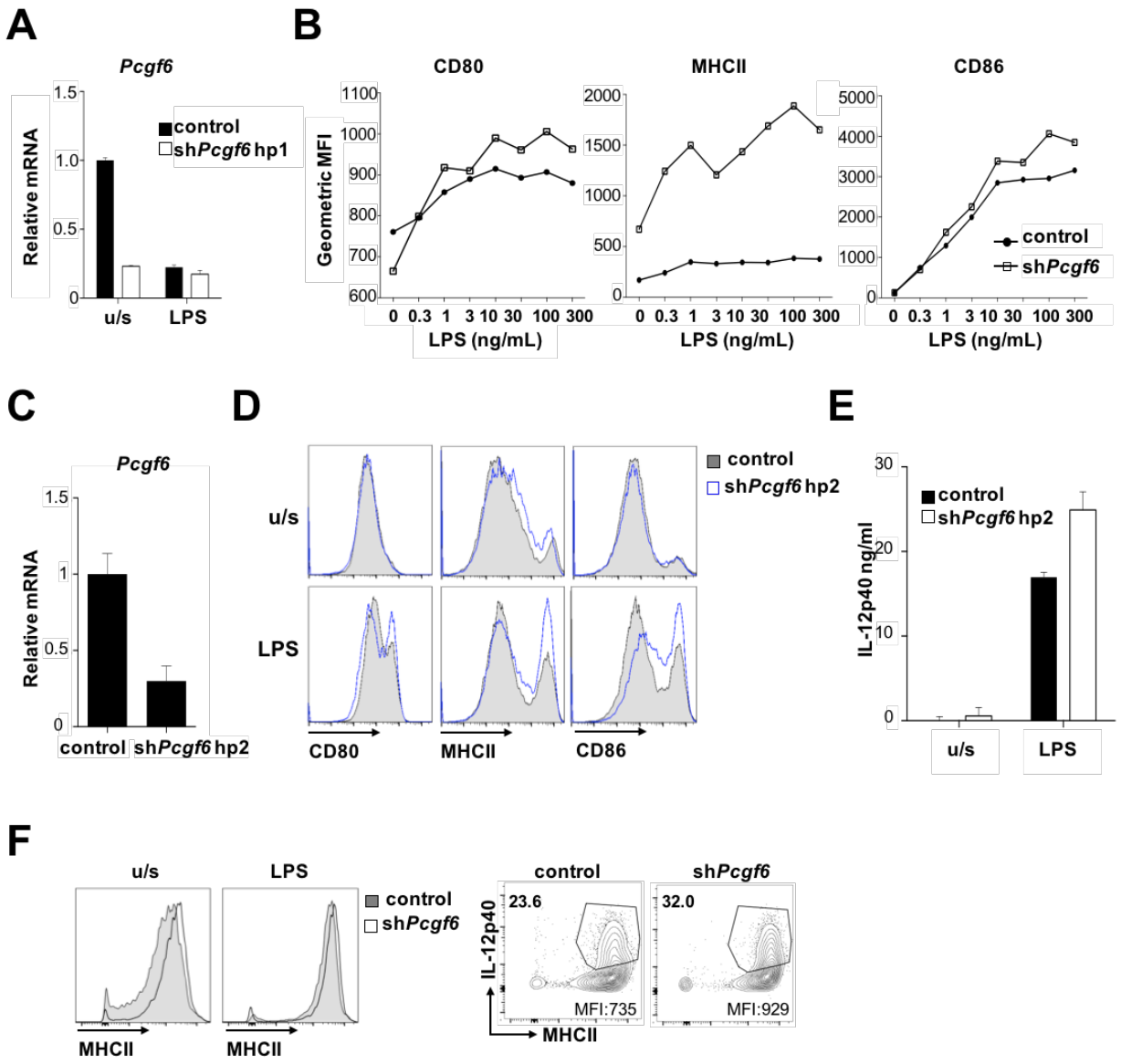

Figure S3, related to Figure 3. A. DC were transduced with a shRNA (hp1) targeting *Pcgf6* or control vector and activated with LPS (10 ng/mL) for 6h. *Pcgf6* expression was determined by qPCR. Error bars indicate the standard deviation of the range of fold change determined by propagating the error between the technical triplicates of the internal control and the target gene. B. Transduced DCs activated for 18h with the indicated amounts of LPS were stained and geometric MFI was measured by flow cytometry for CD80, CD86 and MHCII. C-E. DCs were transduced with a second shRNA targeting *Pcgf6* (hp2) as in Figure 3.D. DCs were stained and activation markers were measured by flow cytometry. E. Supernatants for DCs transduced and activated as in C were saved and IL-12 levels were measured by ELISA. F. DCs generated from B16-Flt3L supernatants were transduced and activated as previously described. MHCII and IL-12 expression were determined by flow cytometry.

Figure S4

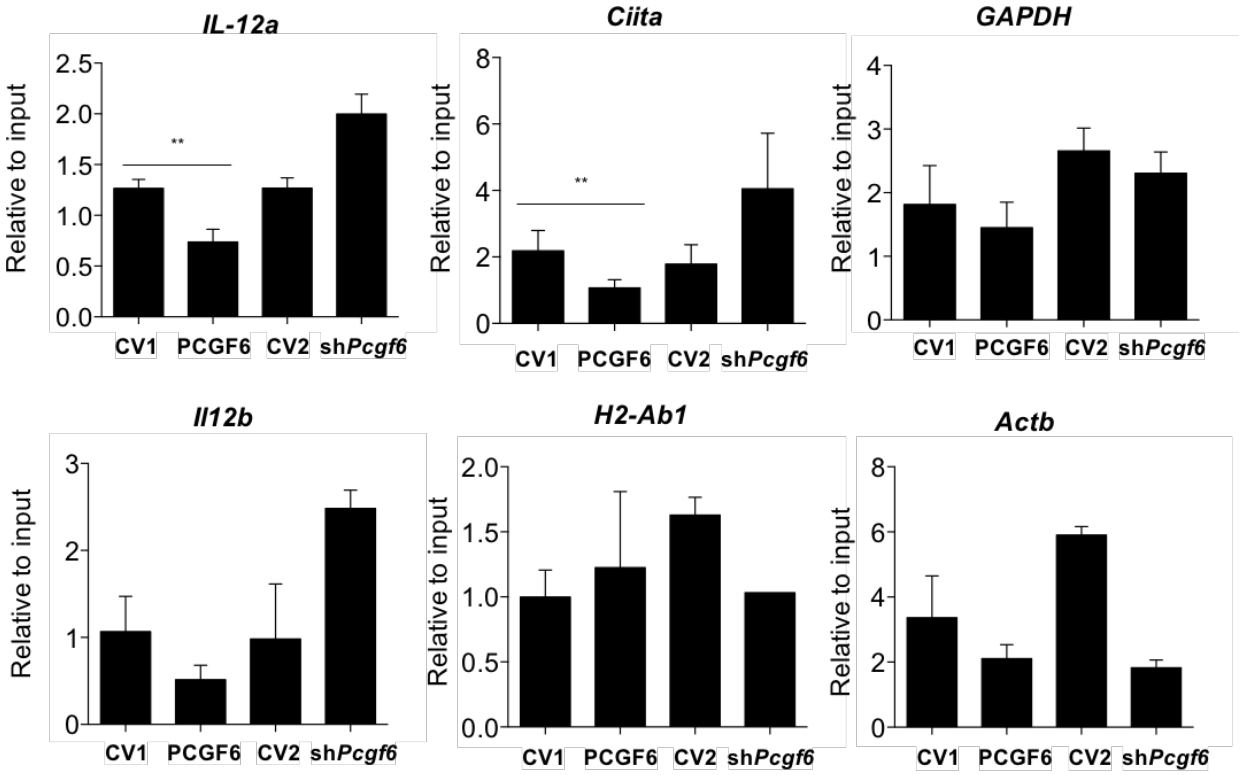

Figure S4, related to Figure 4. Transduced DCs (CV1: MSCV; PCGF6: MSCV-PCGF; CV2: LMP-hCD8; sh*Pcgf6*: LMP-hCD8-sh*Pcgf6*) were sorted based on hCD8 and CD11c expression and were subjected to a transposase reaction to obtain open regions of chromatin. Levels of open chromatin near the transcription start sites of the indicated genes were determined by qPCR and normalized to an input control. Graphs are pooled data from 2-3 biological replicates. Error bars represent standard error of the mean, ** represents $p < 0.05$.

Figure S5

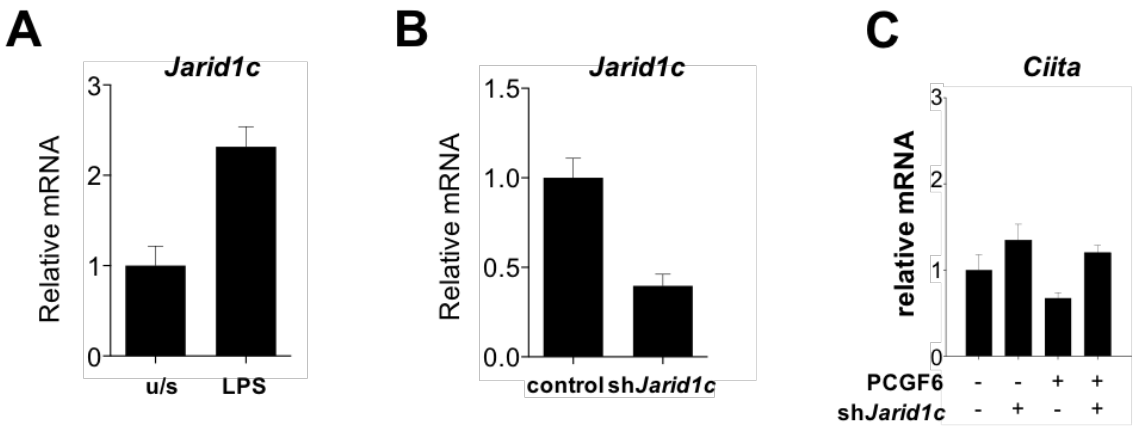

Figure S5, related to Figure 5. A. DCs were stimulated with LPS for 18 hours and *Jarid1c* expression determined by RT-qPCR. B. DC were transduced with a shRNA targeting *Jarid1c* or control vector and *Jarid1c* expression was determined by RT-qPCR C. DCs were transduced as in Figure 5 and *Ciita* levels were determined by RT-qPCR.

Preface to Chapter 3

In Chapter 2 we found that PCGF6 was necessary to maintain the steady state of DCs. Furthermore, we found evidence through ATAC-seq and H3K4me3 ChIP that manipulating expression of PCGF6 in steady state DCs is sufficient to modulate the chromatin accessibility at specific genes involved in the inflammatory process. The objectives of the next study were to perform a more in depth study into the mechansims by which PCGF6 regulates gene expression in DCs. We examined the effect of PCGF6 expression on the epigenetic landscape at the genome-wide level, on the regulation of specific gene pathways and on the availability of specific transcription factor binding sites.

Chapter 3: PCGF6 dynamically regulates the availability of Egr1 transcription factor binding sites to fine-tune TLR-mediated maturation

PCGF6 dynamically regulates the availability of Egr1 transcription factor binding sites to fine-tune TLR-mediated maturation

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3.1 Short Summary

Dendritic cells (DCs) integrate cues from their microenvironment through chromatin remodeling and transcriptional reprogramming. Pathogen and danger recognition by DCs triggers an inflammatory response and improves their capacity to communicate with T cells. At steady state, the chromatin state and transcription factor binding dynamics can prime inflammatory genes for transcription. While the transcriptional networks that coordinate DC activation and function are well characterized, less is known about the specific factors that mediate chromatin modification. PCGF6 is a polycomb group factor that is required to maintain DC quiescence at steady state through transcriptional repression. PCGF6 regulated the chromatin accessibility at several key gene promoters involved in inflammation. Here we demonstrate that PCGF6 regulates chromatin accessibility genome-wide. PCGF6 downregulation revealed binding sites for the immediate-early transcription factor EGR1. EGR1 target genes include pathways in immunity and lipid metabolism. PCGF6 deficiency enhances DC activation as well as $CD8^+$ T cell cross-priming, in part through permitting EGR1 binding.

3.2 Introduction

The chromatin landscape dictates the development, differentiation and function of cells by enabling and restricting the expression of subsets of genes that specify cell fate and function (Bannister and Kouzarides, 2011; Bracken et al., 2006). Chromatin remodeling plays a significant in role in instructing innate immune responses to pathogenic stimuli (Huang et al., 2012; Saeed et al., 2014). Dendritic cells (DCs) are innate immune cells that rely on signals from their microenvironment to direct their function. Both activating and suppressive stimuli are accompanied by transcriptional

reprogramming; in part mediated through genome-wide redistribution of histone methylation, acetylation as well as DNA methylation (Huang et al., 2012; Kaufmann et al., 2018; Saeed et al., 2014). The combination of histone modifications in a genomic region determines whether that region is accessible or not for transcription factor binding. Histone modifications can serve as docking sites for transcription factors, but also regulate chromatin architecture, and the formation of heterochromatin and euchromatin. DNA-binding transcription factors cannot localize to heterochromatin, leading to transcriptional repression of compacted regions of chromatin.

Hierarchical transcription factor networks control transcriptional reprogramming of DCs in response to stimulation (Garber et al., 2012). Lineage-specifying factors recruit chromatin modifying enzymes to activate cell-type specific enhancers. Priming factors then prime lineage-specific genes and determine their steady state levels of expression. Finally, dynamic factors are activated in a context-dependent manner, thereby instructing the expression of gene programs in response to specific stimuli. One such dynamic factor, early growth response 1 (EGR1) rapidly increases in expression and binding of target sites within the first two hours of LPS stimulation of DCs and induces the expression of proinflammatory genes (Garber et al., 2012; Hancock et al., 2014). EGR1 also regulates the expression of a number or tumour suppressor genes, and has an established role in growth and proliferation of cells. Dysregulation of EGR1 has been implicated in myelodysplastic disorders, a heterogeneous group of disorders characterized by impaired differentiation of hematopoietic stem cells (Adema and Bejar, 2013). In humans, *Egr1* is localized within the chromosome 5q region that is commonly deleted in patients with

myelodysplastic disorder. Studies have confirmed that haploinsufficiency of EGR1 in mice accelerates progression of this disease (Stoddart et al., 2014).

It is well-established that chromatin modification underlies the transcriptional reprogramming that occurs during DC activation. However, the specific chromatin-modifying complexes that balance chromatin states and regulate the binding site availability of transcription factors are largely unidentified. Characterizing the specific factors and complexes that regulate chromatin accessibility in DCs is crucial to gain a better understanding of the mechanisms underlying DC function.

Polycomb group factor 6 (PCGF6), is a member of the PRC1.6 complex that mediates transcriptional repression through chromatin modification (Akasaka et al., 2002). We have shown in DCs that PCGF6 cooperates with JARID1C to facilitate demethylation of H3K4me3 at the promoters of specific genes involved in inflammation. PCGF6 also regulates chromatin accessibility of the same genes (Boukhaled et al., 2016). Through this regulation, PCGF6 maintains DC homeostasis, preventing spurious activation of proinflammatory genes. Mechanistically, the breadth of chromatin regulation by PCGF6 and whether it contributes to regulation of dynamic transcription factors is unknown.

Through analysis of ATAC-sequencing data, we found that PCGF6 globally regulates chromatin accessibility in DCs. Loss of PCGF6 leads to a gain in accessibility of EGR1 binding sites in the promoters of genes involved in inflammatory responses and lipid metabolism. PCGF6 deficiency partially compensates for EGR1 haploinsufficiency in DCs with respect to T cell priming. However, EGR1 is still required for enhanced upregulation of CD86 observed upon LPS stimulation of PCGF6 deficient DCs. We

demonstrate a complex relationship between chromatin-modifying factors and the activity of dynamic transcription factors.

3.3 Results

Global impact on chromatin accessibility in DCs with altered PCGF6 expression

Overexpressing PCGF6 resulted in a significant loss of regions of accessible chromatin (Fig. 1A). 68503 peaks genome-wide were unique to control DCs, suggesting that over-expressing PCGF6 promotes the compaction of those regions. Calculating the average peak intensity within a 300-bp range from the center of all the peaks demonstrated a genome-wide loss of signal in DCs overexpressing PCGF6 (Fig. 1B, top panel). The loss of signal was particularly profound across peaks that are unique to control cells (Fig. 1B, middle panel). However, there was no difference in average signal intensity for PCGF6-specific peaks (Fig. 1B, bottom panel), suggesting that the calling of those regions are more representative of noise than biological changes within the cell.

PCGF6-deficiency lead to an overall gain in accessible regions of chromatin (Fig. 1C). 40683 peaks were gained genome-wide (Fig. 1C, top panel). Furthermore, the average signal intensity over the shared regions and gained regions was significantly higher in PCGF6 deficient DCs compared to control (Fig. 1D, top and middle panel). Again, the regions associated with a relative loss in signal with PCGF6 deficiency showed no change in signal intensity and so can be attributed to noise.

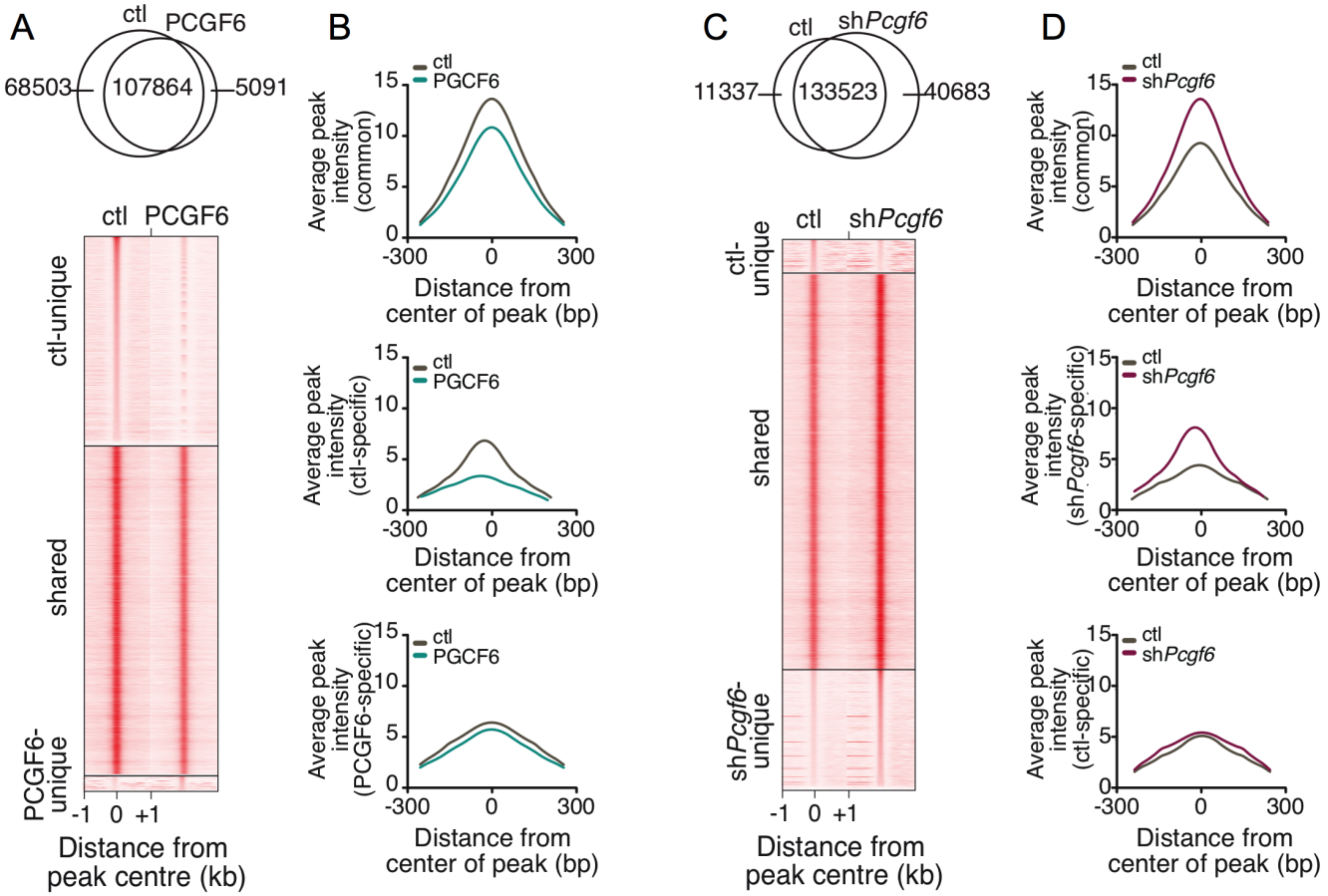

Figure 1. Global impact on chromatin accessibility in DCs with altered PCGF6 expression. Transduced, resting DCs were sorted based on reporter and CD11c expression. Cells were subjected to ATAC-sequencing. Reads were aligned to the mouse genome (mm10) and downsampled to allow for comparison between groups. Significant peaks were called using MACS 2.0. A, C. Venn diagram (top panel) displaying the number of significant peaks called in DCs transduced with a vector control only (ctl), DCs that over-express PCGF6 (PCGF6) or a hairpin to reduce its expression (shPcgf6) and peaks that were shared between their respective controls. The heatmap (bottom panel) shows all aligned peaks flanked by a 1kb region upstream and downstream of the centre of the peak. B, D. The average peak intensity derived from the three distinct clusters in A and C.

Together these data demonstrate a global effect of PCGF6 on chromatin accessibility of DCs. Furthermore, gain or loss of PCGF6 led to changes in accessibility of many genomic elements, with a particular enrichment of intergenic regions (Fig. S1A). This suggests that PCGF6 may be more ubiquitously localized across the genome, but may be particularly concentrated at enhancer elements. This is consistent with a recent

study which demonstrated that the pattern of PCGF6 localization is not restricted to a particular genomic element, similar to that of their trithorax (TrxG) counterparts (Yang et al. 2016).

Gene pathways enriched or suppressed by modified PCGF6 expression

To explore whether specific gene pathways are associated with gain or loss of open chromatin, the peaks were annotated based on their nearest genes and genomic localization using HOMER. We narrowed in on peaks that were changed specifically in promoters. Using gene set enrichment analysis (GSEA) through the molecular signatures database (MsigDb), we identified the top 10 pathways enriched in the peaks that were lost by over-expressing PCGF6 (Fig. 2A). PCGF6 overexpression resulted in loss of chromatin accessibility in the promoters of genes enriched in matrix remodeling, hemostasis, immune system, and various signaling pathways. Importantly, PCGF6 deficiency led to a gain in accessibility of similar gene pathways (Fig. 2B). In addition, several specific genes that were found to lose accessibility in DCs over-expressing PCGF6 (Fig. 2C, S2A) also gained accessibility in PCGF6-deficeint DCs (Fig. 2D, S2A). Thus despite global changes in chromatin accessibility, PCGF6 regulates specific pathways important for DC function.

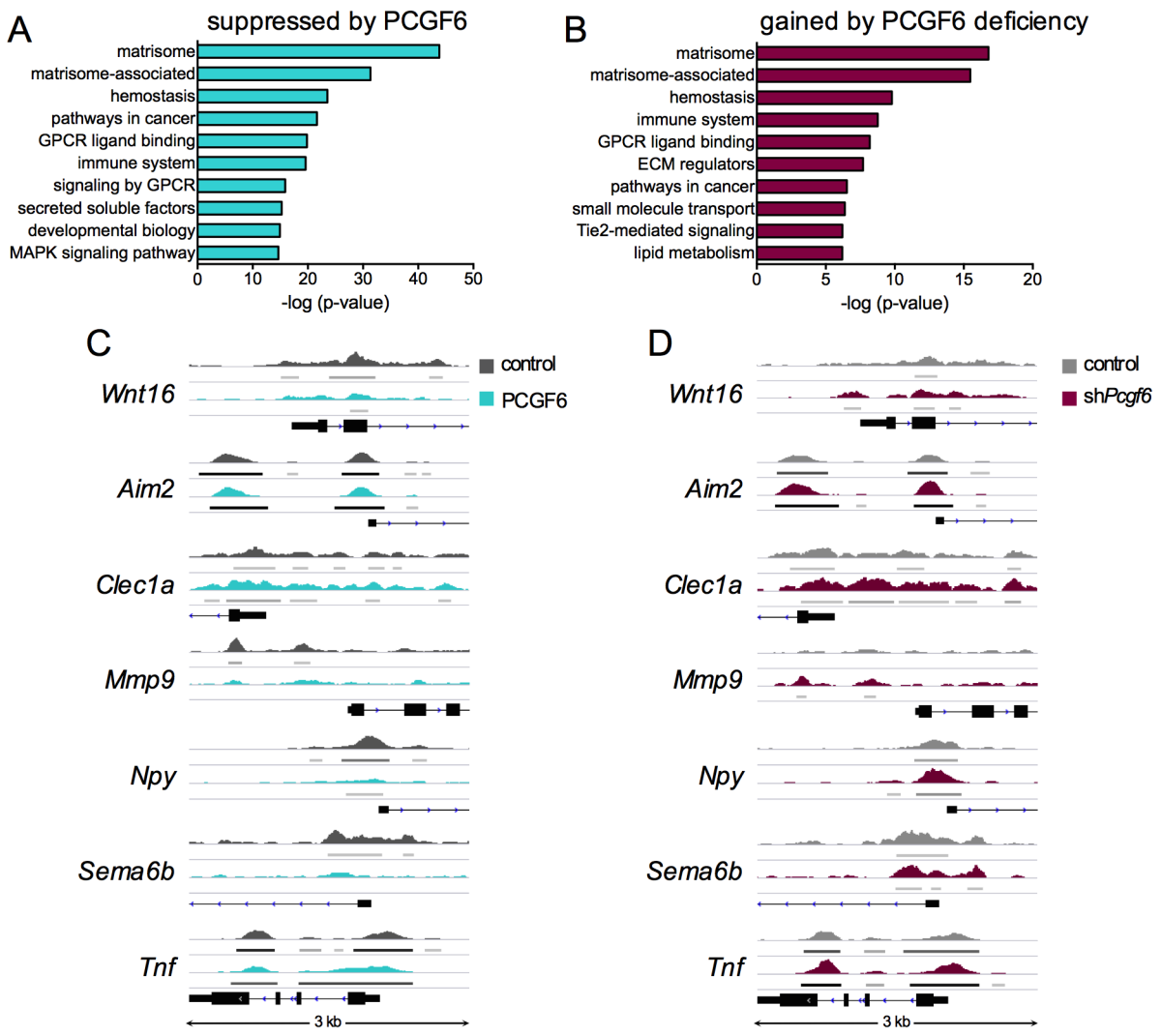

Figure 2. Gene pathways enriched or suppressed by modified PCGF6 expression. A, B. GSEA of annotated gene lists of promoter-specific peaks that are unique to control DCs (suppressed by PCGF6), or unique to PCGF6-deficient DCs (gained by PCGF6 deficiency). p-value was calculated by MSigDb. The KEGG and REACTOME gene signature databases were used for analysis. C, D. Screenshots from IGV genome browser of reads aligned to the mouse genome (mm10). Data show a 3kb region flanking the transcription start site of the indicated genes (gene track, shown in black). Grayscale bars indicate indicate reads that are significantly enriched and the darker the colour the lower the p-value.

EGR1 binding sites are enriched in regions made accessible by PCGF6-deficiency

Regulation of chromatin accessibility through chromatin modification is an important mechanism to control transcription factor accessibility to specific genes. Rapid DC responses to environmental cues relies on the activation and DNA binding of dynamic transcription factors (Garber et al., 2012). To determine whether PCGF6 deficiency regulates the accessibility of dynamic transcription factors to their target genes, we used HOMER analysis to identify enriched TFBS in DCs with altered PCGF6 expression. The top enriched binding site was for EGR1 (Fig. 3A).

EGR1 is an immediate-early gene that is known to regulate the expression of genes important for inflammatory responses and cholesterol metabolism (Garber et al., 2012; Gokey et al., 2011). GSEA of genes with EGR1 binding sites in their promoter regions identified immune system and lipid metabolism pathways, consistent with processes known to be regulated by EGR1 (Fig. 3B). In Pcgf6-deficient cells, EGR1 sites became more accessible in immune system genes involved in pattern recognition, signalling, antigen presentation and type I IFN response (Fig. 3C). Within the lipid metabolism cluster, transcription factors, transporters and scavenger receptors were identified to gain EGR1 binding sites in the absence of PCGF6 (Fig. 3D). This highlights that many processes important for the induction and regulation of inflammation are controlled by PCGF6, possibly by regulating the accessibility of EGR1 binding sites.

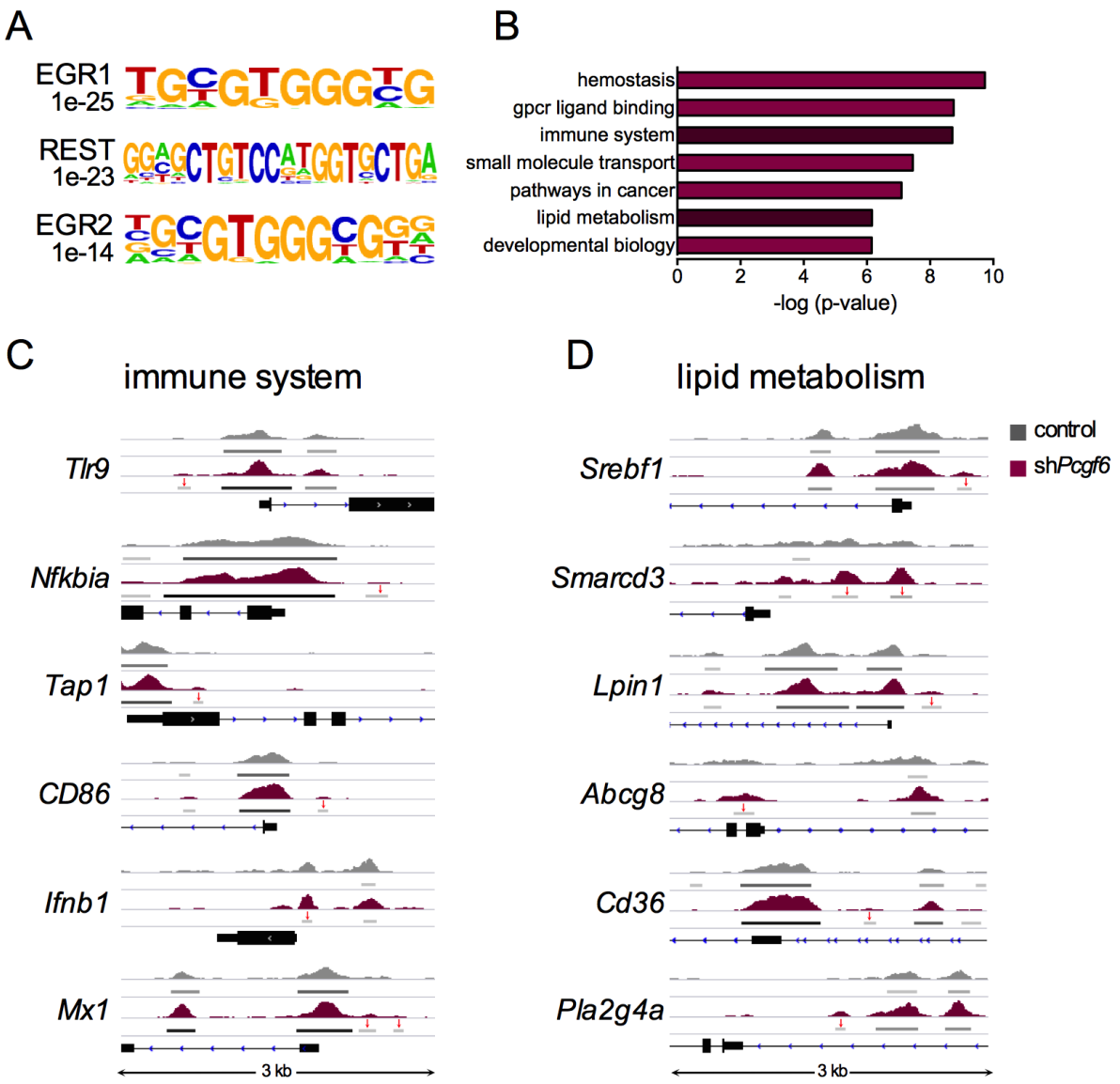

Figure 3. EGR1 binding sites are enriched in regions made accessible by PCGF6-deficiency. A. Transcription factor binding site enrichment analysis was performed using HOMER analysis software. Shown are the top 3 transcription factor binding motifs enriched in the peaks that are unique to PCGF6 deficient DCs (compared to control). B. GSEA was performed as described in figure 2 using annotated gene lists of the peaks containing EGR1 binding sites near promoters, unique to PCGF6 deficient DCs. C, D. IGV genome browser screenshots of 3kb region flanking genes with an EGR1 binding site in their promoters. Red arrows indicate the open region containing an EGR1 binding site in PCGF6 deficient DCs.

PCGF6 downregulation promotes DC maturation by regulating EGR1 target genes

In order to determine whether the loss of PCGF6 promotes DC activation by permitting the induction of EGR1 target genes, EGR1 haploinsufficient (*Egr1*$^{-/+}$) DCs with reduced PCGF6 expression were stimulated with either LPS or IFNβ and compared to wild-type littermate controls. CD86, was identified as an EGR1 target gene and its expression was used to assess the role of PCGF6 in regulating EGR1 target genes (Fig. 4A).

Similar to previously reported results, we found that PCGF6 deficiency increased the steady-state surface expression of CD86. *Egr1*$^{+/-}$ DCs deficient in PCGF6 also displayed increased CD86, indicating that EGR1 does not participate in establishing steady state levels of CD86. This is consistent with literature demonstrating that EGR1 expression and genomic localization to target promoters only increases significantly with LPS stimulation. PCGF6 deficiency increased the expression of CD86 to a greater extent than control in wild-type littermates (Fig. 4A, B). The enhanced CD86 in response to LPS depended on EGR1 since *Egr1*$^{+/-}$ DCs deficient in PCGF6 do not increase CD86 expression to the same extent. Therefore, it is possible that the increased CD86 observed with loss of PCGF6 may be attributed to a gain in EGR1 binding sites. PCGF6-deficient *Egr1*$^{+/-}$ DCs treated with IFNβ still significantly increase CD86 compared to wild-type DCs. There was however, a partial decrease with loss of EGR1 suggesting that though not required for IFNβ-stimulated CD86, EGR1 potentiates its expression in that context.

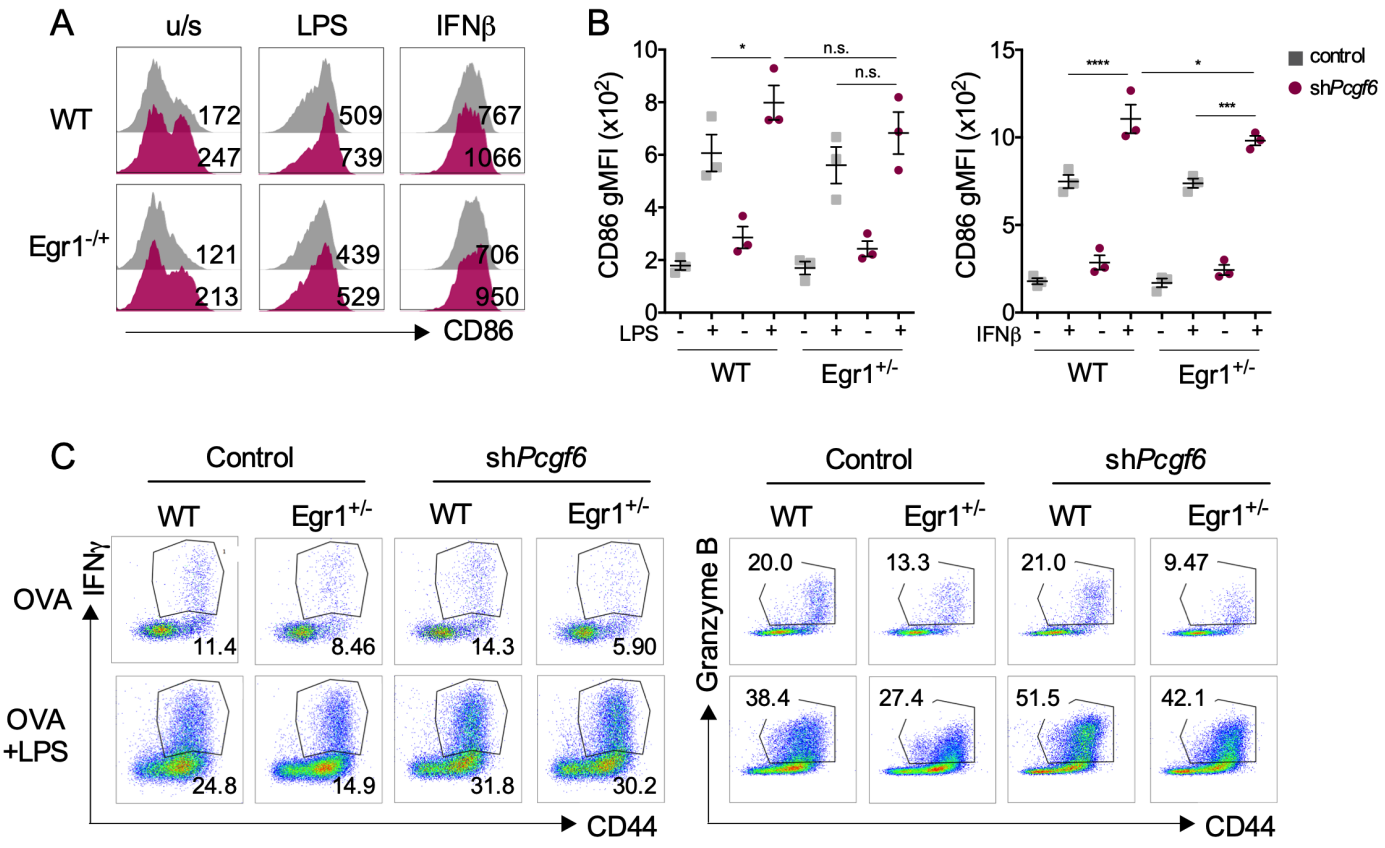

Figure 4. PCGF6 downregulation promotes DC maturation by regulating EGR1 target genes. A, B. Transduced DCs from *Egr1*$^{+/-}$ mice or littermate controls (WT) we treated or not with 10 ng/mL LPS or 1000 U/mL IFNβ. CD86 expression was measured by flow cytometry. A. Representative histograms of CD86 expression on CD11c^{+}Gr-1^{-}reporter^{+} cells. Numbers indicate corresponding geometric MFI (gMFI). B. Quantification of A. Each point is the average of triplicates from a single experiment. Data are pooled from 3 independent experiments. * p<0.05, **** p<0.001, n.s. not significant, One-way ANOVA. Relevant comparisons between WT and Egr1$^{+/-}$ were not significant except for those reported in the figure. C. DCs were pulsed with whole Ovalbumin (2 mg/mL) and stimulated or not with LPS (5 ng/mL). DCs were then co-cultured with purified CD8^{+} OTI TCR transgenic T cells. 4 days later, T cells were restimulated with PMA and ionomycin and intracellular cytokines and Granzyme B production were measured by flow cytometry. Plots show CD44 and IFNγ (left) or Granzyme B (right) production by CD8^{+} T cells. Numbers indicate the percentage of CD8^{+} cells within the gates shown. Data are representative of 2-3 independent experiments.

DCs are specialized to present antigen to T cells through MHCI and MHCII. Cross-presentation is the process of taking up exogenous antigens for antigen presentation on MHCI without first becoming infected; DCs have a unique capacity for cross-presentation (Merad et al., 2013). Another EGR1 target gene identified was *Tap1* an important transport protein involved in antigen cross-presentation. In order to determine whether

regulation of EGR1 target genes by PCGF6 affects the $CD8^+$ T cell priming capacity of DCs, DCs were pulsed with whole ovalbumin protein and co-cultured with antigen specific OTI T cells. The resulting T cell response was measured four days later by IFNγ and Granzyme B production (Fig. 4C). *Egr1*$^{+/-}$ DCs demonstrate a decreased capacity to cross-prime $CD8^+$ T cells compared to wild-type littermate controls (Fig. 4C). Furthermore, IFNγ and Granzyme B production were increased in T cells primed by WT and *Egr1*$^{+/-}$ DCs deficient in PCGF6 (Fig. 4C).

3.4 Discussion

Here we show that epigenetic modification by PCGF6 has a global effect on chromatin accessibility in DCs contributing to the regulation of a variety of genes in signaling, immune system and metabolism. PCGF6 regulates the accessibility of EGR1 binding sites, and downregulation of PCGF6 facilitates efficient $CD8^+$ T cell priming and DC activation by LPS.

Over-expression of PCGF6 was associated with a significant loss of open regions of chromatin. In addition, we found that the overall ATAC signal intensity was lower in DCs overexpressing PCGF6. These data were complemented by data from PCGF6-deficient DCs. Several studies have shown that PRC2 is involved in the spreading of suppressive signals around sites of repression (Lee et al., 2018; Oksuz et al., 2018). It has been proposed that PRC1 complexes may work in a similar way. Therefore, PRCs may be involved in maintaining repression or fine-tuning the activity of specific sites. Since PCGF6 over-expression decreased signal intensity of even the shared peaks between control DCs and PCGF6 over-expressing DCs, it is possible that PCGF6 fine-tunes gene activity in addition to promoting suppression of specific sites. This is further supported by

a study in the literature demonstrating that JARID1C, a PCGF6 binding partner, tempers the activity of enhancers through RACK7 (Shen et al., 2016).

While the sites gained or lost by altered PCGF6 expression were not associated with specific genomic elements, there was an enrichment of intergenic regions. This strongly suggests regulation of enhancer regions. Clusters of enhancers, called “super-enhancers” often regulate sets of related genes (Hah et al., 2015; Hnisz et al., 2013). Therefore, through regulating enhancer activity PCGF6 may achieve specificity; despite the global effect of altered PCGF6. There is growing evidence for dynamic regulation of enhancer elements over the course of an immune response (Kaufmann et al., 2018; Pacis et al., 2015). Therefore, determining the role of PCGF6 in regulating enhancers warrants further investigation.

The gene pathways associated with increased or decreased PCGF6 expression were not surprising given our previous study establishing the role of PCGF6 in maintaining DC homeostasis. PCGF6 regulates a number of genes from different signaling pathways, therefore in addition to chromatin remodeling, PCGF6 could contribute to signal integration by regulating the levels of signaling molecules in particular pathways.

Our analysis reveals that PCGF6 controls a widespread program in DCs that dictates their function. This is further supported by our observation that EGR1 binding sites are enriched in regions regulated by PCGF6. EGR1 has been shown to dynamically relocalize to new binding sites during the first few hours of LPS stimulation. Genes implicated in proinflammatory responses were strongly represented among target genes that were identified with novel EGR1 binding sites made accessible in PCGF6 deficient

DCs. These include *Tlr9*, which is activated by unmethylated CpG, *Tap1*, a gene involved in antigen cross-presentation and *Ifnb1* and *Mx1* which are IFN-β stimulated genes (ISGs). We had previously found that type I IFNs also lead to the downregulation of PCGF6, however the consequence of PCGF6 downregulation in that context remains unexplored. The identification of ISGs and genes involved in cross-presentation in our ATAC-seq data implicates a possible role for PCGF6 in regulating early viral responses in DCs.

Furthermore, another significant pathway enriched among the EGR1 target genes was lipid metabolism. EGR1 has an established role in regulation cholesterol metabolism in liver cells (Gokey et al., 2011). Consistent with this, our study identified *Srebf1* as an EGR1-target gene regulated by PCGF6. *Srebf1* encodes the sterol regulatory binding protein, SREBP1 which controls the transcription of genes involved in cholesterol metabolism. Similarly, we also identified *Abcg8*, a gene encoding a cholesterol transporter important for vesicular export of cholesterol. Several studies have established that cholesterol balance in DCs is crucial to maintain homeostasis. Namely, cholesterol accumulation in DCs *in vivo* leads to anti-nuclear antibody production and features similar to systemic lupus erythematosus (SLE) (Ito et al., 2016; Westerterp et al., 2017). However, SREBP1 activity has been linked to the activation of anti-inflammatory lipid mediators in the late phase of TLR stimulation, contributing to the resolution of inflammation (Oishi et al., 2017). Further studies have shown a reciprocal relationship between type I IFN signatures and cholesterol metabolism (York et al., 2015). Since PCGF6 maintains DC homeostasis, it is possible that downregulation of PCGF6 activates novel regulatory mechanisms as negative feedback, to contribute to maintaining

homeostasis. Whether the lipid metabolism signature contributes to the inflammatory phenotype observed with PCGF6 deficient DCs or whether it tempers DC activation remains to be determined.

Finally, we found that EGR1 haploinsufficiency prevents the increased CD86 observed with PCGF6 deficiency. This occurs in response to LPS stimulation but only modestly in response to IFNβ stimulation. It is possible that TLR4 stimulation depends on EGR1 to activate the IFN response which is known to act in a positive feedback loop and promotes DC activation (Ma et al., 2015). The increased CD86 observed in PCGF6 deficient LPS-stimulated DCs may be due to activation of the IFN response by EGR1. In contrast IFNβ treatment likely employs a different signaling pathway to enhance DC activation.

In terms of priming T cell responses, we found that Egr1 haploinsufficiency in DCs decreased IFNγ production and Granzyme B production by $CD8^+$ T cells. Interestingly, PCGF6 deficiency in DCs restored the T cell response to wild-type levels or greater. LPS stimulation leads to rapid PCGF6 downregulation and yet *Egr1*$^{+/-}$ DCs are impaired in their $CD8^+$ T cell priming capacity. This suggests PCGF6 downregulation promotes T cell priming through EGR1. It also suggests that the steady state level of PCGF6 can be predictive of DC function by promoting a chromatin landscape that favours immune activation. Low steady state levels of PCGF6 may also prime ISGs which favours positive feedback by type I and type II IFNs and boosts the resulting response. Indeed, we showed that type I IFNs can bypass the requirement for EGR1, demonstrating that EGR1 contributes to, but is not essential for hyper-activation mediated by deficiency of PCGF6.

3.5 Contributions

Conceptualization, C.M.K., G.M.B.; Methodology, C.M.K., G.M.B., G.D.; Validation, G.M.B.; Formal Analysis, S.D.B., G.D., G.M.B; Investigation, C.M.K., G.M.B., G.D.; Resources, M.L.; Data Curation, G.D.; Writing- original draft, C.M.K., G.M.B.; Writing – Review and editing, C.M.K., G.M.B.; Visualization, C.M.K., G.M.B.; Supervision, C.M.K., M.L; Project administration, C.M.K., G.M.B.; Funding acquisition, C.M.K

3.6 Experimental Procedures

Mice and Materials

C57Bl/6 mice were obtained from Charles River Laboratories, Egr1-/+ (Jackson stock 012924) mice were obtained from Jackson Laboratories and were bred in house at the Goodman Cancer Centre animal facility. OTI (Jackson stock 003831) mice were provided by Dr. Martin J. Richer and bred in house. All mice were treated according to protocols approved by the institution's Animal Care Committee (ACC), in accordance with standard operating procedures of the Canadian Council on Animal Care (CCAC). Tissue culture reagents: RPMI, β-mercaptoethanol (Corning). Dulbecco's modified Eagle's media (DMEM), Iscove's Modified Dulbecco's media, Hank's balanced salt solution (HBSS), Phosphate buffered saline (PBS) (Wisent). LPS (Sigma, *E. coli* O111:B4). Ovalbumin (OVA) was derived in-house by isolating egg white under sterile conditions. Concentration of OVA was determined by spectrophotometry. Endotoxin levels tested in-house were below the detectable limit. Cytokines: GM-CSF (Peprotech), IFNβ (R&D Systems). Commercial Kits: EasySep CD8+ T cell negative selection kit, with RapidSpheres (STEMCELL Inc.), Intracellular Fixation/Permeabilization Buffer kit (eBioscience/Fisher). Antibodies: eBioscience/Fisher, see supplementary Table 1.

BMDC culture and transduction

Bone marrow cells were differentiated and transduced as described (Boukhaled et al. 2016). Briefly, cells were cultured in the presence of 20ng/mL GM-CSF (Peprotech) for 8-10 days. On day 2 of culture, cells were spin infected at 30°C with virus containing MSCV- or LMP-based vectors to over-express or knock-down PCGF6. Vectors express human CD8 as a reporter. At the end of the culturing period, $2x10^5$ cells were seeded in 96-well plates. DCs were left untreated, activated with 10ng/mL LPS (*E. coli* O111:B4) or 1000 U/mL IFNβ.

ATAC-sequencing and bioinformatic analyses

The experimental procedure for the ATAC-seq data presented in this report, as well as the sequence mapping and downsampling procedure were previously published in Boukhaled et al. 2016. The transposase reaction and sample preparation protocols were adapted from (Buenrostro et al., 2013). Peak annotation and transcription factor binding sites were identified using HOMER (http://homer.ucsd.edu/homer/ngs/). Gene set enrichment analysis (GSEA) was performed using the molecular signatures database (MSigDb; http://software.broadinstitute.org/). KEGG and REACTOME databases were used for GSEA.

T-cell priming assay

$1x10^4$ DCs were treated with 2 μg/mL of whole ovalbumin and stimulated or not with 5 ng/mL LPS. 4-6 hours later, DCs were co-cultured with $1x10^5$ purified $CD8^+$ OTI TCR transgenic T cells. 4 days after co-culture, T cells were re-stimulated with PMA (500 ng/mL) and ionomycin (50 ng/mL) for 4 hours. Brefeldin A was added (1:1000) 2 hours before the end of re-stimulation.

Intracellular staining

Cell surface markers were stained for 30 min at 4°C in FACS buffer (PBS, 2% FCS, 2 mM EDTA, 0.05% azide). Cells were then fixed for 20 min at 4°C with Intracellular (IC) Fixation Buffer then permeabilized with 1X Permeabilization Buffer (eBioscience/Thermo Fisher). Finally intracellular markers were stained for at least 1h in permeabilization buffer.

Statistical Analysis

Statistical analysis of flow cytometry data was performed using Prism 6.0 software. Statistical significance was determined by One-way ANOVA and indicated by the following: n.s., not significant; * $p<0.05$; ** $p<0.05$. Error bars represent either standard error of the mean.

3.7 Accession

ATAC-sequencing data is deposited in the Gene Expression Omnibus database (GEO accession: GSE83640)

3.8 Acknowledgements

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2.10 Supplemental Information

Supplemental Tables

Table S1. List of antibodies used for flow cytometry

Antigen/Fluorophore	Fluorophore	Clone
CD86	PE/Cy7	GL1
human CD8	APC/eFluor-780	SK1
CD11c	PerCP/Cy5.5	N418
Gr-1	eFluor 450	1A8-Ly6G
CD44	PE/Cy7	IM7
mouse CD8	APC/eFluor 780	53-6.7
IFNγ	APC	XMG1.2
Granzyme B	FITC	NGZB

Light blue – Antibodies used for DC staining panel
White – Antibodies usd for T cell staining panel

Supplemental Figures

Figure S1

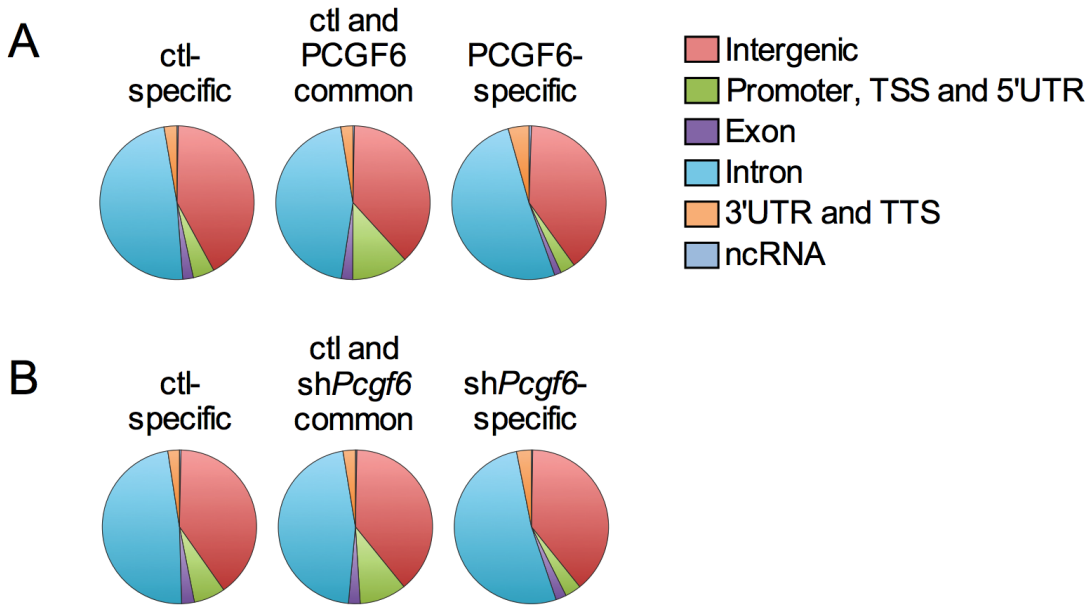

Figure S1, related to Figure 1. A, B. Distribution of open chromatin within specific genomic elements in DCs transduced to either over-express (PCGF6) or reduce (sh*Pcgf6)* the expression of PCGF6.

Figure S2

IFNB1	*GRB2*	*HRH2*	*ADAMTS14*	*SPN*
PLG	*ITGA2B*	*LPAR4*	*LOX*	*GATA6*
F13A1	*GNG4*	*HRH1*	*ASTL*	*MAFF*
WNT16	*GNAS*	*NPY*	*SERPINB9*	*SMO*
WNT6	*RASGRP2*	*CCK*	*TGM5*	*PTCH2*
FGF2	*PRKCD*	*FFAR2*	*SEMA6B*	*ZBTB16*
IGF1	*SHC1*	*GPR68*	*SEMA3D*	*SKP2*
MMP9	*YWHAZ*	*GPR65*	*MUC13*	*PDGFRA*
TNF	*RAPGEF3*	*TAS1R1*	*C1QTNF1*	*GLI1*
ANGPTL4	*LAT*	*RASGRP3*	*CLEC1B*	*RARA*
NRTN	*KIF2C*	*SH3KBP1*	*CLEC1A*	*ADORA2A*
EREG	*SH2B1*	*GRAP2*	*COLEC12*	*BDKRB2*
GDF7	*PLA2G4A*	*RAET1E*	*PAPLN*	*OPRM1*
GDF6	*ABCC4*	*RILP*	*MATN3*	*GALR1*
GDF11	*PRKCZ*	*RNF25*	*CRISPLD2*	*SSTR3*
SCUBE3	*CAV1*	*GBP4*	*LGI1*	*GALR3*
SCUBE2	*DOCK9*	*RIPK1*	*VCAN*	*MC1R*
SEMA6A	*DOCK10*	*LBP*	*IFIH1*	*ANK3*
C9	*GNAO1*	*DLG3*	*AIM2*	*GFRA1*
RGS20	*SMARCD3*	*CNTNAP1*	*CFB*	
NKX6-1	*CACNA1C*	*PTPN5*	*PAX6*	

Figure S2, related to Figure 2. List of genes that lose chromatin accessibility in PCGF6 overexpressin DCs and gain chromatin accessibility in PCGF6-deficient DCs.

Preface to Chapter 4

Chapters 2 and 3 established the central role of PCGF6 in regulating chromatin accessibility in DCs in order to maintain DC quiescence. The goal of the next study was to further explore the consequence of aberrant PCGF6 expression in DCs on their main functional capacity: the priming of T cell responses. In Chapter 2 we found that PCGF6 had a profound effect on the capacity of DCs to prime functional T cell responses. In Chapter 4, we delve further into the mechanism by which PCGF6 in DCs controls T cell responses and more specifically how it participates in establishing an antigen threshold for naïve T cell activation.

Chapter 4: Cooperative and independent control of immunosuppressive programs in DCs by PCGF6 and PDL1

Cooperative and independent control of immunosuppressive programs in DCs by PCGF6 and PDL1

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4.1 Short Summary

Dendritic cells orchestrate T cell responses through antigen presentation, co-stimulation and cytokine production. In order to initiate a robust T cell response to invading pathogens, the T cell antigen receptor should be finely tuned to sense low doses of antigen. Several lines of evidence indicate that $CD8^+$ T cell antigen sensitivity is modulated by the inflammatory milieu, however the specific contribution of DCs is unclear. DCs are also shaped by their microenvironment and integrate cues through epigenetic modification. We had previously identified the transcriptional repressor, PCGF6 for its role in maintaining DC homeostasis. We hypothesized that PCGF6 expression levels at steady state dictate whether they initiate immunogenic or immunoregulatory responses. We found that PCGF6 expression in steady state DCs regulates the antigen sensitivity of $CD8^+$ T cells through PDL1. The PDL1/PD-1 axis limits the antigen sensitivity of T cells in contact with DCs at steady state, but not LPS-matured DCs. Instead, PCGF6 downregulation and LPS maturation both increase T cell antigen sensitivity and strengthen the early interactions of DCs and T cells. In cDCs, regulation of antigen sensitivity by PCGF6 was more important for cDC1s than cDC2s. This work contributes to a growing body of evidence demonstrating fine-tuning of DC homeostasis through dynamic regulation of the chromatin landscape.

4.2 Introduction

Dendritic cells (DCs) are cells of the innate immune system that are highly specialized to process and present foreign antigen to $CD4^+$ and $CD8^+$ T cells, thereby triggering their effector function. In addition to antigen presentation, they also provide costimulation and differentiation signals that modulate the type of adaptive response that ensues. Even in the absence of invading pathogens, DCs play critical roles in immune surveillance, peripheral tolerance and wound healing. Self-peptide MHC interactions with the TCR are not only essential for both negative and positive selection in the thymus, but also to maintain peripheral tolerance to self-antigens throughout the lifetime of the host (Stefanová et al., 2002).

On a molecular level, DCs are poised to rapidly alter their gene expression program in response to danger or pathogens. Transcription factors prime genes that are dynamically activated in response to environmental cues (Garber et al., 2012). However, in the absence of stimuli, DCs actively maintain their steady state. Factors such as NF-κB1, which is essential to activate inflammation, also restrains DC activation at steady state (Dissanayake et al., 2011). Other factors, such as the DC lineage-specific ZBTB46 are downregulated in mature DCs to relieve inhibition of inflammatory genes (Meredith et al., 2012). Similarly, PCGF6, a member of the non-canonical polycomb-repressive complex, PRC1.6. Polycomb repressive complexes suppresses transcription through epigenetic modification. Downregulation of PCGF6 is necessary for optimal DC activation and function (Boukhaled et al., 2016).

The contradictory role that factors like NF-κB1 play at steady state and in an inflammatory setting demonstrates that context is crucial in determining DC function. The

factors that determine how DCs stably integrate context specific cues is an active area of research. Given that PCGF6 maintains steady state through epigenetic modification and that it's downregulation promotes proinflammatory responses, we hypothesize that PCGF6 may also play a role in the integration of context-specific cues.

At steady state, interactions between DCs and T cells help to maintain TCR clusters to ensure a robust functional avidity upon encounter of foreign antigen (Stefanová et al., 2002). During an infection, the antigen sensitivity of effector T cells is dynamically modulated by factors in the environment (Richer et al., 2013; Smith et al., 2018). During acute infections type I IFNs greatly decrease the antigen dose required for effector T cell responses (increases the antigen sensitivity) (Richer et al., 2013) while during chronic infection IL-10 decreases antigen sensitivity, contributing to T cell dysfunction (Smith et al., 2018). DCs can also tune the antigen sensitivity of naïve T cells (Garbi and Kreutzberg, 2012), therefore perturbations in the steady state of DCs could influence the antigen threshold required for T cells to initiate a response. Furthermore, while it is known that different subsets of DCs have a differential capacity to process antigen (den Haan et al., 2000), it is unclear whether given equal doses of antigen, DC subsets can differ in their capacity to elicit sensitive T cell responses. Understanding the role DCs play in establishing the antigen sensitivity of T cells is crucial to gain insight into mechanisms that drive T cell dysfunction or breaks in tolerance.

Here we show that PCGF6 expression by DCs regulates the antigen dose required to activate naive T cells and that PDL1 is implicated in this regulation. PDL1 and PCGF6 drive separate but overlapping immunosuppressive programs in DCs. PDL1 decreases MHCII and CD80 and promotes IL-6 production. Activation of DCs downregulates PCGF6

which favours stable interactions of DCs and T cell early during priming. *Ex vivo*, steady state cDC1s require more antigen to elicit a response from T cells than cDC2s. Both cDC1s and cDC2s increase PDL1 upon interaction with T cells, however cDC1s are more dependent on PCGF6 to maintain their steady state than cDC2s.

4.3 Results

PCGF6 in DCs controls the antigen sensitivity of naive CD8$^+$ T cells at steady state through PDL1

We have previously reported that overexpression of PCGF6 leads to an impaired capacity to prime CD8$^+$ T cell responses by DCs (Boukhaled et al., 2016). To further investigate the role of PCGF6 during priming of CD8$^+$ T cells, the functional avidity of T cells primed by PCGF6 over-expressing DCs and control DCs was determined. Briefly, DCs were treated with titrated doses of the processed H2-k^b-restricted Ovalbumin peptide, SIINFEKL, and co-cultured with CD8$^+$ OTI T cells. Three days later, T cell activation was measured by the expression of markers such as CD25 by flow cytometry. We found that at the highest peptide dose, PCGF6 overexpression in DCs does not greatly limit their capacity to activate CD8$^+$ T cells as measured by CD25 induction (Fig. 1A, left panel). However, decreasing the peptide dose dramatically decreased the priming capacity of PCGF6-overexpressing DCs compared to control (Figure 1A, right panel). We quantified the resulting T cell antigen sensitivity by calculating the EC50 value (or the dose required to elicit a 50% response). We found that PCGF6-overexpressing DCs leads to a decrease in the CD8$^+$ T cell antigen sensitivity (Fig. 1B). Conversely, PCGF6 deficiency increased their capacity to prime CD8$^+$ T cell responses as well as the antigen sensitivity of the T cells (Fig. 1C, D). These changes were unlikely due to differences in

peptide loading since MHCI expression was not significantly different in any of the conditions tested (Fig. S1A, B).

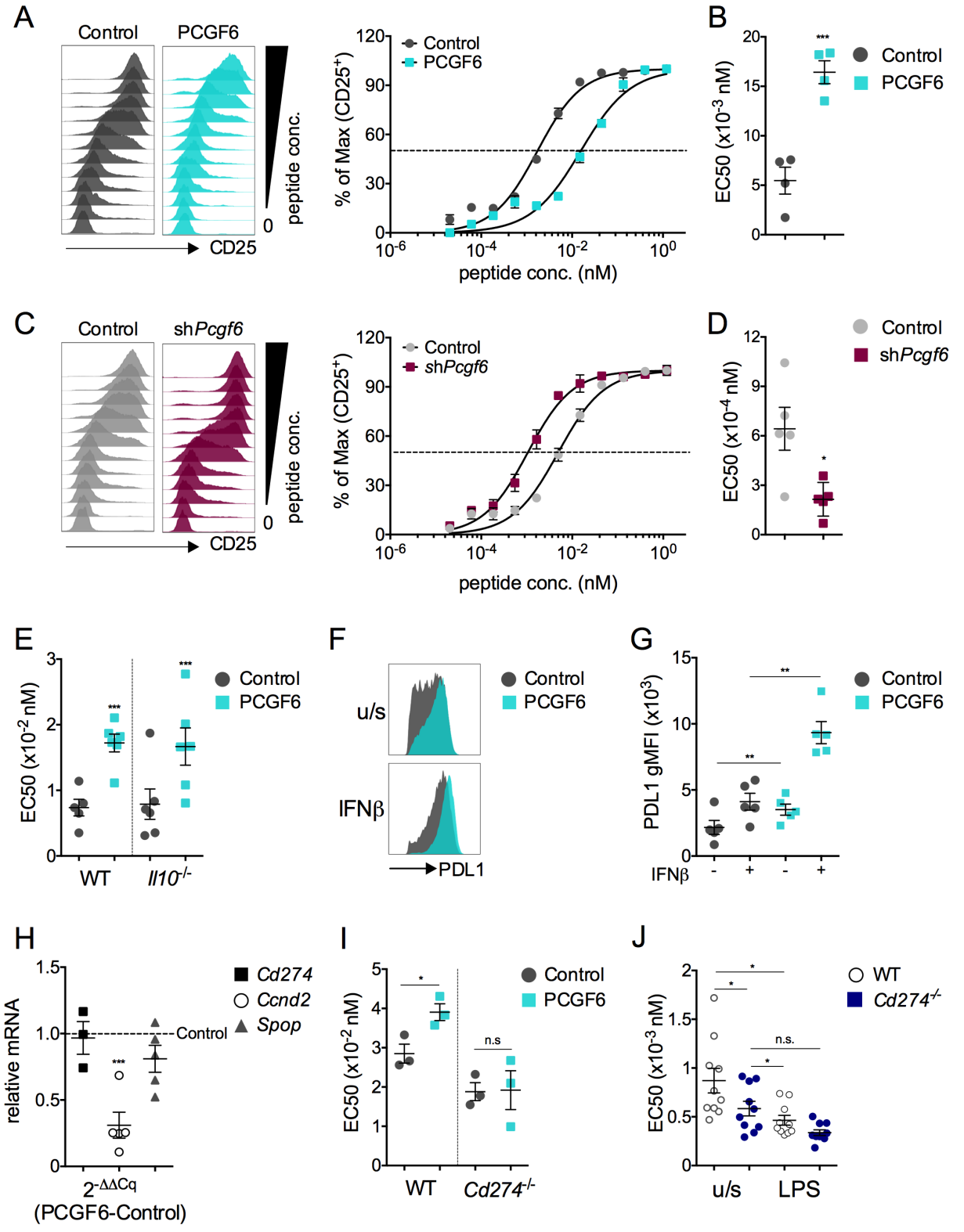


Figure 1. PCGF6 in DCs controls the antigen sensitivity of CD8$^+$ T cells during priming, through PDL1. DCs transduced with a control vector (Control) or a vector to over-express PCGF6 (PCGF6) or reduce the expression of PCGF6 (sh*Pcgf6*) were pulsed with titrated SIINFEKL peptide and co-cultured with purified CD8$^+$ OTI T cells. T cell activation was measured by flow cytometry, three days later. A, C. Representative histograms of CD25 expression (left panel), and sigmoidal dose response curve of the percent of max CD25$^+$ (right panel). B, D. EC50 value of dose response curves. Each point represents a single biological replicate, data are pooled from four independent experiments. * $p<0.05$, ** $p<0.005$, Student's t-test. E. Antigen sensitivity of T cells activated by transduced IL-10 deficient DCs (*Il10*$^{-/-}$) was measured and resulting EC50 values reported. PDL1 expression on unstimulated (u/s) or IFNβ-stimulated (1000U/mL) transduced DCs was measured by flow cytomentry. F. Histograms representative of >5 independent experiments. G. PDL1 geometric MFI (gMFI). Each point is a biological replicate; data are pooled from 5 independent experiments. ** $p<0.005$, One-way ANOVA. H. Relative expression of indicated genes in PCGF6 over-expressing DCs relative to control. Data are normalized to the reference gene *Hprt*. *** $p<0.0005$, Student's t-test. I EC50 values of T cells activated by transduced DCs deficient in PDL1. Each point represents a single biological replicate pooled from three independent experiments. I. EC50 values of T cells activated by PDL1 deficient (PDL1$^{-/-}$) or wild-type (WT) DCs either unstimulated (u/s) or stimulated with 5ng/mL LPS. Each point represents a single biological replicate pooled from 4 independent experiments. * $p<0.05$, One-way ANOVA.

We had previously shown that DCs over-expressing PCGF6 produce more IL-10 than control DCs upon activation (Boukhaled et al., 2016). Chronic infection can lead to a severe decrease in effector CD8$^+$ T cell antigen sensitivity in an IL-10 dependent manner (Smith et al., 2018). To determine whether IL-10 production by DCs mediates the decrease in T cell antigen sensitivity during priming, we measured the antigen sensitivity of T cells following co-culture with IL-10 deficient DCs that either over-express PCGF6 or a control vector (Fig. 1E). PCGF6 over-expression did not require IL-10 to decrease antigen sensitivity. Furthermore, unlike for effector CD8$^+$ T cells, IL-10 does not regulate antigen sensitivity of naïve cells since both WT and IL-10 deficient DCs result in equal EC50 values (Fig. 1E).

We hypothesized that PCGF6 promotes the expression of other factors that mediate immune suppression. For example, programmed cell death ligand 1 (PDL1), is one of two known ligands for the receptor, programmed cell death 1 (PD-1). PD-1 induces T cell exhaustion by inhibiting TCR and CD28 signaling (Hui et al. 2017). We measured

PDL1 expression in unstimulated and IFNβ stimulated DCs that overexpress PCGF6 or a control vector. PCGF6 overexpression led to an increase in cell surface PDL1 both at steady state and with type I IFN stimulation (Fig. 1F, G). However, PCGF6 deficiency had little effect on PDL1 expression (Fig. S1C).

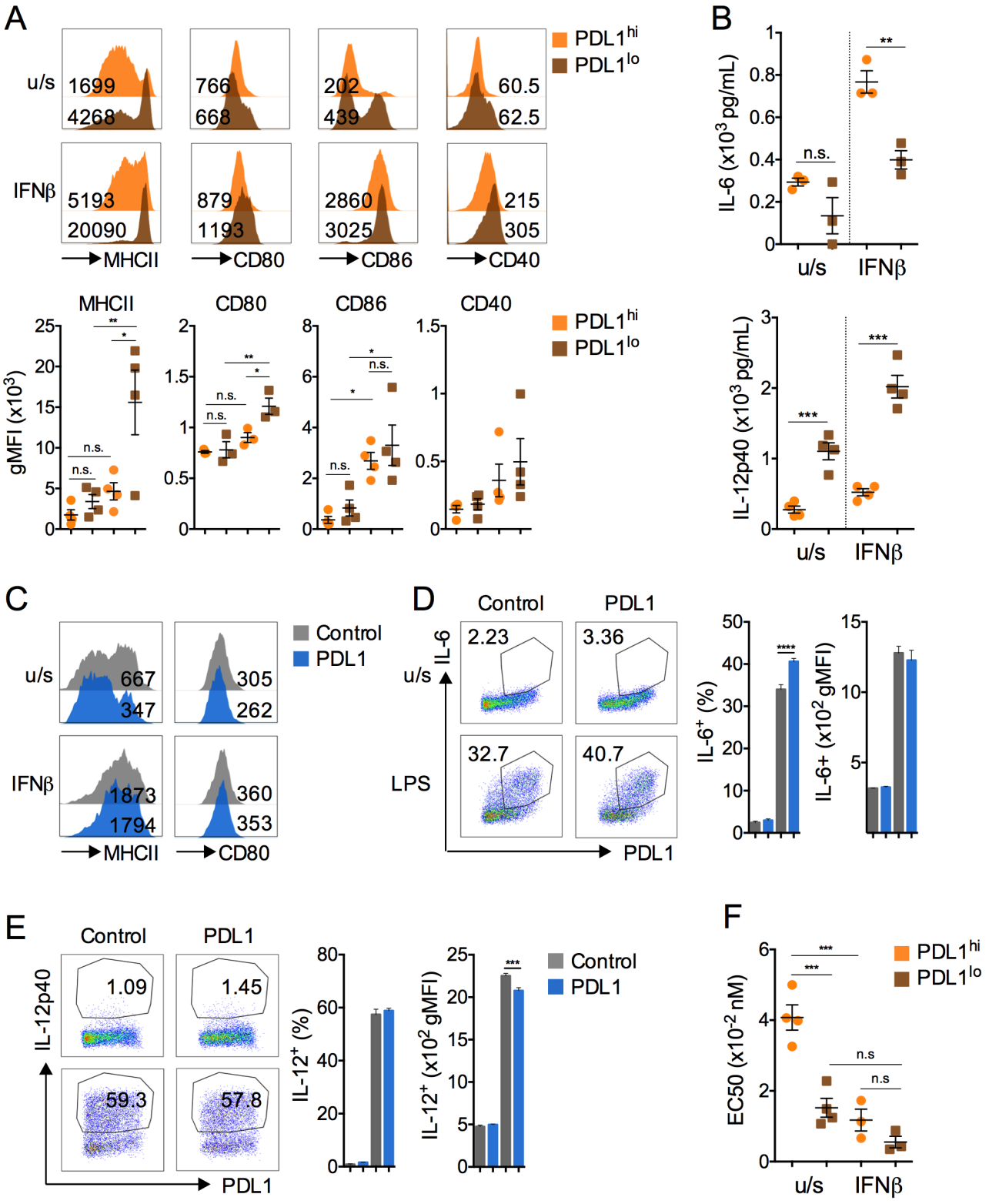
Given its canonical role as a transcriptional repressor, we sought to determine how PCGF6 overexpression results in an increase in PDL1 expression. We found no difference in PDL1 expression at the mRNA level in DCs that overexpress PCGF6, compared to controls (Fig. 1H). Therefore, we hypothesized the PCGF6 may improve the stability of PDL1 protein at the cell surface. Cyclin D2 (CCND2) can destabilize PDL1 protein by activating the E3 Ubiquitin ligase SPOP, which targets PDL1 for degradation (Zhang et al., 2017). We found that PCGF6 overexpression led to a significant decrease in *Ccnd2* expression and a trend towards decreased *Spop* expression compared to control (Fig. 1H). Therefore, PCGF6 may indirectly stabilize PDL1 protein levels through regulating *Ccnd2* expression.

We then sought to determine whether PDL1 expressed by DCs plays a role in regulating the antigen sensitivity of $CD8^+$ T cells. We found that the PCGF6-mediated decrease in $CD8^+$ T cell antigen sensitivity was dependent on PDL1 expression in DCs (Fig. 1I). Furthermore, PDL1 deficiency alone at steady state is sufficient to significantly increase antigen sensitivity (Fig. 1J). LPS stimulation of wild-type DCs significantly increased T cell antigen sensitivity compared to unstimulated DCs (Figure 1J), PDL1 deficiency did not further increase antigen sensitivity induced by LPS-stimulated DCs (Fig. 1J). Therefore, at steady state, PDL1 expression by DCs regulates antigen

sensitivity of T cells. However, once DCs are stimulated with LPS, PDL1 has no impact on antigen sensitivity of T cells, despite its increased expression.

PDL1 intrinsically regulates DC function

It is paradoxical that PDL1 expression is increased on LPS-matured DCs since these matured DCs improve their capacity to prime T cell responses. To determine if PDL1 intrinsically regulates DC function we compared $PDL1^{hi}$ and $PDL1^{lo}$ cells *in vitro* BMDC cultures. Unstimulated cells expressing higher levels of PDL1 show a strong trend to express lower levels of MHCII and costimulatory markers such as CD80 and CD86. CD40 expression was not affected by PDL1 status (Fig. 2A). Treating DCs with IFNβ lead to a significant increase in CD86 for both the $PDL1^{hi}$ and $PDL1^{lo}$ compartments, however $PDL1^{lo}$ cells more readily upregulate MHCII and CD80 in response to stimulation than the $PDL1^{hi}$ (Fig. 2A, bottom panel). This suggests that $PDL1^{hi}$ and $PDL1^{lo}$ DCs respond differently to identical stimuli. To test this further, $PDL1^{hi}$ and $PDL1^{lo}$ cells were sorted by FACS and treated with IFNβ for 18h. $PDL1^{hi}$ cells produced more IL-6 than $PDL1^{lo}$ cells in response to IFNβ. On the other hand, $PDL1^{lo}$ cells produced more IL-12p40 upon stimulation than $PDL1^{hi}$ and also spontaneously produced IL-12p40 even without stimulation (Fig. 2B).


A
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CD86
CD40
PDL1hi
PDL1lo
gMFI (x10³)
B
IL-6 (x10³ pg/mL)
IL-12p40 (x10³ pg/mL)
n.s.
u/s
IFNβ
C
Control
PDL1
667
347
305
262
1873
1794
360
353
D
IL-6
LPS
2.23
3.36
32.7
40.7
IL-6⁺ (%)
IL-6+ (x10² gMFI)
E
IL-12p40
1.09
1.45
59.3
57.8
IL-12⁺ (%)
IL-12⁺ (x10² gMFI)
F
EC50 (x10⁻² nM)
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Figure 2. PDL1 intrinsically regulates DC function. DCs were stimulated with 1000 U/mL of IFNb or left unstimulated (u/s) for 18h. Expression of MHCII, CD80, CD86 and CD40 were measured by flow cytometry. A. Plots show activation markers for $CD11c^{+}Gr\text{-}1^{-}PDL1^{hi}$ and $CD11c^{+}Gr\text{-}1^{-}PDL1^{lo}$ cells. Numbers indicate the corresponding gMFIs (top panel). gMFIs are graphed (bottom panel), each data point representing a single biological replicate. Data are pooled from 4 independent experiments. * $p<0.05$, ** $p<0.005$, One-way ANOVA. B. $PDL1^{hi}$ and $PDL1^{lo}$ cells were sorted by FACS and treated with 1000U/mL of IFNβ or left untreated (u/s) for 18h. IL-6 (left panel) and IL-12p40 (right panel) production were measured in the supernatants by ELISA. ** $p<0.005$, *** $p<0.0005$, **** $p<0.0005$, One-way ANOVA. C. DCs were transduced to over-express PDL1 or control. C. Transduced DCs were treated as in A. Representative histograms of $CD11c^{+}Gr\text{-}1^{-}reporter^{+}$ DCs are shown. Data are representative of 3 independent experiments. Transduced DCs were stimulated with 10ng/mL of LPS for 6h; brefreldin A was added for the last 2h of treatment. Cytokine production was measured by ICS. D, E. Representative plots show expression of IL-6 and IL-12p40 by PDL1 in $CD11c^{+}Gr\text{-}1^{-}reporter^{+}$ cells. Inset numbers indicate the percent of cells expressing IL-6 or IL-12p40. Graphs show averaged triplicates of the percent $cytokine^{+}$ and the gMFI. Data are representative of 2 experiments. ** $p<0.005$, *** $p<0.0005$, One-way ANOVA. F. EC50 value of T cells activated by DCs sorted as in B and stimulated with 50 U/mL IFNβ or not (u/s). Each point represents a single biological replicate. Data are pooed from 4 independent experiments. ** $p<0.005$, n.s., not significant, One-way ANOVA.

Though not well studied, PDL1 has the capacity for retrograde signaling through its cytoplasmic domain (Brogden et al., 2016; Gato-Cañas et al., 2017). This retrograde signalling has been shown to protect cancer cells from IFNɣ-mediated killing (Gato-Cañas et al., 2017). To determine if PDL1 itself promotes suppression of DCs at steady state we overexpressed PDL1 in DCs and measured MHCII and CD80 expression. We found that MHCII expression was lower on DCs overexpressing PDL1 compared to controls both at steady-state and in response to LPS (Fig. 2C, S2A). Furthermore, overexpression of PDL1 led to a significant increase in the production of IL-6 and a modest but significant decrease in the expression (gMFI) of IL-12 per cell (Fig. 2 D, E). PD-1 is the most widely recognized receptor for PDL1, although reports indicate that it can also interact with CD80 (Butte et al., 2007). Interestingly, PD-1 is not appreciably expressed by BMDCs, suggesting that the effect of PDL1 over-expression on DCs is independent of PD-1 (Fig. S2B, C).

We hypothesized that differential cytokine production, particularly the diminished IL-12 production by PDL1^{hi} and PDL1^{lo} cells, might influence the antigen sensitivity in response to type I IFNs. While the antigen sensitivity induced by PDL1^{hi} cells was lower compared to PDL1^{lo}, IFNβ-stimulation of DCs resulted in similar EC50 values (Fig. 2F). The difference in antigen sensitivity is not attributed to differences in MHCI expression given that MHCI was in fact higher on PDL1^{hi} cells compared to PDL1^{lo} (Fig. S2D, E). Furthermore, there was no difference in the expression of markers *Mertk* (macrophages) or *Zbtb46* (DCs) between the two subpopulations of cells. This suggests that macrophage contamination of our cultures likely does not disproportionately contribute to the behaviour of either population (Fig. S2F).

PCGF6 governs early interactions of DCs and T cells

Priming of T cells by DCs occurs in multiple phases of interaction of varying stability (Mempel et al., 2004; Scholer et al., 2008). Interactions of increasing stability over the course of priming dictate the priming efficiency with more stable interactions favouring memory formation (Mempel et al., 2004; Scholer et al., 2008). One study demonstrated that the interaction of PDL1 and PD-1 during priming limits stable interactions between DCs and T cells leading to reduced effector T cell function (Fife et al., 2009). Since PCGF6 downregulation is required for optimal DC activation and triggers an increase in T cell antigen sensitivity, we wanted to determine whether PCGF6 downregulation promotes more stable DC:T cell interactions. To test this, DCs were labelled with violet proliferation dye, pulsed with peptide and co-cultured with CFSE-labelled OTI T cells at a 1:1 ratio. 12 min later DCs interacting stably with T cells (conjugates) were measured by flow cytometry by gating on co-labelled events (Fig. 3A). We found that overexpression

of PCGF6 destabilized early interactions of DCs and T cells (Fig. 3A, B) while PCGF6 downregulation favoured the interaction of DCs and T cells (Fig. 3C, D). Importantly, reporter$^-$ cells serve as an internal control for cells with normal expression of PCGF6. Interaction of reporter$^-$ DCs with T cells is strongly favoured compared to the reporter$^+$ PCGF6 over-expressing DCs (Fig. 3A, B). This indicates that when permitted to compete for binding, T cells prefer to interact with cells expressing lower levels of PCGF6.

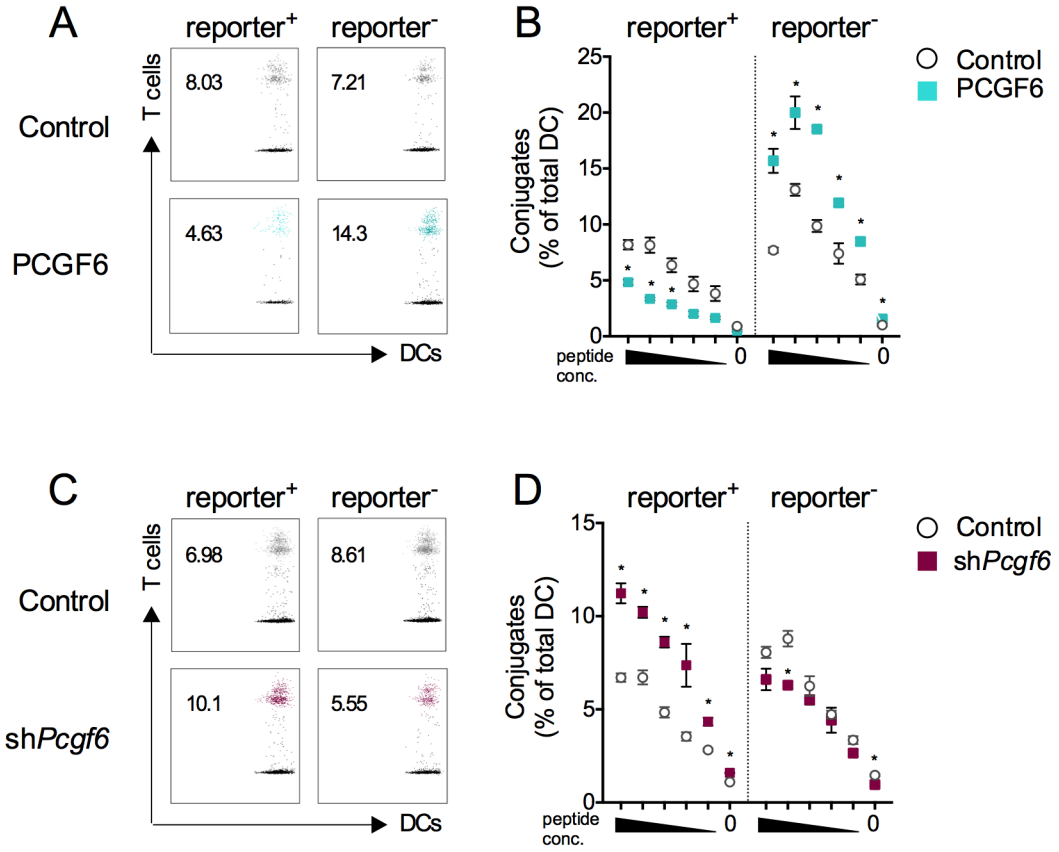

Figure 3. PCGF6 governs early interactions of DCs and T cells. Transduced DCs were labelled with Violet Proliferation Dye (VPD), pulsed with titrated SIINFEKL peptide and co-cultured with purified CFSE-labelled CD8$^+$ T cells at a 1:1 ratio. Conjugate formation was measured 12 min later by flow cytometry by measuring the percent of VPD and CFSE double positive cells. A. Plots show CD11c$^+$reporter$^+$ or reporter$^-$ cells labelled with VPD (DCs) interacting with CFSE-labelled T cells (T cells). Numbers indicate the reporter$^+$ or reporter$^-$ DCs forming conjugates as a percent of the total DCs. B, C. Quantification of conjugate formation by transduced DCs. Points are the average of triplicates from an independent experiment, representative of three independent experiments. Error bars represent standard error of the mean.

PDL1 does not require PCGF6 to suppress DCs

We found that PCGF6 can stabilize PDL1 expression, therefore we examined whether PDL1^{hi} cells reciprocally require PCGF6 to mediate suppression of DCs. Surprisingly, we found that PDL1^{hi} cells express lower levels of *Pcgf6* mRNA compared to PDL1^{lo} (Fig. 4A). To determine whether the low levels of PCGF6 in PDL1^{hi} cells still contribute to the maintenance of their homeostasis, we characterized the PDL1^{hi} and PDL1^{lo} cells in PCGF6 deficient or over-expressing DCs. Consistent with previous data (Fig. 2A) cells with low PDL1 expressed higher levels of MHCII and CD86 (Fig 4B-C, S3). As previously reported, loss of PCGF6 led to a significant increase in steady state levels of MHCII and CD86 (Fig. 4B, S4A).

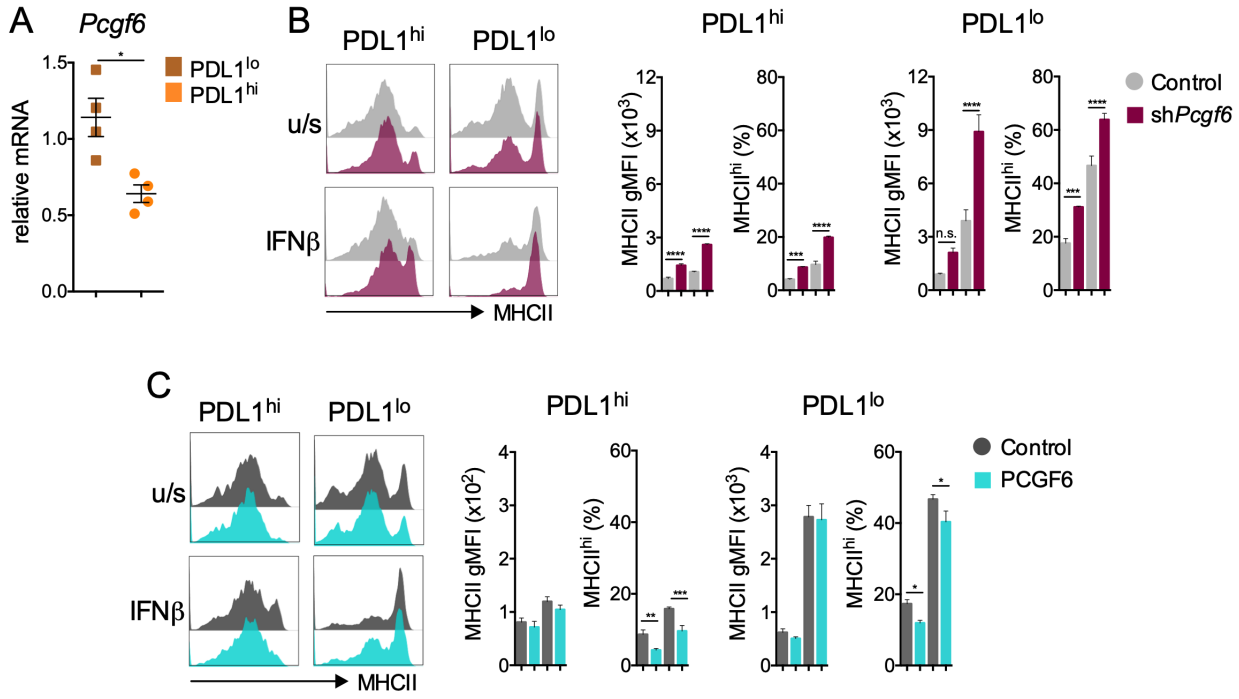

Figure 4. PDL1 does not require PCGF6 to suppress DCs. A. *Pcgf6* mRNA expression was measured by qRT-PCR in sorted PDL1^{lo} and PDL1^{hi} cells. Each point represents an individual biological replicate and data are pooled from 4 independent experiments. * p<0.05, Student's t-test. B, C. Transduced DCs were treated with IFNb (1000 U/mL) for 18h. Histograms show MHCII expression on CD11c^{+}Gr-1^{-}reporter^{+} PDL1^{hi} or PDL1^{lo} cells. The average triplicate gMFI and % MHCII^{hi} from one representative experiment is graphed. * p<0.05, ** p<0.005, *** p<0.0005, One-way ANOVA.

PCGF6 deficiency profoundly enhanced IFNβ-stimulated expression of MHCII and CD86 in $PDL1^{lo}$ cells (Fig. 4B, S3A, S3B). However, the effect of decreasing PCGF6 expression only modestly increased expression of activation markers in $PDL1^{hi}$ cells compared to $PDL1^{lo}$ cells (Fig. 4B, S3A, S3B). The fold increase in MHCII expression on PCGF6 deficient DCs compared to control was similar for both $PDL1^{hi}$ and $PDL1^{lo}$ cells (Fig. S3C). However, the fold increase of CD86 on $PDL1^{lo}$ PCGF6 deficient DCs is higher compared to $PDL1^{hi}$ (Fig. S3D). Interestingly, overexpression of PCGF6 in cells that express high levels of PDL1 led to further suppression of MHCII, suggesting that the mechanisms by which PCGF6 and PDL1 control suppression of DC function are additive (Fig. 4C).

cDC1s maintain steady state through PCGF6

To determine whether PCGF6 in *in vivo*-derived DCs plays a similar role in regulating T cell antigen sensitivity, we transduced fetal liver cells with the hairpin construct targeting PCGF6, or a control vector. We then transferred the transduced cells into lethally irradiated hosts. Similar proportions of cells among different DC subsets expressed the human CD8 reporter (Fig. S4A) Following successful reconstitution, we sorted the splenic DCs by pan-DC negative selection (MACS), and performed the antigen sensitivity experiments. We found that PCGF6 deficiency in the splenic DC compartment recapitulated the results from *in vitro*-derived DC cultures; PCGF6 deficiency significantly increased T cell antigen sensitivity (Fig. S4B).

Recent evidence indicates that cDC1s and cDC2s employ separate mechanisms to maintain homeostasis and respond to stimuli, however crosstalk between the two also influences their function (Gargaro et al., 2018; Macdougall et al., 2018). For example, in

the visceral adipose tissue of mice of a healthy weight, cDC2s engage PPARɣ signaling and cDC1s engage Wnt5a signaling in order to establish an anti-inflammatory microenvironment (Macdougall et al., 2018). Additionally, it is thought that cDC1s preferentially cross-present antigen to $CD8^+$ T cells while cDC2s preferentially activate $CD4^+$ T cells (den Haan et al., 2000; Hildner et al., 2008). Other studies have shown that targeting antigen to specific endocytic pathways leads to equally efficient cross-presentation for both cDC1s and cDC2s (Kamphorst et al., 2010). However, whether cDC1s and cDC2s have a differential effect on the antigen sensitivity of $CD8^+$ T cells has not been reported. We sorted cDC1s and cDC2s based on the expression of XCR1 (cDC1) and CD172α (cDC2) in the DC compartment (Fig. 5A, S4C). Despite their established role in antigen cross-presentation, we found that T cells stimulated by cDC1s were less sensitive to antigen than T cells stimulated by cDC2s (Fig. 5B, C). Intriguingly, LPS-stimulated cDC1s but not cDC2s, significantly increased T cell antigen sensitivity compared to steady state (Fig. 5B, C).

In contrast to our earlier results demonstrating that PDL1 on DCs regulates antigen sensitivity at steady state, cDC2s expressed more PDL1 at steady state than cDC1s (Fig. 5D). However, upon LPS stimulation cDC1s upregulated PDL1 to a greater extent than cDC2s (Fig 5D). Furthermore, while interacting with antigen-specific T cells, both cDC1s and cDC2s increase PDL1 expression in an antigen dependent manner. (Fig. 5E, F). Because T cells can also express PDL1 we examined whether the increase in PDL1 in conjugates was contributed by T cells. Co-culture of antigen-specific T cells with PDL1 deficient DCs demonstrated that PDL1 is also significantly induced on T cells, early after co-culture (Fig. 5F). Calculating the difference in PDL1 gMFI between $PDL1^{-/-}$ DCs and

WT DCs shows the overall contribution of DCs to the total expression of PDL1 is equivalent between cDC1s and cDC2s (Fig. S4D). Therefore, PDL1 may have an equal contribution in determining the priming capacity of cDC1s and cDC2s.

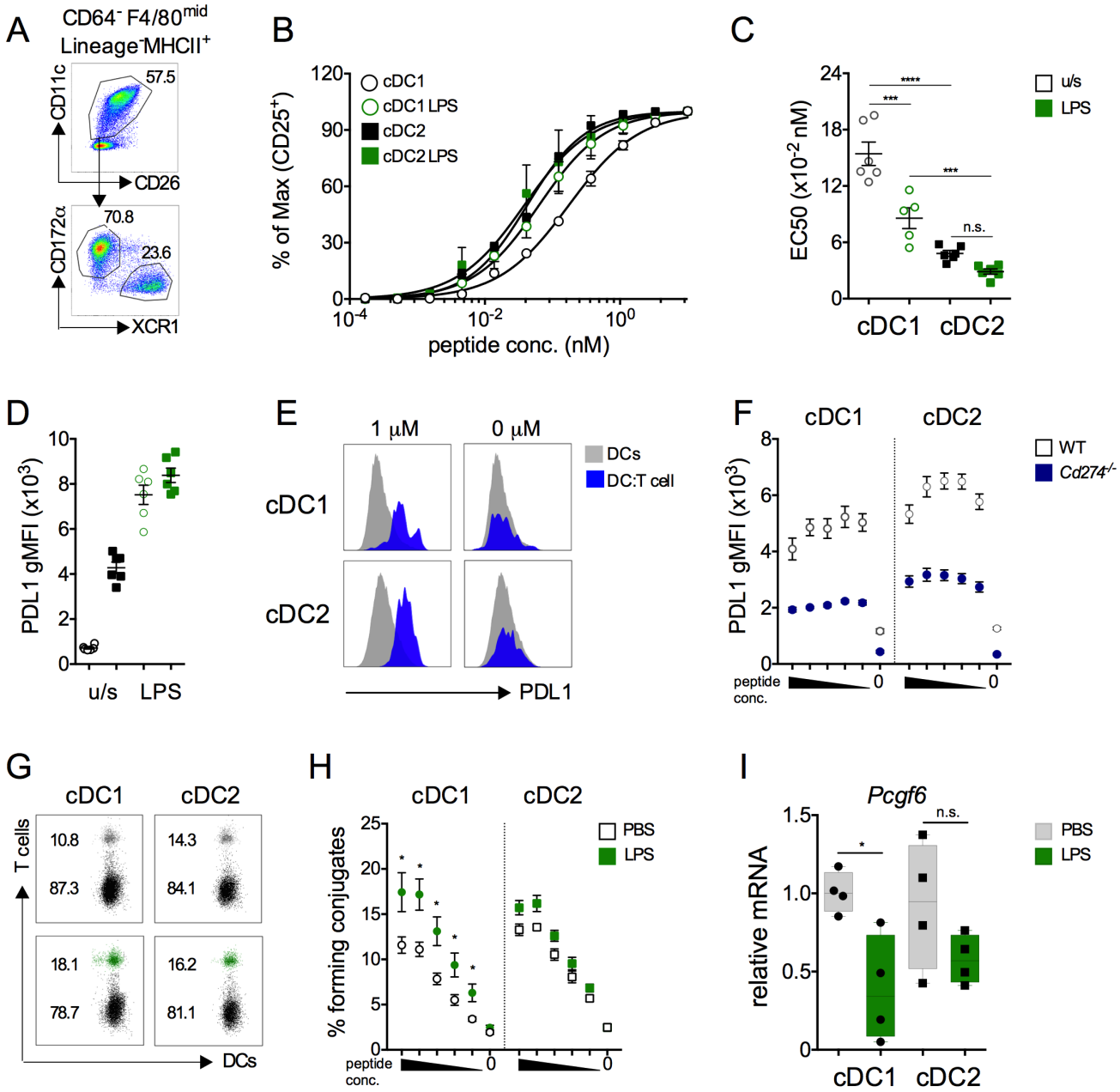

Figure 5. cDC1s maintain steady state through PCGF6. C57Bl/6 mice were injected with 2µg LPS or PBS. 2h later, Splenic cDCs were sorted by FACS and pulsed with titrated doses of SIINFEKL peptide. T cell antigen sensitivity was determined as described in Figure 1. A. Representative gating strategy for FACS sorting and phenotyping. B. Representative sigmoidal dose response curves. Data are the average of 3 biological replicates and error bars represent standard error of the mean. C. EC50 values corresponding to B. Data are pooled from two independent experiments. *** $p<0.0005$, n.s., not significant, One-way ANOVA. D. PDL1 gMFI measured by flow cytometry in cDCs from mice injected with LPS or PBS. Data are pooled from two independent experiments, representative of >3 experiments. E, F. Splenic DCs were sorted by MACS negative selection and labelled with VPD. DCs were co-cultured at a 1:1 ratio with purified CFSE-labelled $CD8^+$ T cells. 14h later, PDL1 expression was measured on DCs interacting with T cells. E. Histogram of PDL1 expression on conjugates from cDC1s or cDC2s, classified according to A. Data are representative of >3 experiments. F. Average PDL1 gMFI induced on conjugates formed by wild-type DCs (WT) and PDL1 deficient DCs (*Cd274*$^{-/-}$) DCs. Shown is the average of 6 biological replicates pooled from 2 independent experiments. G. Plots show conjugate formation of splenic cDCs 12 min after co-culture with $CD8^+$ T cells. H. Average conjugate formation by splenic cDCs from 9 biological replicates pooled from 3 independent experiments. Significance relative to PBS control, * $p<0.05$, t-test. I. *Pcgf6* expression in sorted splenic cDCs, measured by RT-qPCR. Each point represents a single biological replicate. Data are pooled from two independent experiments. * $p<0.05$, One-way ANOVA.

To determine whether the increased antigen sensitivity induced by cDC1s with activation was related to an increase in early interactions of DCs with T cells, we measured conjugate formation. We found that LPS-stimulated cDC1s but not cDC2s significantly increase their stable conjugate formation (Fig. 5G, H). LPS injection also significantly downregulated *Pcgf6* mRNA in cDC1s but not in cDC2s (Fig. 5I). These data suggest that PCGF6 levels in cDC1s versus cDC2s correlates with antigen sensitivity and that downregulation of PCGF6 in response to LPS facilitates the increase in antigen sensitivity observed in T cells primed by cDC1s.

PD-1 induced by steady-state DCs regulates T cell antigen sensitivity

These data suggest that PCGF6 downregulation in response to activating stimuli could counterbalance the elevated expression of PDL1 to elicit an increase in T cell antigen sensitivity. In order to determine whether the regulation of antigen sensitivity by

PDL1 at steady state is dependent on PD-1 expression on T cells we measured the antigen sensitivity of PD-1 deficient T cells activated by WT or PDL1 deficient DCs. Antigen sensitivity of PD-1 deficient T cells was equal when primed by either WT DCs or PDL1 deficient DCs at steady state. Therefore, despite direct regulation of DC activation, expression of PD-1 on T cells is required to decrease antigen sensitivity. (Fig. 6A). These data suggest PD-1 dependent and independent roles for PDL1 in mediating DC function. PD-1 is known to increase briefly during the early phases of T cell activation, but it is then downregulated and only becomes highly expressed days later, in response to chronic antigen stimulation (Agata et al., 1996; Staron et al. 2014). However, we found that PDL1 does not significantly affect the antigen sensitivity of T cells primed by LPS-stimulated DCs. We therefore determined whether the expression of PD-1 induced on T cells is different when T cells are primed by steady state, or LPS-stimulated DCs (Fig. 6B). We found that PD-1 increases on T cells in an antigen dose-dependent manner in cells stimulated by both LPS-activated or steady state DCs. However, there was a striking increase in PD-1 expression on as high as 80% of T cells stimulated by steady-state DCs. Therefore, the dependence of PDL1 on antigen sensitivity at steady state is due to the increased levels PD-1 expression on T cells stimulated by DCs at steady state.

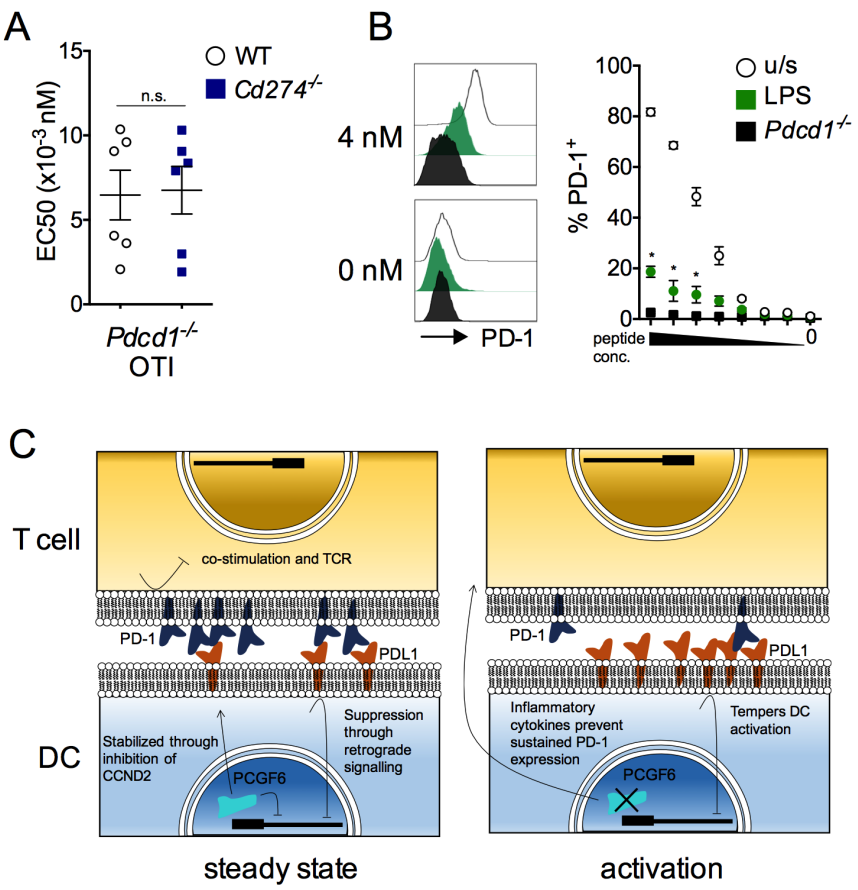

Figure 6. PD-1 induced by steady-state DCs regulates T cell antigen sensitivity. A. EC50 values of PD-1 deficient (*Pdcd1*$^{-/-}$) OTI T cells activated by either wilde-type (WT) or PDL1-deficient (*Cd274*$^{-/-}$) DCs. Each point is one biological replicate, data are pooled from 2 independent experiments. n.s., not significant, Student's t-test. B. PD-1 expression was measured on T cells activated by unstimulated (u/s) or LPS-stimulated (5 ng/mL) DCs pulsed with titrated SIINFEKL peptide. Representative histogram show PD-1 expression on CD8^{+} T cells (left panel). Graph indicates the percent PD-1^{+} averaged from 3 biological replicates. C. Proposed mechanistic model for regulation of T cell priming by PCGF6 and PDL1.

We propose a model by which both PCGF6 and PDL1 expression in DCs affect T cell priming in parallel. At steady state, PCGF6 supports the stabilization of PDL1 protein as well as the suppression of pro-inflammatory genes, which promotes PD-1 expression on T cells. DC activation leads to rapid downregulation of PCGF6, which promotes the induction of inflammation limiting PD-1 upregulation thereby lowering T cell antigen sensitivity (Figure 6C). PDL1 increases upon stimulation, with LPS, but because the T

cells do not upregulate PD-1 to the same extent, PDL1 does not affect antigen sensitivity of T cells.

4.4 Discussion

Here we demonstrate that PCGF6 and PDL1 cooperatively and independently regulate programs of immune suppression in DCs that ultimately modulate the antigen sensitivity of $CD8^+$ T cells. This work contributes novel mechanisms by which DCs fine-tune their responses to stimuli such as type I IFNs and LPS, as well as the factors that regulate their interactions with T cells at steady-state.

Chronic antigen stimulation, for example as in chronic infection and cancer, leads to T cell dysfunction which exacerbates disease. T cell dysfunction occurs in part due to the emergence of a regulatory DC subset that is impaired in its capacity to prime T cell responses (Cunningham et al., 2016; Osokine et al., 2014). Interestingly, type I and type II IFNs drive the formation of these regulatory DCs (Cunningham et al., 2016). Understanding the molecular underpinnings of dichotomous DC responses is crucial to develop appropriate therapeutic strategies. Intrinsic and extrinsic factors contribute to the fate decisions DCs make; for example, types and timing of cytokines, subset specification and epigenetic programming can all contribute to the gene signatures that dictate DC responses (Huang et al., 2012; Kerkar et al., 2014; Norris et al., 2013). Importantly, epigenetic programming can be dynamically modulated by environmental factors, representing an important mechanism by which DCs can commit context-specific information (Huang et al., 2012; Kaufmann et al., 2018). We demonstrated that the transcriptional repressor PCGF6 in DCs favours PDL1 expression in response to stimulation with type I IFNs. Changes in steady state levels of PCGF6 not only regulated

their behaviour and priming capacity at steady state, but also modulated their response to stimulation. PCGF6 expression is dynamically regulated by environmental stimuli, however we show that only certain DC subsets were susceptible to regulation by PCGF6. It is possible that PCGF6 programs regulatory immune responses in susceptible DC populations.

Another important finding that emerged from this study is that PDL1 can regulate aspects of DC biology even in the absence of PD-1. PD-1 was not significantly expressed by DCs, and yet PDL1 overexpression was sufficient to promote IL-6 production upon stimulation and to suppress the expression of CD80 and MHCII. Furthermore, PCGF6 maintained homeostasis of $PDL1^{lo}$ cells while $PDL1^{hi}$ cells have only a modest requirement for PCGF6. These data suggest that PDL1 can control the steady state of DCs independently of PD-1 signaling. Studies demonstrate that PDL1 has a cytoplasmic signaling domain, where it can inhibit mTOR and signal through STAT3 (Gato-Cañas et al., 2017; Zhao et al., 2017). PDL1 protects cancer cells from the toxicity of IFNɣ (Gato-Cañas et al., 2017). Intriguingly PDL1 interacts with CD80; in fact, CD80 competes for binding with PD-1 (Butte et al., 2007). A recent study observed that PDL1 and CD80 can only interact in cis (Chaudhri et al., 2018). Therefore, it is possible that through cis-interactions of PDL1 and CD80, PDL1 signaling can contribute to the maintenance of DC quiescence.

PD-1 was negligibly expressed on DCs; T cells however upregulated PD-1 in an antigen dose-dependent manner. PD-1 is known to increase on naïve T cells during priming and its upregulation correlates with TCR signal strength (Agata et al., 1996). Interestingly PD-1 expression is associated with effector function early on, however

sustained expression contributes to T cell exhaustion and dysfunction (Staron et al., 2014). In our study, elevated PD-1 expression on T cells primed by steady state DCs lead to decreased T cell antigen sensitivity. T cell antigen sensitivity also correlated with the stability of early interactions between DCs and T cells. The first phase of interaction between DCs and T cells during priming is thought to be the phase where T cells detect antigen dose (Mempel et al., 2004; Ozga et al., 2016). Very strong TCR stimulation causes T cells to spend a longer time in the lymph node interacting with DCs, which favours memory formation (Ozga et al., 2016; Scholer et al., 2008). During chronic infection, PD-1 blockade reinvigorates T cells sequestered in secondary lymphoid organs (Freeman et al., 2006). Conflicting reports have ascribed opposite roles for PD-1 in regulating the stability of interactions between DCs and T cells (Fife et al., 2009; Honda et al., 2014). Our study suggests that DCs may deliver a signal during priming to either sustain or delay the expression of PD-1 which in turn impacts their interaction behaviour and the sensitivity of the ensuing T cell response. Interestingly, T cells primed by steady state DCs bearing high doses of antigen were not affected in the magnitude of CD25 upregulation compared to T cells primed by LPS-stimulated DCs. This emphasizes that the role of PD-1 during priming is setting a threshold for T cell activation rather than affecting the magnitude of activation.

PDL1 played a central role in regulating the antigen sensitivity of naïve T cells. IL-10 however played no role. A recent study established that elevated levels of IL-10 during chronic infection decreases the antigen sensitivity of effector $CD8^+$ T cells (Smith et al., 2018). This suggests that antigen sensitivity is regulated by separate mechanisms for naïve and effector T cells. It is also possible that IL-10 would play a role if antigen

processing were taken into account. Whether the separate roles of PDL1 and IL-10 represent different mechanisms to regulate naïve and effector T cells or whether it is due to the use of processed antigen warrants further study.

Regulation of naïve T cell antigen sensitivity is necessarily complex, with several factors including PDL1 and stability of early DC interactions with T cells playing a role. We discovered that PCGF6 regulates the antigen sensitivity of naive T cells and the early interactions of DCs and T cells. Interestingly, cDC1s, but not cDC2s, downregulated PCGF6, increased antigen sensitivity and increased their interactions with T cells in response to LPS. Therefore, it is possible that PCGF6 specifically maintains the homeostasis of cDC1s. This is consistent with studies that demonstrate separate mechanisms employed by cDC1s and cDC2s to maintain homeostasis (Macdougall et al., 2018; Meredith et al., 2012). Paradoxically, steady state cDC2s expressed higher levels of PDL1 than cDC1s but induced increased T cell antigen sensitivity. Similarly, PCGF6 deficiency increased T cell antigen sensitivity compared to control DCs, despite no difference in PDL1 expression. Therefore, the steady-state expression of PDL1 is not the only determinant of naïve T cell antigen sensitivity. Consistent with this observation, DCs matured by LPS or type I IFNs both increased the antigen sensitivity of T cells. The role of PDL1 in naive T cell antigen sensitivity likely depends on availability of its receptor PD-1. Other factors such as timing of initial encounter also likely plays a role in not only the antigen sensitivity but also the functionality of the resulting T cell response. For example, $CD4^+$ T cells primed during chronic infection differentiate into T_{fh} cells at the expense of T_h1, exacerbating T cell dysfunction (Snell et al., 2016). Interestingly, a recent study demonstrated that after priming T cells require a fourth signal from monocyte-

derived DCs to maintain their survival and promote T cell accumulation (Chang et al., 2017). This highlights the importance of context and timing for determining the quality of a T cell response.

4.5 Contributions

Conceptualization, C.M.K., G.M.B.; Methodology, C.M.K., G.M.B..; Validation, G.M.B. H.G.; Formal Analysis, G.M.B..; Investigation, G.M.B., H.G., L.K.S.; Resources, D.G.B, H.E.; Data Curation, G.M.B.; Writing- original draft, C.M.K., G.M.B.; Writing – Review and editing, C.M.K., G.M.B., H.G.; Visualization, C.M.K., G.M.B.; Supervision, C.M.K., M.J.R., D.G.B.; Project administration, C.M.K., G.M.B.; Funding acquisition, C.M.K

4.6 Experimental Procedures

Mice and reagents

C57Bl/6 mice were obtained from Charles River Laboratories. *Pdcd1*$^{-/-}$ (Jackson stock 028276) mice were obtained from Jackson Laboratories and bred in-house. *Cd274*$^{-/-}$ mice were obtained from Genentech and bred in-house. OTI (Jackson stock 003831) mice were generously provided by Dr. Martin J Richer, and bred in house. Animals were treated according to protocols approved by McGill's Animal Care Committee in agreement with standards set by the Canadian Council on Animal Care. Tissue culture: RPMI, β-mercaptoethanol, non-essential amino acids (NEAA, 100X) and Fetal Calf Serum, NuSerum IV (Corning). DMEM, HEPES, Iscove's DMEM, L-Glutamine (100X), Penicillin/streptomycin (100X), Phosphate buffered saline (PBS) and Hank's balanced salt solution (HBSS) (Wisent). Lipofectamine 2000 (Invitrogen). Cytokines: GM-CSF, IL-3, Stem Cell Factor (SCF), IL6 (Peprotech), IFNβ (R&D Systems). LPS (Sigma, *E. coli* O111:B4). Commercial kits and assays: Ready-set-go ELISA kits: IL-12p40 and IL-6

(eBioscience/Fisher). Pan-DC negative selection kit, mouse and PE positive selection kit (Miltenyi Biotec). $CD8^+$ T cell netgative selection kit with RapidSpheres (STEMCELL, Inc.). IC Fixation/Permeabilization buffer (eBioscience/Fisher). ABM 5X All-in-One RT kit and 2X SensiFast SYBR No ROX Mix (Bioline/Froggabio). Enzymes: Collagenase D and DNase I (Sigma-Aldrich). Antibodies: See supplementary Table S1. Primers: See supplementary table S2.

Bone marrow derived dendritic cell culture and transduction

Cells were cultured, transduced and differentiated as described in Boukhaled et al. 2016. Briefly, bone marrow is extracted and seeded at $7.5x10^5$ – $1x10^6$ cells per well in 6-well non-tissue culture treated plates containing 3mL/well of CDCM (10% FCS, 2mM L-Glu, Pen/Strep, 1:1000 β-ME, Non-essential amino acids) supplemented with 20ng/mL of GM-CSF. Cells are cultured for 8-9 days and CDCM+GM-CSF is replenished on days 3, 6 and 8. On day 2 of bone marrow cell culture, media is removed completely from the wells and replaced with 1mL/well of virus-containing supernatant. Cells are spin infected for 1.5 hrs, 2500 rpm, 30°C then virus is immediately removed and replaced with CDCM containing 20ng/mL GM-CSF.

Transfection of 293Ts for virus production

293Ts are plated at $2x10^6$ cells/6cm dish. The next morning the media is replaced with DMEM containing 10% FCS. Lipofectamine, helper (pCL-ECO) and MSCV or LMP-based plasmid mix is prepared in OPTIMEM and added to the 293Ts (1mL/plate). 5-6h later media is replaced with complete DMEM (10% FCS, 2mM L-Glutamine, Pen/Strep). The next day media is replaced with 2mL of complete DMEM for virus collection. Each plate yields 2 mL of high titer virus.

Flow cytometry staining

Cells are washed once with flow wash (PBS, 2% FCS, 2mM EDTA, 0.5% sodium azide). Staining mix is prepared with the appropriate dilutions of antibodies. Cells are typically stained in 96-well plates, at a staining volume of 50 μL/well. Cells are stained for 30-45 min at 4°C. Cells are washed again with flow wash before acquiring.

Antigen sensitivity assay

DCs are pulsed with SIINFEKL peptide for at least one hour and co-cultured with purified $CD8^+$ OTI T cells at a 1:10 ratio. The top concentration of peptide is 4nM and subsequent doses were determined by a 3-fold dilution series. A total of 12 points (including no peptide) are used. T cell activation is measured 3 days later by flow cytometry.

Conjugate assay

DCs are labelled with 1 μM of violet cell proliferation dye for 9 min at 37°C. $1x10^5$-$2x10^5$ labelled cells were seeded per well of a flat, 96-well plate and pulsed for 1-2h with SIINFEKL peptide. Media was removed and replaced with a 1:1 ratio of purified CFSE-labelled $CD8^+$ OTI T cells in complete Iscove's media. Cells were spun quickly to promote conjugate formation for 12 min or 14 hrs at 37°C. Cells are immediately spun down and stained on ice for 15 min. Conjugate formation and PDL1 expression were then measured immediately by flow cytometry.

RNA extraction, RT-qPCR

RNA was extracted according to the Trizol RNA extraction protocol with one modificication; the isopropanol step is carried out at -80°C, overnight. cDNA was prepared

using ABM 5X All-in-One RT kit. qPCR was performed using SYBR-Green (Froggabio). *Hprt* is used as a reference gene unless otherwise indicated.

Splenic cDC treatments and isolation

For LPS studies, 2µg of LPS per mouse were delivered i.v. 2h prior to sacrifice. Spleens were digested with 1 mg/mL collagenase D and 10 µg/mL DNase I for 30 min at 37°C. Spleens were homogenized through a 70 mm filter and thoroughly washed with HBSS. Splenic cDCs were then sorted by negative selection (Pan-DC negative selection kit, mouse, Miltenyi Biotec), according to the protocol provided by the manufacturer.

Generation of fetal liver chimeras

Fetal liver chimeras were generated as described (Schmitt et al., 2002). Briefly, fetal livers were harvested from embryos between 14 and 17 days of gestation. Livers were homogenized and transduced with high titer virus. To transduce fetal liver cells, cells were subjected to 2 rounds of spin infection separated by 6-8 hours on the day of the harvest and another two rounds the next day. Fetal liver cells were cultured with a cocktail of IL-3 (20 ng/mL), IL-6 (2 ng/mL) and SCF (20 ng/mL) and were allowed to recover for about 18h following transduction. Mice were lethally irradiated with 2 doses of 450 rad separated by 4 hours the day prior to reconstitution. Finally, recipients were injected i.v. with approximately $3x10^6$ transduced fetal liver cells. Bone marrow reconstitution was verified by tail bleeds greater than four weeks post-reconstitution. Finally, mice were used approximately three months post-reconstitution.

Statistical Analysis

All statistical analysis was performed in Prism 6.0. Where appropriate, One-Way ANOVA and Student's t-tests were used for analysis. * $p<0.05$, ** $p<0.05$, *** $p<0.005$,

**** $p<0.001$, n.s. not significant. Error bars indicate either the standard deviation or the standard error of the mean as indicated in the figure legends. Antigen sensitivity was calculated by determining the % of maximum $CD25^{+}$. EC50 values were calculated by non-linear regression analysis of a sigmoidal dose-response curves. The points in dose response curves correspond to the actual data and the lines represent the fit of the regression analysis.

4.7 Acknowledgements

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4.9 Supplemental information

Supplemental information inventory

Supplemental Tables 1-2

Table S1. List of antibodies used in flow cytometry experiments

- blue cells indicate antibodies used exclusively for splenic DC staining
- orange cells indicate antibodies used for T cell assays
- green cells indicate antibodies used for intracellular cytokine staining

Table S2. List of primers used for qPCR

Supplemental Figure S1-S4

- Figure S1, related to Figure 1
- Figure S2, related to Figure 2
- Figure S3, related to Figure 3
- Figure S4, related to Figure 5

Supplemental tables

Table S1. List of flow cytometry antibodies used

Antigen/Fluorophore	Fluorophore	Clone	Source
CD26	PerCP/Cy5.5	H194-112	eBioscience/Fisher
XCR1	PE	ZET	BioLegend
CD172α	APC	P84	eBioscience/Fisher
CD3	FITC	17A2	eBioscience/Fisher
B220	FITC, PE	RA3-6B2	eBioscience/Fisher
NK1.1	FITC	PK136	eBioscience/Fisher
CD64	BV711	X54-5/7.1	BioLegend
PDL1	PE/Cy7	10F.9G2	BioLegend
PDL1	PE	MIH5	eBioscience/Fisher
CD86	PECy7, PE	GL1	eBioscience/Fisher
CD80	FITC, PE, APC	16-10A1	eBioscience/Fisher
CD40	APC	1C10	eBioscience/Fisher
human CD8	PE, APC/eFluor 780, APC	SK1	eBioscience/Fisher
Gr-1	FITC, eFluor-450	1A8-Ly6g	eBioscience/Fisher
CD11c	eFluor-450, PerCP/Cy5.5	GL1	eBioscience/Fisher
MHCII	PE, APC, APC/eFluor-780	M5/114.15.2	eBioscience/Fisher
CD25	PE, PerCP/Cy5.5	PC61.5	eBioscience/Fisher
CD8	APC/eFluor780	53-6.7	eBioscience/Fisher
PD-1	PE/Cy7	J43	eBioscience/Fisher
CD44	PE/Cy7	IM7	eBioscience/Fisher
IL-6	eFluor-450	MP5-20F3	eBioscience/Fisher

Table S2. List of primers used for qPCR

Target gene	Forward Primer	Reverse Primer
Ccnd2	TCAAGTGTGACCCGGACTG	ATGTCCACATCTCGCACGTC
Cd274	CTCGCCTGCAGATAGTTCCC	GGGAATCTGCACTCCATCGT
Hprt	AGGACCTCTCGAAGTGTTGG	GGCTTTGTATTTGGCTTTTCC
Mertk	AAACTGCATGTTGCGGGATG	CCACATGGTCACGCCAAAAG
Pcgf6	GGAGAAGCAACTATCGGGCA	CCAGTAAGTGATCCCCACAGA
Spop	TCTTAGCAGCTCGGTCTCCA	AGTCGTCAGCCATCTTGTCG
Zbtb46	AGTTCAAGTGCCCCTACTGC	TGTGGACCAGAGTATGTCGC

Supplemental Figures

Figure S1

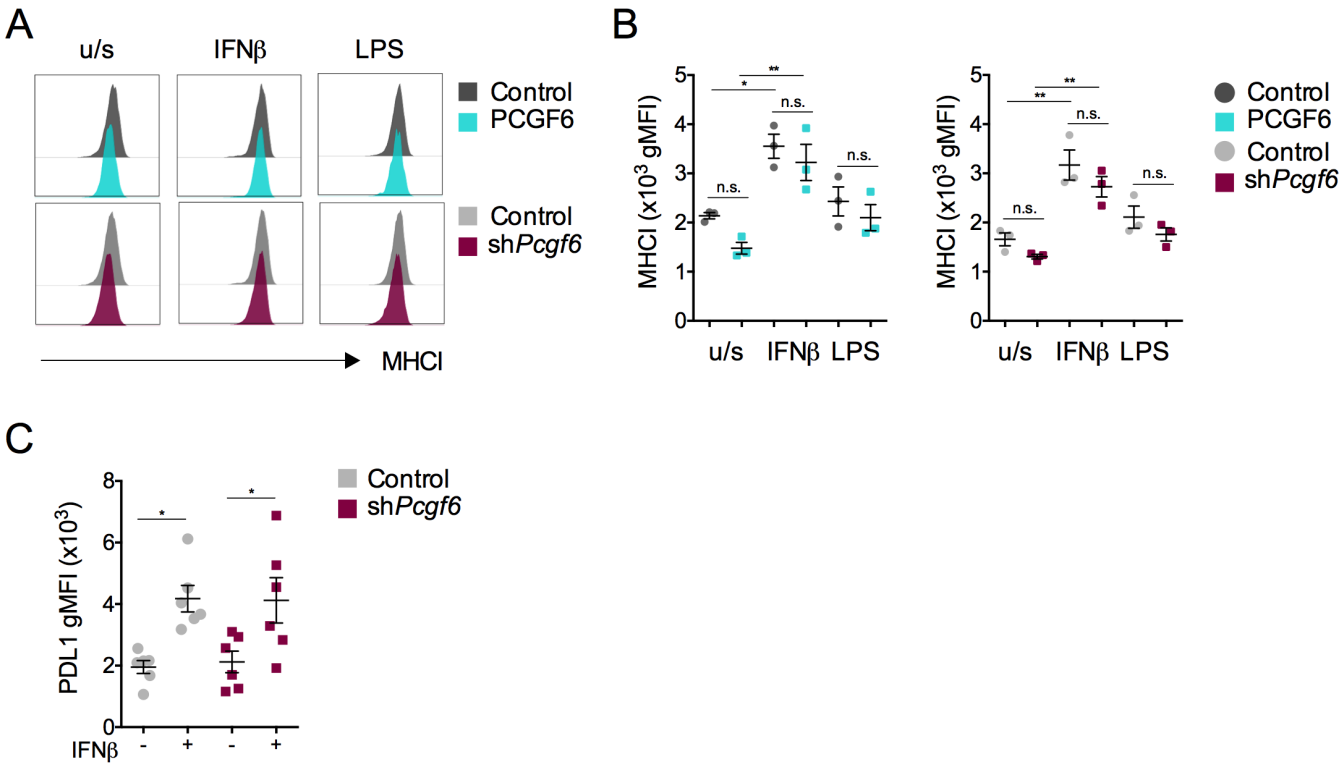

Figure S1, related to Figure 1. Transduced DCs were either left untreated (u/s), treated with 1000 U/mL IFN or treated with 10 ng/mL LPS. A. Representative histograms of MHCI expression measured by flow cytometry in either control vector (Control), PCGF6 over-expressing (PCGF6) or PCGF6-deficient (sh*Pcgf6*) DCs. B. Quantification of geometric MFI corresponding to A. Each point represents an individual biological rep, data are pooled from 3 independent experiments. * $p<0.05$, ** $p<0.005$, n.s., not significant, One-way ANOVA. C. PDL1 gMF1 measured in transduced DCs treated as in Figure 1F. Each point represents the average of triplicates pooled from indiviual experiments. * $p<0.05$, One-way ANOVA.

Figure S2

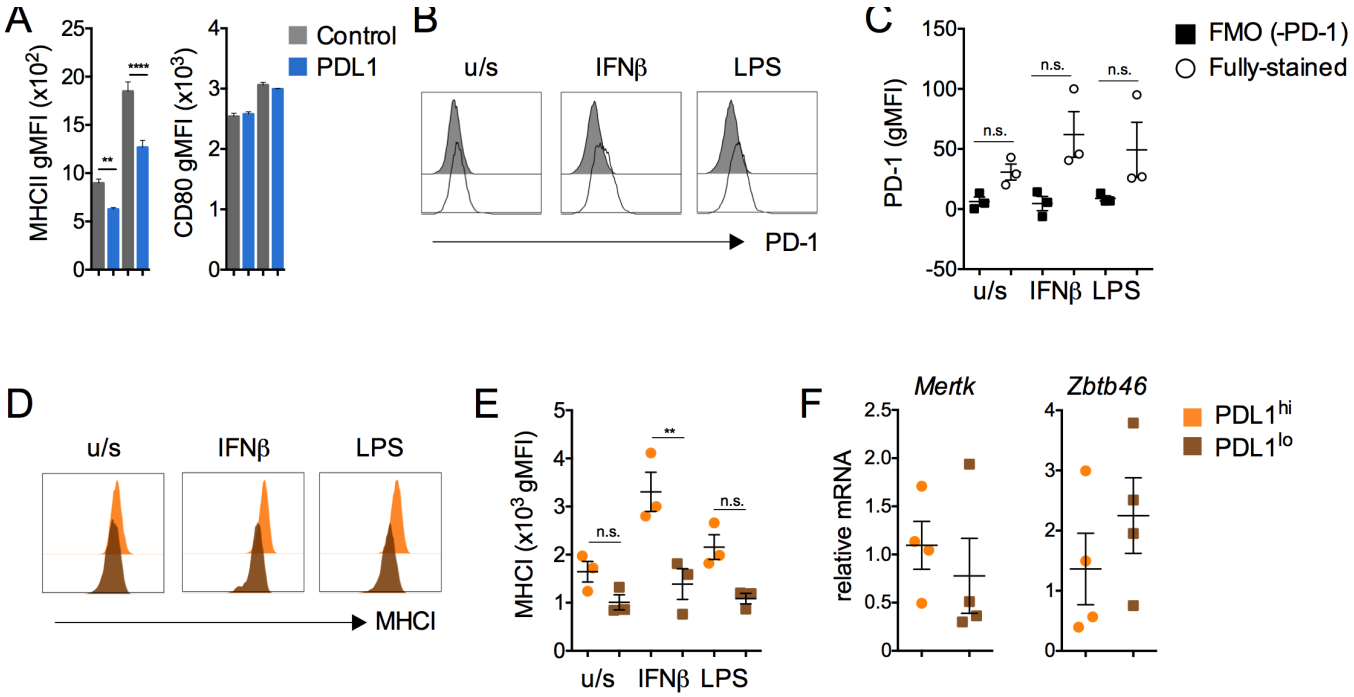

Figure S2, related to Figure 2. A. gMFI of MHCII and CD80 in DCs overexpressing PDL1 or a control vector, stimulated or not with 1000U/mL IFNβ for 18h. Bars represent average of triplicates from a single experiment, representative of 3. Error bars represent standard deviation. ** p<0.005, **** p<0.0001, One-way ANOVA. PD-1 expression in DCs stimulated as in S1. Shown are $CD11c^{+}Gr\text{-}1^{-}$ cells. B. Quanitification of A. Each point is the average from one independent experiment, data are pooled from three independent experiments. n.s., not significant, One-way ANOVA. C. MHCI expression in DCs stimulated as in A. Shown are $CD11c^{+}Gr\text{-}1^{-}PDL1^{hi}$ and $CD11c^{+}Gr\text{-}1^{-}PDL1^{lo}$. D. Quantification of C. Each point is the average from one independent experiment, data are pooled from three independent experiments. *, p<0.05, n.s., not significant, One-way ANOVA. E. mRNA expression of *Mertk* and *Zbtb46* by qRT-PCR in sorted $CD11c^{+}Gr\text{-}1^{-}PDL1^{hi}$ and $CD11c^{+}Gr\text{-}1^{-}PDL1^{lo}$ populations.

Figure S3

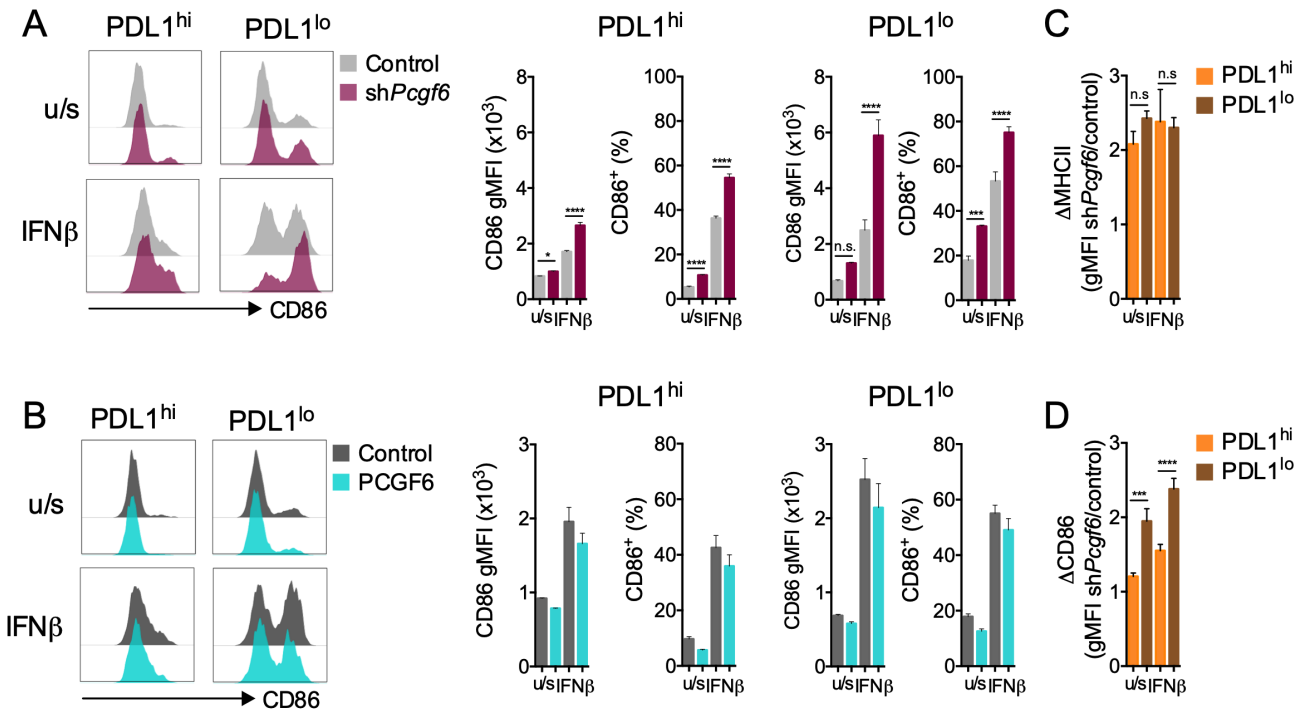

Figure S3, related to Figure 4. A,B. CD86 expression of transduced DCs treated as in Figure 4. Representative histograms (left panel) and average triplicate gMFI and percent $CD86^+$ from a single experiment (right panel). Data are representative of >3 experiments. *p<0.05, ***p<0.005, ****p<0.0001, One-way ANOVA. C,D. Fold change in expression of MHCII (C) and CD86 (D) between PCGF6 deficient DCs compared to Control DCs in $PDL1^{hi}$ and $PDL1^{lo}$ cells stimulated or not with IFNβ. ***p<0.005, ****p<0.0001, n.s not significant, One-way ANOVA.

Figure S4

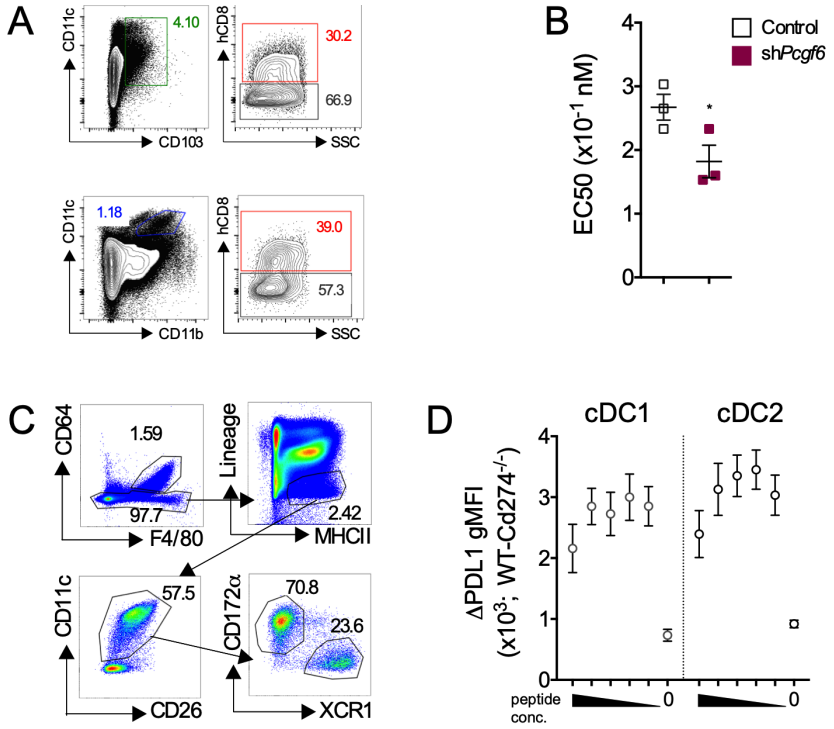

Figure S4, related to Figure 5. A, B. Lethally irradiated mice were reconstituted with fetal liver cells transduced with a vector to reduce PCGF6 expression (sh*Pcgf6*) or a control vector (Control). Greater than 4 weeks post-reconstitution, splenic DCs were sorted through negative selection by MACS. A. Representative data showing reporter expression (hCD8) in $CD103^+$ DCs and $CD11b^+$ DCs. B. EC50 values of $CD8^+$ T cells activated by splenic DCs is reported. Each point is one biological rep and data are pooled from 3 independent expeirments. *$p<0.05$, Student's t-test. C Complete gating strategy corresponding to figure 5A. C. The difference in PDL1 gMFI between WT splenic DCs and PDL1-deficient (*Cd274*$^{-/-}$) splenic DCs from Figure 5F.

Chapter 5: Discussion

In the absence of stimulation, DCs actively maintain their steady state to avoid unwarranted activation and stimulation of immune responses (Dissanayake et al., 2011; Meredith et al., 2012). The molecular mechanisms by which they restrain their activation are not well understood. The work described in this thesis identifies regulation of the chromatin landscape as a central mechanism for restraining DC activation. I found that the transcriptional repressor, PCGF6, is a central regulator of DC homeostasis and function. Data presented in Chapter 2 establishes that PCGF6 maintains DC quiescence through transcriptional repression. Experiments described in Chapter 3 expand on PCGF6-mediated control of chromatin accessibility and reveals that PCGF6 specifically regulates the accessibility of EGR1 binding sites. Finally, data presented in Chapter 4 demonstrates that PCGF6 expression in DCs regulates the antigen sensitivity of naïve T cells through PDL1. This regulation is particularly important for cDC1s, which dynamically modulate the antigen sensitivity of T cells, in response to stimulation. In summary, this work contributes several important findings to our understanding of DC biology: that DC homeostasis, especially that of cDC1s, is maintained through PCGF6, that balancing chromatin states through PCGF6 programs DC responses, and that PCGF6 downregulation serves as a checkpoint to promote inflammation (Figure 1).

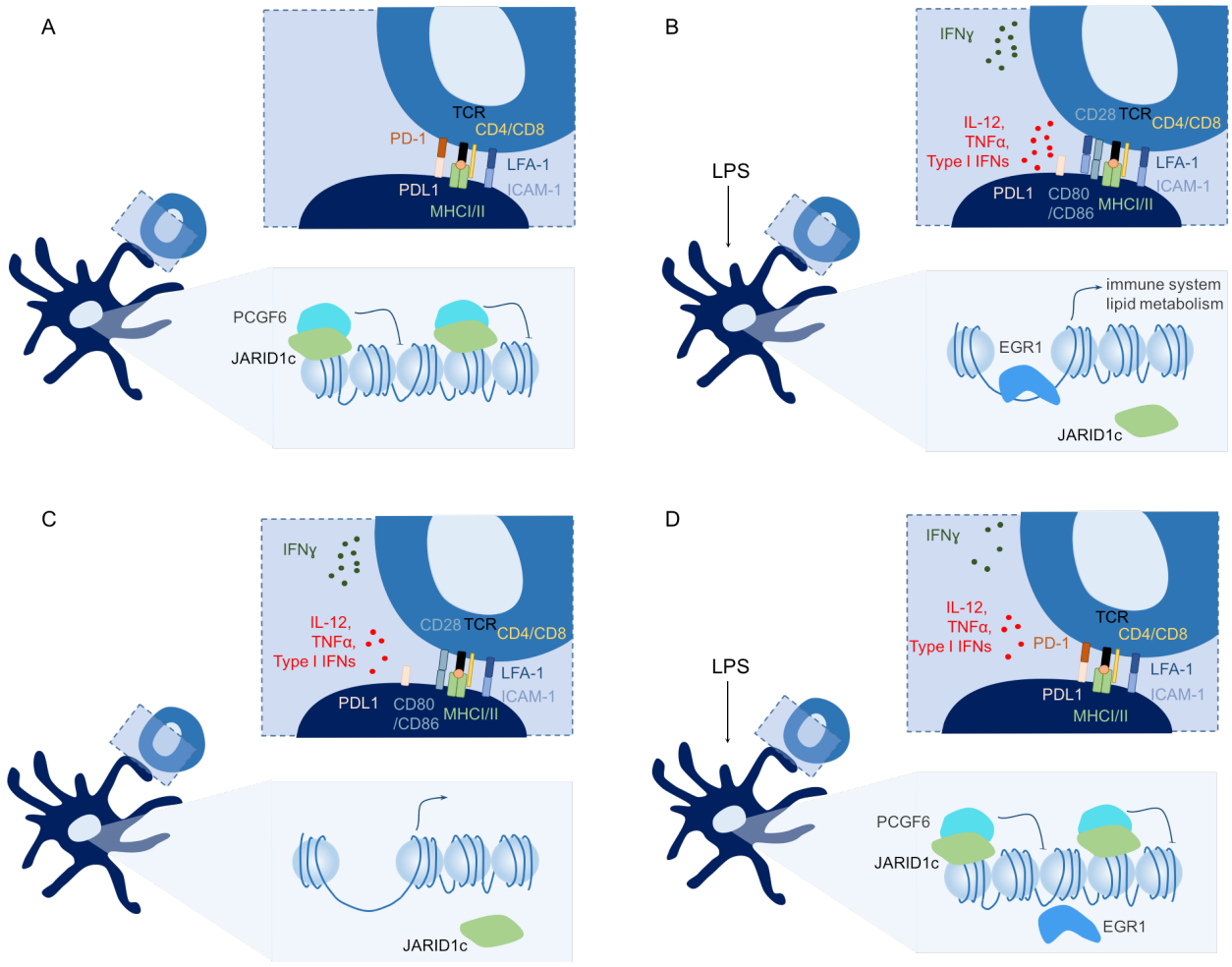

Figure 1. Proposed mechanism by which PCGF6 instructs immunity through transcriptional silencing. A. Under normal physiological conditions at steady state, PCGF6 and JARID1c target genes involved in inflammation for transcriptional silencing. DCs that encounter antigen specific T cells do not induce sensitive T cell responses due to PD-1. PD-1 expression may be sustained by a signal delivered by DCs or it may be a default state. B. LPS stimulated DCs rapidly downregulate PCGF6 which promotes the induction of pro-inflammatory genes by increasing their chromatin accessibility. PCGF6 downregulation also permits EGR1 binding, leading to the induction of immune system and lipid metabolism genes in response to LPS. JARID1c is not downregulated, and may find other targets for suppression in the absence of PCGF6. DC activation in turn leads to more sensitive T cell responses, due to lower levels of PD-1 expressed on T cells. C, D. Proposed ways that dysregulation of PCGF6 could lead to inappropriate immune responses. C. Loss of PCGF6 in the absence of activating signals leads to a chromatin state that favours stimulatory responses as well as an increased capacity to prime sensitive T cell responses. EGR1 has to be induced by TLR stimulation therefore, other transcription factors may play a role at steady-state. D. Failure to downregulate PCGF6 in response to stimulation would dampen the resulting immune response by limiting the accessibility of factors like EGR1. This may in turn favour the upregulation of anti-inflammatory pathways, promoting immune suppression.

5.1 PCGF6 is a central regulator of DC activation and function

PCGF6 maintains DC quiescence

PCGF6 is downregulated in response to PRR stimulation and cytokines such as type I IFNs. Loss of PCGF6 relieves tonic inhibition of proinflammatory genes and promotes DC proinflammatory function. There is a growing body of evidence that suggests multiple levels of regulation are required to maintain DC quiescence. For example, the signaling molecule NF-κB, the transcription factor ZBTB46, and the E3 ubiquitin ligase A20, all restrain DC activation at steady state (Dissanayake et al., 2011; Kool et al., 2011; Meredith et al., 2012). PCGF6 regulates DC steady state through chromatin modification. Mechanistically, PCGF6 mediates the demethylation of key inflammatory gene promoters, through its interaction with JARID1C, a H3K4me3 lysine demethylase. PCGF6 regulated many cellular processes including cytokine production, early induction of glycolysis as well as T cell priming.

Data presented in Chapter 4 suggests that cDC1s and cDC2s maintain homeostasis through separate mechanisms. cDC1s and cDC2s differentially expressed PDL1, and only cDC1s dynamically modulated the antigen sensitivity of T cells and the stability of their interaction with T cells in response to LPS. Furthermore, only cDC1s significantly downregulated PCGF6 in response to LPS. This highlights that PCGF6 may be an important regulator for cDC1s but less so for cDC2s. Interestingly, several mechanisms to regulate the homeostasis of cDC1s have been identified. For example, similar to PCGF6, downregulation of ZBTB46 in cDC1s permits their activation (Meredith et al., 2012). In addition, homeostatic maturation occurs at a low frequency in cDC1s in the periphery. In the thymus, homeostatic maturation of cDC1s plays a role in central

tolerance (Ardouin et al., 2016). Aberrantly increasing the rate of homeostatic maturation contributes to SLE-like pathology (Kool et al., 2011). Therefore, balancing the state of cDC1s is crucial to maintain self-tolerance. In contrast to homeostatic maturation, in visceral adipose tissue, both cDC1s and cDC2s establish an anti-inflammatory environment (Macdougall et al., 2018). Importantly, cross-talk between adipose tissue and cDCs mediate immune suppression, but in obesity, the cross-talk is abrogated and inflammation occurs. cDC1s and cDC2s engaged separate signalling pathways to mediate immune suppression. Input from resident tissues participate in regulating cDC homeostasis, but cross-talk between cDCs also affects their responses. However, our data suggests that cDC2s are intrinsically stimulatory. Despite the increased antigen sensitivity induced by cDC2s compared to cDC1s, we found no significant difference in the capacity of cDC1s or cDC2s to form stable interactions with T cells at steady state. Given that cDC2s induce more sensitive $CD8^+$ T cell responses, it is possible that their reduced capacity for antigen cross-presentation might be an adaptation to protect the host from self-reactivity. This could reconcile reports where under certain contexts cDC2s acquire adequate cross-priming capacity (Kamphorst et al., 2010; Sheng et al., 2017). Our data shows that functional differences between cDC1s and cDC2s occurs in part through a differential requirement for PCGF6.

Balancing chromatin states by PCGF6 programs DC responses

Polycomb group proteins have been associated with the maintenance of chromatin states rather than the establishment of chromatin states (Endoh et al., 2017; Lee et al., 2018; Oksuz et al., 2018; Yang et al., 2016). PRC2, for example mediates spreading of repressive H3K27me3, which serves to reinforce transcriptional repression (Lee et al.,

2018; Oksuz et al., 2018). Consistently, loss or gain of PCGF6 caused widespread changes in chromatin accessibility. Furthermore, the average ATAC signal intensity was decreased in DCs that overexpress PCGF6; this could indicate either a lower proportion of cells with open chromatin in a given region, or it could indicate spreading of repressive epigenetic modifications. One report has found that PCGF6 is not specifically localized to particular genomic elements (Yang et al., 2016). Our data supports this, as the regions with altered chromatin accessibility were not restricted to promoters or enhancers. However, there was an enrichment of intergenic regions suggesting that PCGF6 may play a role in regulating enhancer elements.

Our data suggests that rather than regulating DC lineage, PCGF6 specifically regulates DC function. It has been shown that pioneer factors such as PU.1 and C/EBP specify lineage by recruiting chromatin modifying factors to lineage-specific enhancers (Garber et al., 2012). Meanwhile priming factors set the baseline levels for lineage-specific genes and dynamic factors alter transcriptional signatures in response to environmental cues. Our transcription factor binding analysis identified EGR1 and EGR2 which are two related dynamic transcription factors. This suggests PCGF6 specifically regulates the availability of priming factors or dynamic factors. EGR1 occupies upwards of 2000 sites over the course of LPS-maturation, the majority of which are located within promoters (Garber et al., 2012). Our study identified approximately 900 EGR1 binding sites that become available in PCGF6 deficient DCs. The gene pathways enriched among EGR1 binding sites were largely overlapping with the overall pathways enriched by PCGF6 deficiency. It is intriguing that despite a global regulation of accessibility, PCGF6 still achieves specificity in regulation of transcription factor binding and gene pathways.

PCGF6 may regulate the accessibility of specific priming factors at enhancers. It is thought that priming factors can act at enhancers and promoters to prime lineage specific genes by maintaining the availability of dynamic transcription factor binding sites (Garber et al., 2012; Mullen et al., 2011). Environmental cues activate dynamic transcription factors which then replace priming factors belonging to the same family. Therefore, PCGF6 may regulate the accessibility of a priming factor necessary to prime EGR1 target genes. Enhancer elements, particularly super-enhancers can regulate sets of related genes, including genes that are separated by long distances (Hah et al., 2015; Shlyueva et al., 2014) (Figure 2). Since PCGF6 is thought to maintain chromatin states, it may be bound by previously established, lineage specific enhancers. As a result, PCGF6 may target specific gene pathways by regulating sets of lineage-specific enhancers that govern their expression (Mousavi et al., 2013). Given that its binding partner, JARID1C, fine-tunes the activity of enhancers through RACK7, it is possible that PCGF6 plays a similar role (Shen et al., 2016) (Figure 2).

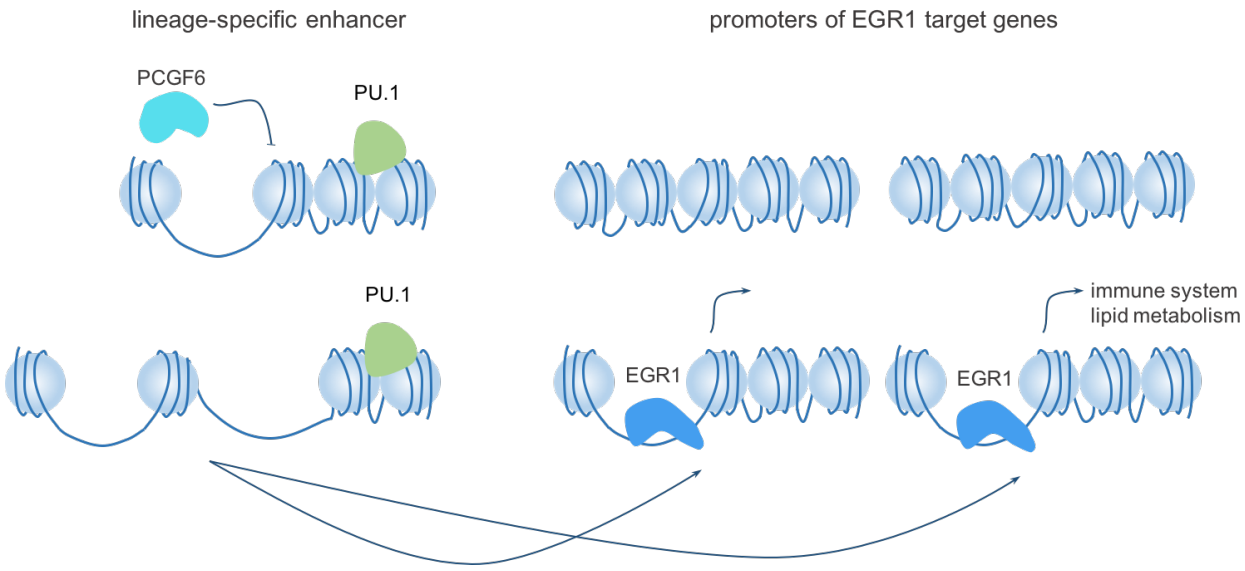

Figure 2. Proposed model for PCGF6-mediated regulation of enhancer activity. PCGF6 likely fine-tunes the activity of enhancers, which regulate related gene sets. Loss of PCGF6 results in increased enhancer activity and accessibility. Enhancers in turn increase the activity of their target promoters.

In Chapters 2 and 3 it was shown that changes in chromatin accessibility at steady state affected the capacity of DCs to become activated and prime T cell responses. Interestingly, revealing EGR1 binding sites at steady state through PCGF6-deficiency enhanced CD86 expression in response to LPS, but steady-state levels of CD86 did not depend on EGR1. This is consistent with literature showing that EGR1 increases in expression and promoter binding only following LPS stimulation (Garber et al., 2012). Our data strongly suggest that low levels of PCGF6 at steady state would prime EGR1 target genes for transcription and promote immunity. This suggests a potential role for PCGF6 in trained immunity. However, data from Chapter 4 suggests that loss of PCGF6 may also lead to the activation of compensatory mechanisms to prevent aberrant activation. Indeed, further loss of PCGF6 in $PDL1^{hi}$ cells increased the expression of surface markers at steady state and in response to activating stimuli, but could not match the extent to which PCGF6-deficiency potentiates $PDL1^{lo}$ cells. This supports the notion that other factors contribute to the maintenance of steady state in $PDL1^{hi}$ cells. Our study does not address the kinetics of PCGF6 expression during the development and differentiation of DCs. Given that PCGF6 overexpression increases PDL1, it is possible that PCGF6 expression was higher in $PDL1^{hi}$ cells earlier on during their development. Therefore, while low levels of PCGF6 may prime proinflammatory genes, PCGF6 levels need to be taken into account with other factors such as PDL1 status.

One caveat of the ATAC-seq study is that it was carried out in primary bone-marrow derived DCs. Although these DCs were FACS sorted to exclude known contaminating populations, they are still a heterogeneous population of cells that may have a differential requirement for PCGF6. This became clear in Chapter 4; we

discovered that PDL1^{hi} cells express lower levels of PCGF6 at steady state than PDL1^{lo} cells which are more stimulatory at steady state. PDL1^{hi} cells also produced less IL-12p40 in response to IFNβ which is inconsistent with data presented in chapter 2 demonstrating that PCGF6 deficiency enhanced IL-12p40 production in response to LPS stimulation. It is possible that, similar to cDC2s, PDL1^{hi} cells are not dependent on PCGF6 while PDL1^{lo} cells are. Another intriguing possibility is that PCGF6 regulates a different set of genes in PDL1^{hi} cells than PDL1^{lo}. A recent study demonstrated that PCGF6 localizes to different gene sets depending on its interaction with either E2F6 or L3MBTL2 (Stielow et al., 2018). Whether these factors regulate PCGF6 localization in DCs is unknown. However, with respect to our ATAC-seq data, it is unclear to what extent differences in PCGF6-dependence affected our results. Ideally, a conditional PCGF6 knockout of murine DCs would help to validate and refine the results obtained in chapter 3. The advantage of ATAC-seq is that very few cells are required to get robust data (as few as 5000 cells), therefore ATAC-seq of cDCs and other rare DC subsets would be relatively straight-forward. Finally, to gain a better understanding of cell-type dependence, single cell sequencing of cDCs from a PCGF6 knockout mouse could reveal novel gene pathways and subpopulations of cells regulated by PCGF6.

PCGF6 downregulation serves as a checkpoint to promote inflammation

PCGF6 downregulation accompanies DC activation and was necessary to promote the expression of proinflammatory genes. PCGF6 downregulation favoured an increase in chromatin accessibility and revealed EGR1 binding sites, promoting cross-priming of CD8^{+} T cells. Forced expression of PCGF6, on the other hand, promoted the expression of anti-inflammatory mediators, IL-10 and PDL1. These data suggest that

PCGF6 downregulation is a checkpoint that promotes immunity while sustained PCGF6 expression leads to immunoregulation. They also suggest that PCGF6 regulates context-dependent integration of environmental cues. Regulation of immunity by PCGF6 may be more important for cDC1s than cDC2s.

During chronic infection, continuous type I and type II IFNs drive the differentiation of a regulatory subset of DCs (iregDCs) that express high levels of IL-10, PDL1 and IDO (Cunningham et al., 2016). iregDCs prime inefficient T cell responses, exacerbating T cell dysfunction. Importantly, IFNɣ was necessary to differentiate iregDCs during chronic infection. Treatment of human monocytes with low levels of IFNɣ leads to development of DCs that are impaired in their capacity to respond to secondary stimulation and prime efficient T cell responses (Kerkar et al., 2014). Interestingly, microarray data show that PCGF6 is upregulated in monocytes treated with low levels of IFNɣ (Kerkar et al., 2014). Taken together with data presented in Chapters 2-4, it is likely that PCGF6 programs tolerogenic DC responses that then promote T cell dysfunction in contexts such as chronic infection and cancer.

Role of PCGF6 in immunometabolism

Metabolic reprogramming has emerged as an important process for controlling DC activation and function (Everts et al., 2012; Guak et al., 2018; Krawczyk et al., 2010). Several key experiments have demonstrated a potential role for PCGF6 in regulating metabolism. For example, early glycolytic flux in response to LPS was abrogated in DCs over-expressing PCGF6 (Chapter 2). Furthermore, PCGF6 deficiency led to an enrichment in lipid metabolism genes that become accessible (Chapter 3). Of particular

interest, lipid metabolism was also enriched among EGR1 target genes that are regulated by PCGF6.

As discussed in Chapter 1, epigenetic modifications are controlled in part by substrate availability (Arts et al., 2016b; Deblois et al., 2018). Metabolic pathways such as the TCA cycle and one-carbon metabolism converge directly on epigenetic pathways by providing substrates and co-factors for chromatin-modifying enzymes. In turn, transcriptional regulation of metabolic pathway enzymes can confer stable changes in cellular metabolism. Therefore, PCGF6 may play a role in regulating metabolism on multiple levels: through direct suppression of metabolic enzymes and by changing the availability of substrates needed for histone modification.

In addition, we found in Chapters 2 and 4 that PCGF6 overexpression in DCs favours the production of immunoregulatory molecules IL-10 and PDL1. It is likely PCGF6 can promote the expression of additional anti-inflammatory mediators, and this warrants further exploration. Interestingly, IDO is often expressed by immunoregulatory cells that co-express IL-10 and PD-L1 (Cunningham et al., 2016). IDO catalyses the conversion of tryptophan to kynurenine, which then acts as an immune suppressive signalling molecule. Importantly, LPS-stimulated cDC1s were found to increase IDO, and kynurenine was found to be an important molecule for cross-talk between cDC1s and cDC2s; kynurenine signaling limits cDC2 reactivity to stimuli (Gargaro et al., 2018).

In Chapter 4, we reported that cDC1s at steady state elicited less sensitive T cell responses than cDC2s, which suggests that cDC1s are more restrained than cDC2s. This was supported by the data demonstrating that only cDC1s dynamically increased the antigen sensitivity of T cells in response to stimulation, and significantly downregulated

PCGF6. It is possible that regulation of PCGF6 in cDC1s also regulates cross-talk between cDC1s and cDC2s that contributes to restraining the activation of cDC2s.

Role of PCGF6 in central and peripheral tolerance and autoimmunity

DCs have a proven role in central and peripheral tolerance. In fact, hyperactivation of DCs has been implicated in autoimmune diseases such as type I diabetes and SLE (Gaudreau et al., 2007; Kool et al., 2011). In Chapters 2-4, it is clear that PCGF6 deficiency leads to increased steady state activation, and also sensitizes DCs to further stimulation. PCGF6 deficiency also enhanced the accessibility of gene pathways that promote the inflammatory response (Chapter 3). Importantly, PCGF6 deficiency promotes T cell activation and IFNɣ production, boosting T cell responses even without stimulation (Chapters 2-4). cDC1s are more dependent on PCGF6 to maintain their homeostasis than cDC2s (Chapter 4). As discussed in Chapter 1, homeostatic maturation of cDC1s occurs to facilitate negative selection in the thymus (Ardouin et al., 2016). This points to a potentially very important role for PCGF6 in maintaining tolerance. Perturbations in PCGF6 expression in cDC1s could change the rate of homeostatic maturation which in turn would affect T cell selection and the resulting T cell repertoire.

Similar to PCGF6, deficiency of JARID1C also promotes dysregulation of DC homeostasis, inducing the spontaneous activation of DCs (Chapter 2). The relationship between PCGF6 and JARID1C at steady state is clear; however, it is less clear following stimulation of DCs. Though PCGF6 is rapidly downregulated in response to stimulation, we found JARID1C levels to be increased. The question arises of what role JARID1C plays in the absence of PCGF6. Given that JARID1C deficiency enhanced the activation of DCs it is possible that in the absence of PCGF6, JARID1C binds to a new partner to

fine-tune DC activation. Indeed, JARID1C has been shown to fine-tune, rather than suppress, enhancer activity through its interaction with RACK7 (Shen et al., 2016). Interaction of the Y-linked homolog of JARID1C, JARID1D, with PCGF6 was required for the enzymatic activity of JARID1D (Lee et al., 2007). It is unclear whether JARID1C has a similar requirement, though it is likely that PCGF6 enhances JARID1C activity but is not required for it.

PD-1 independent functions of PDL1

One intriguing finding from Chapter 4 was that PD-1 expression by DCs was negligible, yet PDL1 over-expression still led to a modest but significant suppression of IL-12p40, as well as an increase in IL-6 production. Therefore, it is likely that PDL1 has immunosuppressive function in the absence of PD-1. PDL1 protects cancer cells from IFNɣ cytotoxicity by STAT3 signaling activated downstream of its cytoplasmic signaling domain (Gato-Cañas et al., 2017). Furthermore, PDL1 interferes with mTOR signaling (Zhao et al., 2017a). It will be important to establish whether PDL1 initiates signaling events in DCs to promote immune suppression. In addition, PDL1 and CD80 are known to interact (Butte et al., 2007). In some reports the interaction of PDL1 and CD80 promotes immune suppression while in others it promotes immune activation (Butte et al., 2007; Cassady et al., 2015). CD80 also has a signaling domain and retrograde signaling through CD80 has been shown to activate IL-6 (Orabona et al., 2004). Whether CD80 and PDL1 interact on DCs and the functional consequence of that interaction is unknown.

5.2 Conclusion

In summary, this work contributes novel insights into the epigenetic regulation of DC function. More specifically, we established that the maintenance of DC steady state is an active process that depends in part on chromatin modification. PCGF6 is a master regulator of DC activation and function. Investigating the mechanisms by which DCs maintain homeostasis is crucial to the understanding of processes such as central and peripheral tolerance as well as pathologies such as chronic infection or autoimmunity. Furthermore, this work highlights a previously underappreciated role for PDL1 during the priming of naïve $CD8^+$ T cell responses. Future work should further characterize the role of PCGF6 in regulating DC metabolism and the function of PDL1 signaling in DCs.

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