Identifying mechanisms that regulate the expression of PCGF6, a repressor of DC activation

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ABSTRACT

The immune system is capable of providing an intricate and well-orchestrated defense program against a variety of different threats including microbes, parasites and cancer. Dendritic cells (DCs) are professional antigen presenting cells and as such master regulators of the immune response. DCs are strategically placed in all interfaces between our body and the external environment where they encounter pathogens in their point of entry and rapidly mount an immune response if needed. Once they encounter a pathogen, DCs relay threat specific information to other cells of the immune system in order to dictate the type of immune response elicited. Activation of the immune system however does not always elicit protection. Inappropriate immune responses can lead to immunopathological diseases such as autoimmune diseases and immunodeficiency disorders. Therefore, it is crucial for the immune system to have mechanisms in place to prevent activation of DCs until the appropriate stimulus is present. Negative regulators lead to changes in the transcriptional program of DCs, an important mechanism used to maintain quiescence. Recent work from our lab has identified PCGF6 as negative regulator of DC activation and function. PCGF6 is a component of Polycomb group (PcG) multiprotein PRC1-like complexes, which act via chromatin remodelling to mediate repression of transcription. In response to a variety of stimuli, expression of *Pcgf6* is rapidly downregulated to allow DC maturation. The objective of my work is to investigate the mechanisms that are in place to regulate the expression of *Pcgf6* at rest and following activation. We showed that *Pcgf6* mRNA levels are downregulated upon engagement of a diverse set of pattern recognition receptors (PRRs) and that this downregulation is correlated with the extent of activation. Furthermore, we showed that *Pcgf6* downregulation is mainly dependent on

transcriptional activity of the gene but post-transcriptional mechanisms also play a role. Finally, our data demonstrated that PI3K, Syk and p38 MAPK signaling cascades are responsible for promoting *Pcgf6* downregulation following activation.

RÉSUMÉ

Le système immunitaire est capable de fournir un programme de défense complexe et bien orchestré contre une variété de menaces différentes, incluant les microbes, les parasites et le cancer. Les cellules dendritiques (CDs) sont des cellules présentatrices d'antigènes professionnelles et figurent donc parmi les principaux régulateurs de la réponse immunitaire. Les CDs, situées stratégiquement à tous les interfaces entre notre corps et l'environnement extérieur, peuvent rencontrer des agents pathogènes à leur point d'entrée et ainsi monter rapidement une réponse immunitaire si nécessaire. Lorsque les CDs rencontrent un pathogène, elles relaient l'information spécifique à la menace aux autres cellules du système immunitaire afin de dicter le type de réponse immunitaire à induire. Cependant, l'activation du système immunitaire ne permet pas toujours la protection. Une réponse immunitaire inappropriée peut conduire à des maladies immunopathologiques telles que les maladies autoimmunes et l'immunodéficience. Par conséquent, il est essentiel pour le système immunitaire d'avoir des mécanismes en place afin d'empêcher l'activation des CDs jusqu'à ce que le stimulus approprié se présente. Les CDs sont donc maintenues en état de quiescence par des régulateurs négatifs qui modifient leur programme transcriptionnel. Des travaux récents menés dans notre laboratoire ont identifié PCGF6 comme supresseur de l'activation et de la fonction des CDs. PCGF6 est un composant des complexes multiprotéiques du groupe polycomb (PcG) PRC-1 qui agissent par l'intermédiaire du remodelage de la chromatine afin de médier la répression de la transcription. En réponse à une variété de stimuli, l'expression de *Pcgf6* diminue rapidement pour permettre la maturation des DCs. L'objectif de mon travail est d'étudier les mécanismes en place qui régulent l'expression de base de *Pcgf6*, ainsi qu'après activation. Nous avons montré que les niveaux d'ARN messager de *Pcgf6*

sont régulés à la baisse suivant l'engagement d'un ensemble de divers récepteurs de reconnaissance des motifs moléculaires (pattern recognition receptors; PRRs) et que cette régulation négative est en corrélation avec l'étendue de l'activation. De plus, nous avons montré que la régulation à la baisse de *Pcgf6* est principalement dépendante de l'activité transcriptionnelle du gène, mais que des mécanismes post-transcriptionels jouent aussi un rôle. Finalement, nos résultats démontrent que les cascades de signalisation de PI3K, Syk et p38 MAPK sont responsables de la régulation négative de *Pcgf6* après activation.

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DCs	dendritic cells
MDP	macrophage and DC progenitor
CDP	common DC progenitor
pDC	plasmacytoid DCs
cDC	conventional DCs
MHC	major histocompatibility complex
CXCL10	C-X-C motif chemokine 10
CCL3	C-C motif ligand 3
SLE	systemic lupus erythematosus
TLR	toll like receptor
PRR	pattern recognition receptor
IL	interleukin
TNF	tumor necrosis factor
IFN	interferon
LPS	lipopolysaccharide
Th	T helper
Treg	regulatory T cell
TGF-β	transforming growth factor beta
CTLA-4	cytotoxic T-lymphocyte associated protein 4
PI3K	phosphatidylinositol 3-kinase
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
ERK	extracellular signal-regulated kinase
JNK	c-Jun N-terminal kinase
TCR	T cell receptor
MAMPs	microbial-associated molecular patterns
DAMPs	danger-associated molecular patterns

IRF	interferon regulatory factor
AP-1	activator protein-1
NLR	NOD-like receptor
RLR	retinoic-acid-inducible gene I (RIG-I)-like receptors
ALR	absent-in-melanoma 2-like receptors
TIR	toll/interleukin 1 receptor
My-D88	myeloid differentiation primary response gene 88
IRAK	IL-1 receptor associated kinase
TRAF6	TNFR-associated factor
TAK	TGF-β-activated kinase
FADD	Fas-Associated protein with Death Domain
MAPK	mitogen-activated protein kinase
CREB	cAMP responsive element binding protein
RIP1	receptor-interacting protein 1
TBK1	tank-binding kinase 1
CRDs	carbohydrate recognition domains
DC-SIGN	dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin
CBP	CREB-binding protein
NLRP3	NLR family pyrin domain containing 3
PTM	post-translational modification
HAT	histone acetyl transferase
DNMT	DNA methyltransferase
moDCs	monocyte-derived DCs
HDACI	histone deacetylase inhibitors
TrxG	Trithorax group proteins
PcG	polycomb group proteins
PRC	polycomb repressive complex
SUZ12	suppressor of zeste 12

EZH2	enhancer of zeste 2
EED	embryonic ectoderm development
CBX	chromobox
PCGF	polycomb group RING finger
HPH	human polyhomeotic
JARID	jumonji and AT rich domain
ESC	embryonic stem cells
PLCγ2	phospholipase Cγ2
IKK	IκB kinase
NFAT	nuclear factor of activated T cells
TWIST2	Twist-related protein 2
(ChiP)-Seq	chromatin immunoprecipitation
MINCLE	macrophage inducible Ca^{2+}-dependent (C-type) lectin

PREFACE

My thesis work focuses on exploring the mechanisms that regulate the expression of *Pcgf6*, a polycomb group protein recently identified in our lab as a negative regulator of DC activation and function. Investigating the mechanisms that promote DC quiescence is important in understanding how to maintain immune homeostasis. Multiple stimuli that engage a variety of PRRs lead to *Pcgf6* downregulation to allow DCs to fully mature. A large network of proteins are activated downstream of PRRs which lead to the activation of a variety of transcription factors. The first part of the introduction will review differential activation in DCs and the various signaling networks that are triggered upon stimulation. PCGF6 is one of the subunits of PRC1-like complexes that mediate epigenetic silencing of genes. The second part of the introduction will review the various methods of epigenetic regulation and the role of epigenetics in modulating immune responses. The literature review will be followed by the rationale for my M.Sc. project.

1. INTRODUCTION

1.1 Dendritic cells

Subsets and function

Dendritic cells (DCs) are innate immune cells that are among the first responders in the presence of infection or injury. They were first discovered in 1973 by Ralph Steinman and Zanvil Cohn (Steinman and Cohn, 1973) and were described as professional antigen presenting cells and as such orchestrators in the initiation and regulation of immune responses (Nussenzweig et al., 1980). Macrophage and DC progenitor (MDP) is a bone marrow progenitor cell with the capacity to give rise to both DCs and macrophages (Fogg et al., 2006). Once MDP commits to the common DC progenitor (CDP) it has the capacity to differentiate into all DC subtypes (Naik et al., 2007). Traditionally DCs are classified into two principal subsets derived from the CDP, plasmacytoid DCs (pDCs) and conventional DCs (cDCs) (Merad et al., 2013).

cDCs are a heterogeneous cell population which can be found in both lymphoid organs such as the spleen and lymph nodes as well as the migratory cDCs that are found in many nonlymphoid organs. DCs found in the lymphoid organs are either $CD8^+$ or $CD11b^+$ and those found in non-lymphoid organs are either $CD103^+$ or $CD11b^+$ (Merad et al., 2013). The migratory cDCs are found in all peripheral areas of the body such as the skin, intestinal tract and the lungs (Kushwah and Hu, 2011). (Paludan et al., 2005). cDCs are constantly sampling their external environment and migrate to the lymph nodes to present antigen (Kushwah and Hu, 2011). This process can occur at steady state but increases in the presence of infection. cDCs have high expression of major histocompatibility complex (MHC)-II and specialize in antigen processing

and presentation which is important in priming T cell-mediated adaptive immune responses (Bevan, 1976) cDCs are responsible for the activation of both naïve $CD4^+$ and $CD8^+$ T cells as well as the maintenance of T cell tolerance (Merad et al., 2013). Most cDCs die following they are activated and have presented antigen in the lymph nodes. Some cDCs are able to exit the lymph nodes and modulate tissue immune responses and tolerance (Randolph et al., 2005).

At steady state, pDCs are low in number and characterized by low surface expression of CD11c and MHC-II (Reizis et al., 2011). pDCs express toll like receptor (TLR)7 and TLR9 which are transmembrane PRRs found in endosomal compartments and typically engaged by single-stranded (ss)RNA and DNA respectively (Hemmi et al., 2002) (Krug et al., 2004). As such, pDCs play a significant role in the clearance of viral infections. pDCs are unique in their ability to produce large amounts of type I interferon (IFNα/β) in response to infection (Siegal et al., 1999) . Aside from type I IFN induction however, they can also produce cytokines such as interleukin (IL)-12 and tumor necrosis factor (TNF)-α as well as chemokines such as C-X-C motif chemokine 10 (CXCL10) and chemokine C-C motif ligand 3 (CCL3) (Swiecki and Colonna, 2015). When activated, pDCs also upregulate surface expression of MHC-II and other costimulatory molecules which enhances their ability to present antigen and activate $CD4^+$ T cells, although less efficiently than cDCs (Swiecki and Colonna, 2015). All of these features allow pDCs to play a variety of roles in triggering both innate and adaptive immune responses.

Differential activation of dendritic cells

DCs are present in almost all peripheral areas of the body that are most exposed to the external environment which enables them to encounter pathogens at their point of entry. Once activated, DCs will migrate via afferent lymphatics to reach lymph nodes in order to communicate with other cells of the immune system and initiate an immune response (Förster et

al., 1999) (Mäkelä et al., 2009). The engagement of PRRs by pathogens will activate a series of signalling cascades which leads to changes in the transcriptional program. The transcriptional changes allow the DCs to switch from a quiescent state to an active state which underlies their ability to exert their immunological function (Kawai and Akira, 2010). Their activation is characterized by an upregulation of costimulatory molecules, enhanced ability to present antigen and production of pro-inflammatory cytokines such as interferons (IFNs), ILs and chemokines (Mäkelä et al., 2009).

The immune system has developed mechanisms to tailor the immune response to the type of pathogen it encounters. One such mechanism is the ability of DCs to adopt different activation phenotypes in response to environmental cues such as type of stimulus, type of receptors engaged and the cytokine/chemokine milieu. This phenotypic plasticity allows them to relay stimulus-specific information to other cells of the immune system and orchestrate a response fine tuned to the pathogen. The DC-T cell interaction plays an important role in dictating specificity of the immune response. There are 3 signals that are necessary for stimulating the activation, differentiation and effector functions of T cells. The first signal is antigen presentation in the context of MHC molecules, the second is interaction with co-stimulatory molecules and the third signal, or differentiation signal is generally provided by cytokine production (Kalińsk et al., 1999) . The third signal is what allows DCs to specifically influence the phenotype of T helper (Th) cell responses.

For example, DCs activated by lipopolysaccharide (LPS), a TLR4 agonist have high levels of co-stimulatory molecules and induce a proinflammatory environment by secreting IL-12, IL-6 and IL-1 and in doing so promote the differentiation of Th1 cells (Walsh and Mills, 2013)(Netea et al., 2005). Th1 cells produce IFN-γ and TNF-α and drive B cell production of

immunoglobulin which are important for protection against bacterial and viral infections (Jankovic et al., 2001). Similarly, activation of TLR9 by unmethylated DNA which induces IFN-α production can also prime a Th1 response (Kline and Krieg, 2008).

Alternatively, parasitic infections activate a different signaling pathway in DCs which favours Th2 cell polarization through the inhibition of IL-12p70 (Netea et al., 2005) . Th2 cells produce IL-4, IL-5 and IL-13 to further promote Th2 responses and drives B cell production of IgE and eosinophil recruitment important for clearance of parasitic infections (Allen and Maizels, 2011) (Hussaarts et al., 2014). Although Th2 responses have been extensively studied in the context of parasitic infections, they can also be induced by specific bacterial and viral infections (Pulendran et al., 2010).

In the presence of fungal infections, glucans will bind to C type lectin receptors (CLRs) such as Dectin-1 to promote IL-6 and IL-23 production which facilitates Th17 cell differentiation (Vautier et al., 2010). Some of the cytokines produced by Th17 cells include IL-22 and IL-17 which are important for mediating protective immunity against fungal as well as bacterial infections (Qu et al., 2013). Th17 cells also play a major role in the onset and progression of various autoimmune disorders such as multiple sclerosis, type I diabetes and rheumatoid arthritis, therefore their induction and expansion must be tightly regulated (Esendagli et al., 2013) (Ryba-Stanisławowska et al., 2013) (Hickman-Brecks et al., 2011).

Microbial pathogens have developed mechanisms to manipulate the immune system and improve their chance of survival in the host. They can provide inhibitory signals that induce immunosuppression and allows them to go unnoticed. Pathogens such as *Bordetella pertussis* and *Yersinia pestis* achieve this by releasing virulence factors that promote immune tolerance by facilitating regulatory T cell (Treg) differentiation (McGuirk et al., 2002). Treg polarization is

facilitated by DCs that adopt a less activated phenotype than the fully activated state that is required for the activation of effector T cell responses (Jonuleit et al., 2000). Virulence factors promote IL-10 secretion and suppress interferon regulatory factors which inhibits IL-12 production and promotes Treg differentiation (Hickey et al., 2008). Mechanisms used by Tregs to promote immune tolerance include the production of anti-inflammatory cytokines such as IL-10 and transforming growth factor beta (TGF-β) to dampen the immune response as well as cytotoxic T-lymphocyte associated protein (CTLA)-4-mediated negative regulation of T cell proliferation/activation (Schmidt et al., 2012).

Immunoregulation of DCs

One of the most important roles DCs adopt is as regulators of immune homeostasis. Most studies to date have predominantly focused on investigating the mechanisms that drive DC activation and how activation pathways coordinate the proper immune responses. What has become more apparent over the years however, is their significant role as negative regulators in many contexts such as autoimmunity, transplantation and hypersensitivity diseases (Thomson and Robbins, 2008) (Hu and Wan, 2011). The cytokine milieu plays an important role in determining DC phenotype. Multiple immunoregulators can drive a tolerogenic phenotype in DCs including IL-10 and TGF-β 1 (Steinbrink et al., 1997) (Yamaguchi et al., 1997).

DCs that are pretreated with IL-10 fail to mature in the presence of infection due to their inability to upregulate costimulatory molecules and reduced production of proinflammatory cytokines such as IL-1β and IL-12 (McBride et al., 2002). IL-10-treated DCs are also inefficient at presenting antigen and inducing $CD8^+$ and $CD4^+$ T cell proliferation/activation (Yang and Lattime, 2003). IL-10 mediates its effects by inhibiting phosphatidylinositol 3-kinase (PI3K) and

nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), pathways that are important in mediating DC maturation (Bhattacharyya et al., 2004) (Hoentjen et al., 2005).

Similar to the effect of IL-10, pretreatment with TGF-β 1 blocks the ability of DCs to mature post stimulation and therefore they are unable to induce the appropriate T cell responses (Yamaguchi et al., 1997). TGF-β 1 can also reduce the ability of DCs to recognize certain pathogens by inducing the downregulation of PRRs such as TLR4 (Mou et al., 2004). Upon encountering a pathogen, DCs are required to migrate to lymph nodes and present antigen to T cells to induce their activation. TGF-β 1 impairs this ability by promoting downregulation of CCR7, a chemokine receptor that mediates migration (Ogata et al., 1999).

Role of DCs in central and peripheral tolerance

Central tolerance is required for the immune system to discriminate between self and foreign antigen. The affinity of the T cell receptors (TCRs) for self-antigen in complex with MHC determines the T cell's fate. T cells with low affinity TCRs are positively selected while the ones with high affinity for self-antigen are deleted (Xing and Hogquist, 2012). DCs play a significant role in the establishment of central tolerance in the thymus by mediating deletion of T cells that are highly responsive to self-antigen and promoting Treg differentiation (Proietto et al., 2008). Tregs will go on to suppress the proliferation and activation of self-reactive T cells both by direct contact as well as the production of anti-inflammatory cytokines (Sakaguchi, 2005).

Some self-reactive T cells escape central tolerance because they express TCRs specific for self-antigen that is only found in peripheral tissues and is not be presented in the thymus during negative selection (Anderton and Wraith, 2002). An important aspect in establishing peripheral tolerance is the induction of T cell unresponsiveness in the case of self-reactivity. This

occurs when tolerogenic DCs present self-antigen without costimulatory signals, whereby T cells that receive signal 1 without signal 2 are rendered unresponsive or anergic (Gallucci et al., 1999).

Overall, DCs receive a wide variety of cues from the environment. All of these extracellular factors converge in the activation of intracellular signaling cascades that modulate transcription and dictate DC phenotype and function.

1.2 Signaling networks in DCs

DCs can respond to a variety of threats in their environment which requires them to express a broad repertoire of PRRs (Takeuchi and Akira, 2010). These receptors can recognize microbial-associated molecular patterns (MAMPs) which are structures conserved among microbial pathogens such as lipoproteins and nucleic acids or molecules released by injured or dying cells termed danger-associated molecular patterns (DAMPs) (Palm and Medzhitov, 2009). The engagement of PRRs will initiate downstream signaling cascades that results in the activation of many factors including NF-κB, interferon regulatory factors (IRFs) and activator protein-1 (AP-1). The activation of these transcription factors leads to changes in expression of genes that mediate the immune response (Takeuchi and Akira, 2010).

Based on their localization, PRRs can be classified into membrane bound receptors which include TLRs and CLRs and cytosolic receptors such as NOD-like receptors (NLR), retinoic-acid-inducible gene I (RIG-I)-like receptors (RLR), absent-in-melanoma 2-like receptors (ALR) (Takeuchi and Akira, 2010) (Mullen et al., 2015). The engagement of various PRRs at the same time can provide the diversity that is essential in tailoring the immune response according to the pathogen. This is achieved by the specific combination of PRRs engaged by microbes expressing a wide variety of PAMPS (Geijtenbeek and Gringhuis, 2009).

1.2.1 Toll like receptor signaling

TLRs are the most extensively investigated PRRs. They are transmembrane proteins with leucine-rich repeats in the N terminus and TIR domains in the C terminus located in the intracellular compartment. There are a total of 13 TLRs found in humans and mice. TLR 1-10 are expressed in humans and TLR 1-9 and 11-13 are expressed in mouse (Deng et al., 2014). Immune cells express TLR 1,2,4,5 and 6 on their cell surface which are specialized in recognizing various components of bacterial cells. On the other hand, TLR3, 7, 8 and 9 are predominantly expressed in intracellular vacuoles and respond to nucleic acids (Akira et al., 2003). The engagement of TLRs leads to the recruitment of toll/interleukin 1 receptor (TIR) domain-containing adaptors which set up the appropriate framework to trigger downstream signaling cascades (Lim and Staudt, 2013). Different adaptors will be recruited depending on the ligand and TLR engaged which provides diversity in downstream signaling and ultimately trigger the appropriate immune response (Akira et al., 2003). There are two main pathways activated downstream of TLRs, one is dependent on myeloid differentiation primary response gene 88 (My-D88) and the other dependent on TIR-domain-containing adapter-inducing interferon-β (TRIF) (Medzhitov et al., 1998) (Yamamoto et al., 2003) **(Figure 1)**.

MyD88-dependent signaling

The engagement of MyD88 is required in all TLRs except for TLR3 which uses TRIF as an adaptor (Nardo, 2015) (Lin et al., 2010). Once MyD88 is activated it interacts with IL-1 receptor associated kinase 4 (IRAK4) via death domains (Li et al., 2002). IRAK4 is a serine/threonine kinase which when autophosphorylated recruits and activates other IRAKs, IRAK1 and IRAK2 (Kawagoe et al., 2008). The MyD88-IRAK complex provides a docking site for E3 ubiquitin ligase TNFR-associated factor 6 (TRAF6), and the autoubiquitylation of TRAF6

leads to the activation of TGF-β-activated kinase 1 (TAK1) (Qian et al., 2001). TAK1 then phosphorylates and activates the IKK complex which is made up of three components, NEMO/IKKγ, IKKβ and IKKα (Lim and Staudt, 2013). Phosphorylation of IKKβ causes the degradation of IκB and release of NF-κB, allowing it to translocate into the nucleus and induce the transcription of proinflammatory cytokines and chemokines (Napetschnig and Wu, 2013). Target genes of NF-κB include IL-12, IL-1 and TNF-α (Lawrence, 2009). TRAF6 can also simultaneously bind to and activate IRF5 which is also important in the induction of proinflammatory cytokine gene expression (Takaoka et al., 2005). In addition, TAK1 can activate the mitogen-activated protein kinase (MAPK) pathway mediated by p38, Jnk and Erk which is responsible for the activation of AP-1 and cAMP responsive element binding protein (CREB) (Kawai and Akira, 2010). NF-κB signaling also triggers a negative feedback loop driven by negative regulators such as A20 in order to prevent chronic inflammation resulting in tissue damage (Newton and Dixit, 2012). Other negative regulators of TLR signaling include IRAK1 inhibitor MyD88 short (MyD88s) and MyD88 inhibitor Fas-Associated protein with Death Domain (FADD) (Flannery and Bowie, 2010).

The engagement of TLR7 and TLR9 in pDCs is also MyD88 dependent and induces the expression of type I interferons, IFNα and IFNβ (Hornung et al., 2004). This is achieved by the activation and nuclear translocation of IRF7 following IRAK1-mediated phosphorylation and TRAF6-mediated ubiquitination (Uematsu et al., 2005). When IRAK1 is absent pDCs are not able to induce type I IFN production but instead will promote expression of IL-12 and IL-6 (Uematsu et al., 2005).

TRIF-dependent signaling

Engagement of TLR3 and TLR4 recruits TRIF adapter protein which initiates a unique signaling cascade (Yamamoto et al., 2003). TLR4 is unique because it is able to recruit both adaptors MyD88 as well as TRIF. MyD88-dependent signaling is initiated at the plasma membrane whereas TLR4 needs to be translocated in the endoplasmic compartment prior to initiation of TRIF-dependent signaling (De Nardo, 2015). Upon activation TRIF associates with TRAF6 which polyubiquitinates receptor-interacting protein 1 (RIP1) and activates NF-κB (Gohda et al., 2004). Alternatively, TRIF can also interact with TRAF3 which activates tank-binding kinase 1 (TBK1). TBK1 then triggers the phosphorylation and nuclear translocation of IRF3 and lead to the production of type I interferons (Sato et al., 2003).

1.2.2 C type lectin receptor signaling

CLRs are calcium-dependent lectins that are specialized in binding to glucans but can also bind to proteins and lipids (Zelensky and Gready, 2005). They can be found both as soluble proteins as well as membrane bound. Their binding activity is reliant upon carbohydrate recognition domains (CRDs) (Maglinao et al., 2014). The amino acid sequence of CRDs can dictate sugar specificity. Calcium binds to CRDs and facilitates recruitment of carbohydrates by binding to their hydroxyl groups (Furukawa et al., 2013). Upon encountering ligands the receptors often form homodimers or homotrimers which result in improved avidity. CLRs are able to crosstalk with TLRs and modulate their signaling but they can also induce a TLR

independent response (Geijtenbeek and Gringhuis, 2009). CLRs play an important role in driving immune responses against fungal infections (Hardison and Brown, 2012).

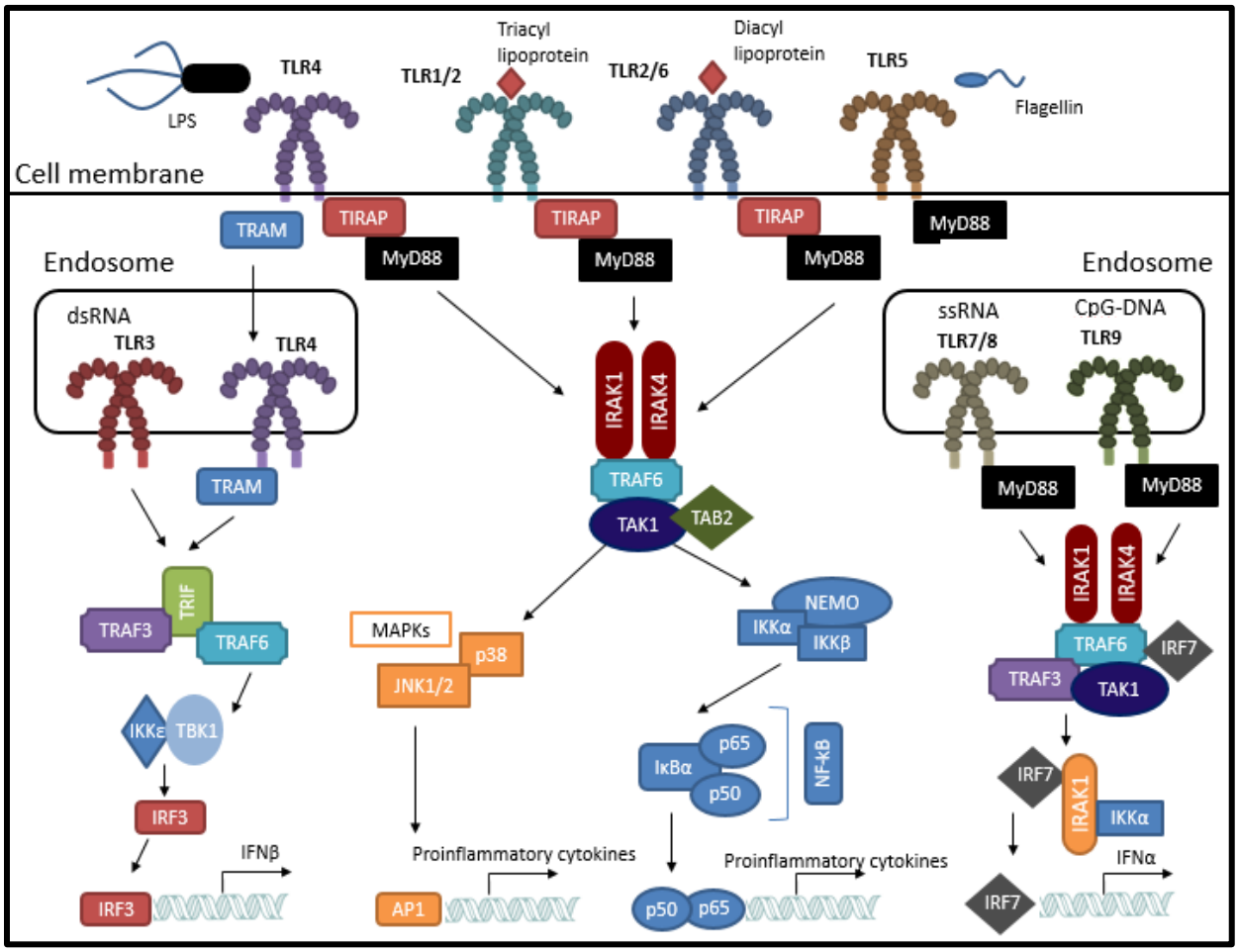


Figure 1. TLR signaling cascades. TLRs are expressed on the cell surface and endosomal membranes. All TLRs aside from TLR3 signal through MyD88 pathway to induce NF-κB and AP1 activation. TIRAP and MyD88 is required for signaling downstream of TLR2 and TLR4. Both TLR3 and TLR 4 can signal through TRIF adapter protein to induce IRF3 activation. TLR7, TLR8 and TLR9 activate IRF7 signaling. TLR signaling results in the transcription of proinflammatory cytokines and type I IFNs. (Figure adapted from Yang L and Seki, E 2012)

TLR-dependent signaling

One of the CLRs that mediates TLR-dependent signaling is dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN). DC-SIGN is a mannose and fucose binding CLR which functions indirectly by modulating the signaling of PRRs that induce NFκB expression such as TLR3, TLR4 and TLR5 (Gringhuis et al., 2007). Upon engagement of DC-SIGN by mannose-expressing pathogens such as HIV-1 and *Leishmenia* species, Ras proteins are activated **(Figure 2)**. Ras proteins induce phosphorylation and activation of RAF1, a serine/threonine protein kinase (Gringhuis et al., 2007). RAF1 then phosphorylates the p65 subunit of NFκB which provides a docking site for histone acetyltransferases, CREB-binding protein (CBP) and p300. The subsequent acetylation of p65 enhances DNA binding and its transcriptional activity (Chen et al., 2002). This CLR-TLR crosstalk is dependent on prior TLR-mediated NFκB activation.

TLR-independent signaling

Dectin-1 is a CLR capable of activating a signaling cascade that is TLR-independent. Dectin-1 binds to glucose polymers, β-1,3-glucans found on the surface on many fungi such as *Candida albicans* and *Aspergillus fumigatus* (Brown et al., 2007). When activated, amino acid residues on Dectin-1 get phosphorylated and provide binding sites for SYK which required to form the CARD9-MALT1-BCL-10 protein complex (Gross et al., 2006). This ultimately induces the activation and nuclear translocation of NFκB. Some studies suggest that in response to certain pathogens this pathway can also lead to the activation of the NLR family pyrin domain containing 3 (NLRP3) inflammasome and induce IL-1β production (Gross et al., 2009).

Alternatively, Dectin 1 can also activate a SYK-independent signaling cascade that involves the activation of RAF1 (Gringhuis et al., 2009). This pathway induces NFκB activation and increased expression of cytokines IL-12 and IL-23 and chemokines CCL22 and CCL17 which leads to recruitment of other leukocytes and induction of Th1 and Th17 responses (Gringhuis et al., 2009).

Overall, signaling cascades activated downstream of PRRs lead to global changes in gene expression to modulate DC maturation and function. Gene expression is ultimately constrained by accessibility of

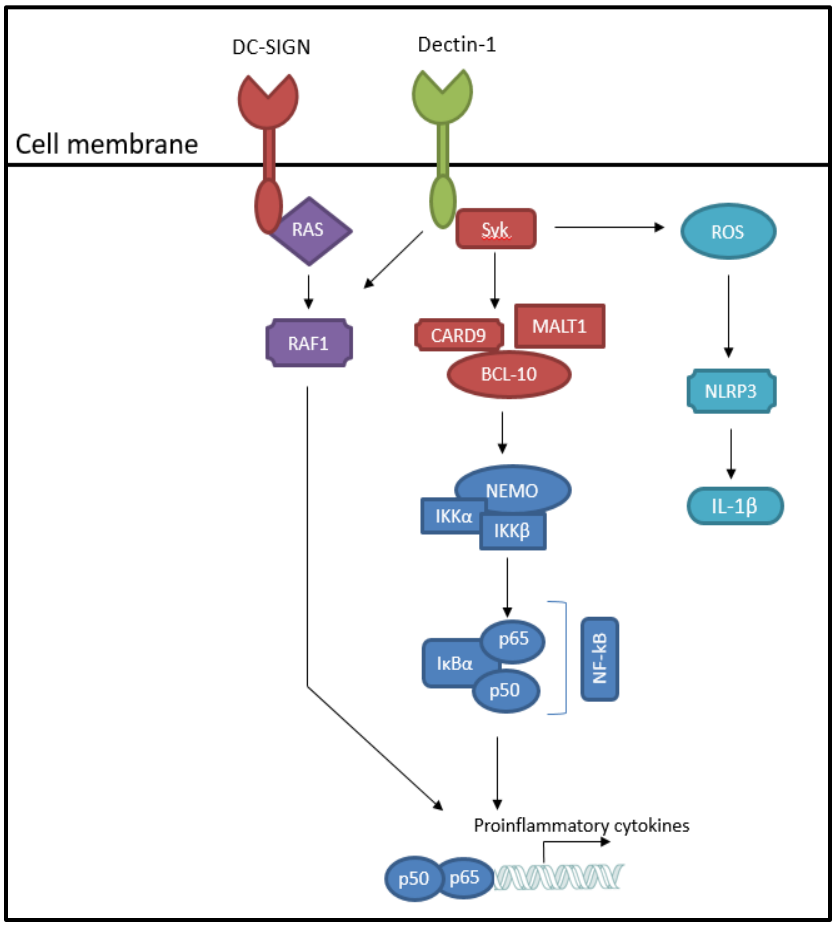


Figure 2. CLR signaling cascades. CLRs can activate TLR-dependent and independent signaling cascades. DC-SIGN activates TLR-dependent signaling. Upon engagement of the receptor Ras proteins are activated which phosphorylate RAF1 and induce NF-κB. Dectin-1 is an example of a CLR involved in TLR-independent signaling. In the context of some infections Syk is activated downstream of CLR signaling followed by CARD9-MALT1-BCL10 protein complex activation and induction of NF-κB. Alternatively, SYK-dependent signaling leads to NLRP3 activation to induce IL-1β production. Dectin-1 can also activate SYK-independent signaling cascade that involves activation of RAF1. (Figure adapted from Geijtenbeek et al, 2012)

chromatin. Epigenetic mechanisms play a key role in mediating changes in the state of chromatin and ultimately modulating the transcriptional state of genes.

1.3 Epigenetic regulation

Epigenetics is defined as variations that result from gene expression by covalent modifications of DNA and proteins but not changes in the DNA sequence itself. The two most significant methods of epigenetic regulation are DNA methylation and histone post-translational modification (Handy et al., 2011).

Mechanisms of epigenetic regulation

DNA methylation:

The methylation of DNA on the 5^{th} carbon of cytosine residues by a family of enzymes called DNA methyltransferases (DNMTs) causes silencing of genes. This method of DNA modification is meiotically stable and therefore heritable (Holliday and Pugh, 1975). It is also generally irreversible and as such an important mechanism for long term silencing of gene expression such as in the case of X chromosome inactivation (Riggs, 1975). DNA methylation has been also been well characterized as a regulator of differentiation by dictating which genes are repressed during cell-fate decisions (Ji et al., 2010).

Histone modification:

Nucleosomes are the fundamental repeating structural units of DNA which consist of stretches of DNA wrapped around 8 histone proteins (Kornberg, 1974). The positive charge of histones allows them to bind tightly to the DNA which has an overall negative charge due to the phosphate groups in its backbone (Cosgrove and Wolberger, 2005). The combination of DNA

and all associated protein is called chromatin, the state of which dictates the level of transcription and replication in the cell (Cosgrove and Wolberger, 2005). Heterochromatin is defined as regions of chromatin that are more compact and therefore less accessible to environmental factors and often transcriptionally silent (Owen-Hughes and Bruno, 2004). Alternatively, chromatin can adopt a lightly packed and decondensed structure, euchromatin which is more accessible and usually transcriptionally active (Owen-Hughes and Bruno, 2004). Histones have flexible tails that project away from the nucleosome core and are accessible to enzymes responsible for post-translational modification (PTM) (Cosgrove and Wolberger, 2005). Histones undergo PTMs including methylation, acetylation, ubiquitination and phosphorylation and various combinations of these PTMs make up the "histone code" (Kouzarides, 2007). The functional consequence of the histone code is to recruit non-histone proteins that either directly or indirectly change the state of the chromatin and ultimately the transcriptional state of genes (Turner, 2000). PTMs such as histone acetylation mediated by histone acetyl transferases (HATs) promote transcriptional activity while histone methylation generally represses gene expression. Histone acetylation changes the interaction between DNA and histones by neutralizing the charge on histones. Various PTMs can also provide binding sites on chromatin for the recruitment of non-histone adaptor proteins responsible for chromatin remodeling and transcriptional regulation (Di Croce and Helin, 2013).

Epigenetic regulation of immune responses

Epigenetic regulation is important in modifying global gene expression of many key players of the immune system including DCs and T cells (Suárez-Álvarez et al., 2013). Epigenetic histone modifications are involved in regulating the function of activated or tolerogenic monocyte derived DCs (moDCs) (Huang et al., 2012). LPS stimulated DCs have

higher levels of H3K4me3 marks in genes that drive maturation such as costimulatory molecules and inflammatory cytokines (Huang et al., 2012). DCs conditioned with TGF-β have a different epigenetic landscape which is associated with a tolerogenic phenotype in DCs. For example, a reduced expression of maturation markers such as CD83 is mediated by an increase in repressive H3K27me3 marks. (Huang et al., 2012). Previous research suggests that histone deacetylase inhibitors (HDACIs) play an anti-inflammatory role for in DCs by reducing the expression of costimulatory molecules and the production of inflammatory cytokines (Nencioni et al., 2007). Although DNA methylation marks are thought to be highly stable, recent studies have shown that various environmental cues can change the methylation patterns. In the context of bacterial infection, demethylation of many loci occurs in DCs particularly in enhancer regions (Pacis et al., 2015). Moreover, recent work has shown that IL-4 modulates DNA methylation landscapes of genes involved in DC differentiation (Vento-Tormo et al., 2016).

Epigenetic modifications at specific loci are responsible for the differentiation of naïve T cells to effector cells or suppressive Tregs. Modulations of epigenetic marks in genes such as IFNγ and IL-4 are responsible for dictating the differentiation of T cells to Th1 and Th2 cells. The IFNγ gene which is highly expressed in Th1 cells has active H3K4me3 marks in Th1 cells and repressive H3K27me3 marks in Th2 cells (Aune et al., 2009). Furthermore, the IL-4 gene which is actively transcribed in Th2 cells, is hypermethylated in naive and Th1 cells and is demethylated in Th2 cells (Santangelo et al., 2009). Demethylation and acetylation of the FoxP3 gene induces its expression to promote Treg differentiation (Lal and Bromberg, 2009; van Loosdregt et al., 2010).

Polycomb group proteins

Two major families of epigenetic regulators involved in transcriptional activation and repression are Trithorax group proteins (TrxG) and polycomb group proteins (PcG), respectively (Lewis, 1978). PcGs were first discovered in Drosophila for their role in the repressing the transcription of the *Hox* gene (Lewis, 1978). They also play a vital role in the regulation of various processes such as cell cycle, senescence, inactivation of the X chromosome and cell differentiation (Kennison, 1995) (Wang et al., 2001) (Oktaba et al., 2008). Their role in regulating cell differentiation makes them a favourable target for mutation in many cancers (Luis et al., 2011) (Richly et al., 2011). PcGs work in multi-protein complexes termed polycomb repressive complex 1 (PRC1) and polycomb repressive complex 2 (PRC2). The three main components that make up PCR2 are suppressor of zeste 12 (SUZ12), enhancer of zeste 2 (EZH2) and embryonic ectoderm development (EED). PRC1 on the other hand is made up of a member from the RING1 family in complex with a member of the chromobox (CBX), polycomb group RING finger (PCGF) and human polyhomeotic (HPH) family (Di Croce and Helin, 2013) **(Figure 3)**. Different combinations of PRC1 proteins will result in complexes of

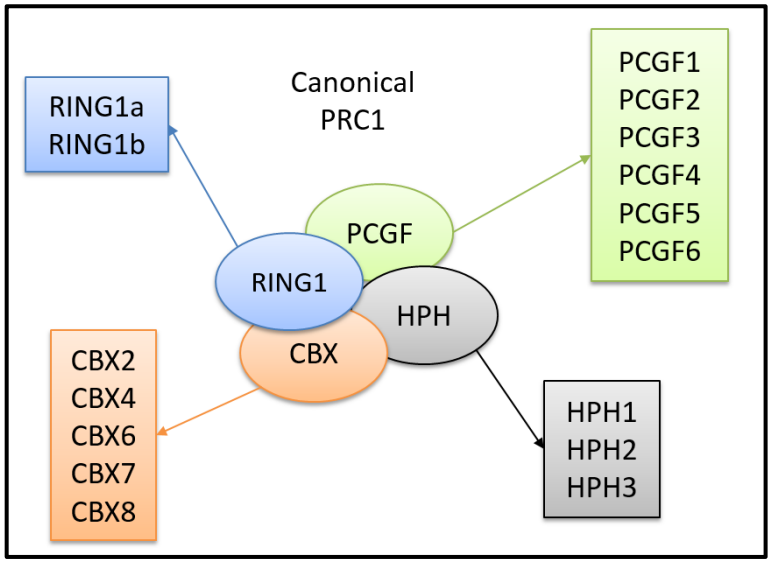


Figure 3. Canonical PRC1 schematic. The canonical PRC1 complex is made up of four core components. Differences in subunit composition accounts for different recruitment mechanisms and function. PRC1 is responsible for H2AK119 ubiquitination, H3K27me3 binding, chromatin compaction and inhibiting RNA polymerase II mediated elongation. (Figure adapted from Croce L and Helin K, 2013)

various biological functions and their composition will dictate the genes targeted for silencing (Di Croce and Helin, 2013). The various subunits of these protein complexes can be categorized according to their function. Writer proteins catalyze the PTMs which are recognized by reader proteins (Molitor and Shen, 2013). The recruitment of polycomb complexes can be mediated by interactions with transcription factors and long non-coding RNAs but they can also bind to regulatory sites in the genome such as CpG islands commonly found in promoter regions (Brockdorff, 2013) (Blackledge et al., 2015). The enzymatic functions of the PcG repressive complexes underlie their ability to maintain a repressive chromatin environment via histone modifications and ultimately inhibit gene expression (Morey and Helin, 2010). For example, PRC1 is responsible for catalyzing lysine 119 mono-ubiquitylation of histone H2A (H2AUb1) while PRC2 catalyzes the di- and tri-methylation of histone H3 lysine 27 (H3K27me2/3) both of which are repressive histone marks leading to transcriptional silencing (Francis et al., 2004).

PCGFs

There are six PCGF proteins (PCGF1-6) currently identified as components of various PRC1 complexes. PCGFs do not bind DNA directly but they enhance the enzymatic activity of other members of polycomb repressive complexes such as EZH2 and jumonji and AT rich domain (JARID) proteins (Richly et al., 2011). The six PCGF proteins can form unique functionally distinct PRC1 complexes termed PRC1.1-1.6 (Gao et al., 2012) .

PCGF6 became of particular interest to the lab because its levels were found to be rapidly downregulated in LPS stimulated DCs compared to immature DCs. PCGF6 acts as a transcriptional repressor, like other PcG proteins and its function is regulated by cell cycle-dependent phosphorylation (Akasaka et al., 2002). PCGF6 is highly expressed in embryonic

stem cells (ESCs) and its expression is reduced upon differentiation (Zdzieblo et al., 2014). PCGF6 interacts with JARID1d, a JmjC-domain-containing protein that regulates gene transcription through demethylation of H3K4me3. PCGF6 has been shown to facilitate the enzymatic activity of JARID1d (Lee et al., 2007). Previous research has also investigated the role of PCGF6 as a negative regulator of mouse male germ cell proliferation (Sun et al., 2015). In contrast to all previous research, the most recent paper has shown that PCGF6 can also function as a transcriptional activator of various genes important for embryonic stem cell identity (Yang et al., 2016).

1.4 Rationale and goals

The immune system has developed mechanisms that are in place to maintain DCs in a quiescent state until the appropriate stimulus is present. DC quiescence is important for preventing defective immune responses that can contribute to acute and chronic inflammation and autoimmune diseases. Negative regulators can lead to changes in the transcription program of DCs maintain quiescence. Epigenetic regulation is an important mechanism used to modulate the transcriptional state of genes via post-translational modification of histones. We have identified PCGF6 as a suppressor of DC activation and function. Recent work from our lab has shown that DCs overexpressing PCGF6 fail to mature in the presence of stimuli shown by reduced expression of costimulatory molecules as well as production of proinflammatory cytokines (Boukhaled et al., 2016). Furthermore, DCs overexpressing PCGF6 have a reduced ability to induce T cell proliferation and activation (Boukhaled et al., 2016). We have demonstrated that *Pcgf6* is downregulated upon stimulation of DCs with various activators that engage multiple PRRs. **We hypothesize that multiple mechanisms that stimulate a pro-inflammatory phenotype in DCs lead to the downregulation of *Pcgf6*.** We have set up three

aims to examine the mechanisms that are in place to drive *Pcgf6* downregulation. My first aim is to characterize *Pcgf6* mRNA expression in DCs following activation with various stimulants. My goal for this aim is to examine whether *Pcgf6* downregulation occurs upon stimulation with activators that vary in the type of PRRs they engage as well as in strength of activation. My second aim is to determine which signaling pathways activated downstream of PRR signaling promote *Pcgf6* downregulation. There is a wide variety of PRRs that can detect a range of stimuli and converge in the activation of signaling cascades that induce a transcriptional response. My goal is to examine which molecules downstream of PRR signaling are responsible for modulating *Pcgf6* mRNA levels. My final aim is to examine the regulation of *Pcgf6* expression by transcription factors that can bind the promoter. My goal for this aim is to identify transcription factors that bind *Pcgf6* and investigate their potential to modulate the mRNA levels of *Pcgf6*.

2. MATERIALS AND METHODS

2.1 Mice and BMDC/BMDM cultures

All the experiments with wild type BMDCs and BMDMs were performed using female C57BL/6 mice aged 6-8 weeks purchased from Charles River Laboratories. STAT6 wild type and knockout Balb/c mice were provided by Dr. Elizabeth D. Fixman (Meakins-Christie Laboratories, McGill). Animals were maintained in a specific pathogen-free environment. All experiments were conducted following the guidelines of the Canadian Council of Animal Care, as approved by the animal care committee of McGill University. For BMDC cultures, bone marrow was extracted from the leg bones and cultured with complete DC media (RPMI supplemented with 10% FCS, 100 U/mL Penicillin /Streptomycin, 2mM L-Glutamine, 1:1000 mM β-ME and 1:100 Non-essential amino acids) and 20 ng/mL of GM-CSF to induce *in vitro* differentiation of DCs. DCs were harvested after 7-9 days and activated with LPS (10 ng/mL), HDM (50 ug/mL), Zymosan (10 ug/mL), Curdlan (50 ug/mL) and Zymosan depleted of TLR ligands (10 ug/mL) for various times from 2 hours-overnight. For BMDM cultures, bone marrow was extracted and cultured with complete MΦ media (RPMI supplemented with 10% FCS, 100 U/mL Penicillin /Streptomycin, 2mM L-Glutamine, 1:100 Non-essential amino acids, 1% Na pyruvate, 2% HEPES and 0.1% NaOH (5N) and 40% L929 M-CSF feeder line supernatant. Cells were fed on Day 3 with complete MΦ media and 25% L929 supernatant and on Day 6 with complete MΦ media and 5% L929 supernatant. Cells were harvested on Day 7 and stimulated overnight with 10ng/mL of LPS and 50ng/mL of IFN-γ to induce *in vitro* M1 differentiation and with 20ng/mL of IL-4 to induce *in vitro* M2 differentiation.

2.2 PRR inhibitor experiments

BMDCs were pretreated with indicated inhibitors for approximately 1h and then activated for 2h with various PRR ligands including LPS, Zymosan, HDM and Curdlan. The inhibitors used include: MEK inhibitor PD0325901 (1 uM), NFκB inhibitor BAY 11-7082 (10 uM), Syk inhibitor R406 (5 uM), PI3K inhibitor Ly294002 (20 uM) and p38 MAPK inhibitor SB202190 (15 uM) were purchased from Selleckchem. The STAT3 inhibitor (S3I-201) was purchased from Sigma.

2.3 RNA extraction and qPCR

Total RNA of BMDCs was extracted using TRIZOL reagent (Life Technologies). Concentration and quality of RNA was measured by Nanodrop. cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) which uses random primers and MultiScribe reverse transcriptase. qRT-PCR was then performed using SYBR Select Master mix (Applied Biosystems) and ran on a CFX96 Real-Time PCR system (BioRad). Relative fold change was calculated using the $\Delta\Delta C_q$ method, normalized to *Hprt*. qPCR bar graphs represent the average fold change from pooled biological replicates and the error bars indicate the standard error of the mean. The primer sequences used are listed below:

Gene	Forward primer	Reverse Primer
Hprt	AGG ACC TCT CGA AGT GTT GG	GGC TTT GTA TTT GGC TTT TCC
IL-12p40	CTG GAG CAC TCC CCA TTC CT	CGC CTT TGC ATT GGA CTT CG
Pcgf6	GGA GAA GCA ACT ATC GGG CA	CCA GTA AGT GAT CCC CAC AGA

2.4 Statistical Analysis

All the statistical analyses were performed using GraphPad Prism 7. In order to determine significant differences between various conditions the unpaired *t* test and one-way ANOVA were carried out. $*p < 0.05$ as indicated.

3. RESULTS

3.1 Stimulation of DCs with various activators induces *Pcgf6* downregulation.

To examine the expression of *Pcgf6* following activation, DCs were stimulated with a variety of activators: LPS, HDM, Curdlan, Zym and ZymD **(Table 1)**. These activators were chosen because they engage a variety of PRRs and therefore induce different DC phenotypes. LPS triggers TLR signaling, Zym activates both TLR and CLR signaling while Curdlan engages CLRs only. These activators also differ in activation strength whereby Zym and LPS induce a highly inflammatory phenotype and HDM and ZymD induce a weaker or less activated phenotype in DCs. DCs were stimulated for 2 hours and relative mRNA expression of *Pcgf6* and *IL-12p40* were determined by qRT-PCR **(Figure 4A,B)**. *Pcgf6* mRNA levels were found to be significantly downregulated following activation with LPS, Zym and HDM. *IL-12p40* levels were measured as a positive control for activation. Our data suggests a negative correlation between *Pcgf6* expression and activation strength. In the presence of strong activators such as Zym and LPS, *Pcgf6* was reduced to a greater extent compared to weak activators such as HDM and ZymD. The strength of the different activators was determined via expression of *IL-12p40*.

To examine the kinetics of *Pcgf6* downregulation DCs were stimulated over a period of 36 hours. *Pcgf6* levels were measured at 2, 6, 24 and 36 hours. *Pcgf6* downregulation was seen at 2 hours and persisted over the 36 hour period **(Figure 4C)**.

Our data shows that *Pcgf6* downregulation occurs in the presence of a variety of activators that engage many different PRRs which suggests that multiple pathways regulate

Pcgf6 expression. Furthermore, stimulants that induce a more inflammatory phenotype in DCs downregulate *Pcgf6* to a greater extent than weaker activators.

3.2 *Pcgf6* downregulation occurs independently of MEK and NFκB signaling.

Although DCs express a wide variety of PRRs, they all initiate downstream signaling cascades that converge in PI3K, NFkB and MAPK pathways that drive transcriptional activation by factors such as AP-1, CREB, and NF-κB. Pharmacological inhibitors of signaling molecules were used to determine which molecules downstream of PRRs play a role mediating *Pcgf6* downregulation following stimulation. DCs were pre-incubated with indicated inhibitors for approximately 1 hour and then activated for 2 hours with different activators.

MEK1/2-ERK pathway is a MAPK signaling pathway typically activated downstream of TLR signaling in a MyD88-dependent manner (Kawai and Akira, 2010). The MEK1/2-ERK pathway can also be induced upon engagement of CLRs that activate RAF1 (Geijtenbeek and Gringhuis, 2009). PD0325901 was used to inhibit MEK1/2 signaling in resting DCs and DCs stimulated with LPS which engages TLR signaling. As expected, in the presence of LPS *Pcgf6* mRNA levels were downregulated and the levels of *IL-12p40* were upregulated indicating that the DCs are activated **(Figure 5A)**. Inhibition of MEK1/2 signaling prior to activation did not have an effect on *Pcgf6* expression.

Another pathway we examined, NF-κB pathway is typically activated upon engagement of various TLRs in a MyD88-dependent manner (Napetschnig and Wu, 2013). The NF-κB pathway can also be activated downstream of CLR signaling both in a TLR-dependent and independent manner (Geijtenbeek and Gringhuis, 2009). Once activated, NF-κB translocates into the nucleus and activates the transcription of proinflammatory cytokines and chemokines

(Napetschnig and Wu, 2013) . BAY 11-7082 was used to inhibit NF-kB signaling in resting DCs as well as DCs stimulated with LPS. Inhibition of NF-κB signaling did not have an effect on *Pcgf6* mRNA expression at steady state **(Figure 5B)**. In the presence of LPS *Pcgf6* mRNA levels were significantly downregulated. Inhibition of NF-κB signaling prior to activation did not have an effect on *Pcgf6* expression **(Figure 5B)**. IL-12p40 is one of the target genes activated downstream of NF-κB and as expected DCs treated with the NF-κB inhibitor prior to activation had decreased levels of *IL-12p40* mRNA. Overall, our data suggests that MEK1/2 and NF-κB are not responsible for mediating *Pcgf6* downregulation post LPS stimulation.

3.3 Syk signaling is partially responsible for mediating *Pcgf6* downregulation in response to Zym stimulation.

Syk is a tyrosine kinase that is activated downstream of TLR-independent CLR signaling cascades which result in the activation of NFκB as well as NLRP3 inflammasome in some cases (Geijtenbeek and Gringhuis, 2009) (Gross et al., 2009). DCs were treated with R406 to specifically inhibit Syk signaling in resting and stimulated DCs. DCs were activated with Zym in order to induce CLR-dependent Syk activation. Inhibition of Syk did not have an effect on *Pcgf6* mRNA levels at steady state **(Figure 6)**. *Pcgf6* mRNA levels were significantly downregulated post Zym stimulation. DCs pre-treated with Syk inhibitor prior to stimulation had higher levels of *Pcgf6* compared to DCs stimulated with Zym alone **(Figure 6)**. More specifically, treatment of DCs with Zym alone reduced *Pcgf6* mRNA levels by 65% but in the presence of R406 there was only a 54% reduction. The increase in *IL-12p40* mRNA levels was significantly greater in DCs pre-treated with Syk inhibitor prior to stimulation. This data is consistent with previous research which has shown that Syk activation leads to production of IL-10 which in turn suppresses IL-12 production (Slack et al., 2007). Following inhibition of Syk, IL-10 production

is suppressed and therefore IL-12 production is enhanced. Overall, this data suggests that Syk is partially responsible for mediating Zym-dependent *Pc*gf6 downregulation.

3.4 PI3K is partially responsible for mediating *Pcgf6* downregulation post LPS stimulation.

PI3K/Akt signaling can be activated upon engagement with various TLRs as well as downstream of CLR/Syk signaling (Plato et al., 2013) (Troutman et al., 2012). Cells were treated with Ly294002 to inhibit PI3K in unstimulated DCs and DCs activated with LPS. Inhibition of PI3K signaling did not have an effect on *Pcgf6* mRNA expression at steady state. DCs pre-treated with PI3K inhibitor prior to stimulation had significantly higher levels of *Pcgf6* compared to DCs stimulated with LPS alone **(Figure 7)**. More specifically, LPS stimulation reduced *Pcgf6* mRNA levels by approximately 70% whereas in the presence of the PI3K inhibitor there was only a 34% reduction. Our data suggests that PI3K plays a role in regulating LPS-mediated downregulation of *Pcgf6*.

3.5 p38 MAPK signaling plays a significant role in regulating *Pcgf6* expression at steady state and following differential activation.

One of the pathways triggered by MyD88-dependent TLR signaling is the p38 MAPK pathway and it is responsible for the activation of AP-1 and CREB (De Nardo, 2015). Syk-independent signaling also activates MAPKs via activation of Raf1 (Gringhuis et al., 2009). SB202190 was used to inhibit p38 MAPK in resting DCs and DCs stimulated with LPS, Zym and Curdlan. DCs treated with the p38 MAPK inhibitor had significantly higher levels of *Pcgf6* mRNA compared to control suggesting that p38 MAPK is responsible for regulating *Pcgf6* expression at steady state **(Figure 8A)**. *Pcgf6* mRNA levels were significantly reduced following stimulation of DCs with LPS, Zym and Curdlan. When DCs are pre-treated with p38 MAPK

inhibitor prior to stimulation *Pcgf6* is no longer significantly downregulated. More specifically, LPS stimulation significantly reduced the *Pcgf6* mRNA levels by 68% whereas in DCs pre-treated with p38 MAPK inhibitor there was only a 37% reduction. Zym stimulation significantly reduced *Pcgf6* mRNA levels by 60% but in the presence of p38 MAPK inhibitor there was only a 30% reduction. Finally, stimulation with Curdlan resulted in an average of 44% downregulation in *Pcgf6* mRNA levels but treatment with p38 MAPK inhibitor reduced this downregulation to 20%. Overall our data shows that inhibition of p38 MAPK signaling induces expression of *Pcgf6* at steady state and blocks significant downregulation of *Pcgf6* following differential activation.

In order to further examine the regulatory role of p38 MAPK we investigated p38 MAPK targets that could potentially regulate *Pcgf6*. One of the candidates we examined was glycogen synthase kinase 3 (GSK3), a serine/threonine protein kinase. GSK3 was of interest was because it regulates many transcription factors that are activated downstream of TLR and CLR signaling such as NF-κB, AP-1 and CREB. GSK3 is also implicated in many diseases including inflammatory diseases, diabetes and cancer (Beurel et al., 2015). Finally GSK3 is inactivated by p38 MAPK by direct phosphorylation (Thornton et al., 2008). In order to test its regulatory effect on *Pcgf6* we pretreated unstimulated and LPS stimulated cells with GSK3 inhibitor. While there was a trend, GSK3 inhibitor did not have a significant effect on *Pcgf6* mRNA levels **(Figure 9)**. Therefore, our data suggests that GSK3 may have a minor effect in mediating *Pcgf6* downregulation following LPS stimulation.

3.6 Downregulation of *Pcgf6* post stimulation is mainly dependent on transcriptional activity of the gene.

mRNA downregulation can be mediated by several mechanisms such as a reduction in transcription activity of the gene or decay of the mRNA. To examine whether the downregulation of *Pcgf6* post stimulation is dependent on transcriptional activity of the gene, DCs were treated with actinomycin D (AcD), an inhibitor of global transcription. AcD was added to unstimulated and LPS-stimulated DCs and *Pcgf6* mRNA levels were measured over a period of 6 hours. *Pcgf6* mRNA levels decreased approximately 80% after a 6-hour treatment with AcD **(Figure 10A)**. The half-life of *Pcgf6* mRNA at steady state is 2.19 hours. The stability of *Pcgf6* was not significantly affected by treatment with LPS (half-life=2.54) **(Figure 10A)**. This data suggests that the downregulation of *Pcgf6* post stimulation is mainly dependent on transcriptional activity of the gene.

Treatment with AcD could be blocking transcription of a factor that is typically upregulated upon activation. Therefore, a second experiment was performed whereby DCs were pre-incubated with LPS for 2 hours before the addition of AcD to allow the upregulation of factors that may regulate *Pcgf6* expression. Relative mRNA levels of *Pcgf6* were measured every hour for 4 hours after the addition of AcD. The half-life of *Pcgf6* is 2.29 hours **(Figure 10B)**. LPS stimulation slightly destabilizes *Pcgf6* mRNA demonstrated by the reduction in half-life to 1.74 hours. This effect, although not statistically significant (p=0.06), suggests that post-transcriptional mechanisms may also play a role in maintaining *Pcgf6* levels **(Figure 10B)**.

3.7 Transcription factors predicted to bind the promoter of *Pcgf6*, p53 and STAT3 do not regulate its expression.

To investigate potential regulators of *Pcgf6* we identified transcription factors with predicted binding sites on the *Pcgf6* gene via ECR browser (www.ecrbrowser.dcode.org) and the encyclopedia of DNA elements, ENCODE (www.encodeproject.org) **(Figure 11)**. The first transcription factor of interest was p53 which is most well-known for its role as a tumor suppressor. Some of its functions include induction of apoptosis and angiogenesis inhibition (Shao et al., 2000). p53 reduces NFκB-dependent gene expression in TLR4-stimulated DCs (Dijsselbloem et al., 2007). This led us to hypothesize that p53 may be mediating its anti-inflammatory role by promoting *Pcgf6* expression. At steady state, p53 is kept in an inactive state by interacting with its inhibitor, mouse double minute 2 homolog (MDM2) which mediates its ubiquitination and targets it for degradation (Brooks and Gu, 2006). Following various stress stimuli such as oxidative stress or DNA damage, signaling pathways are activated that lead to the dissociation of mdm2 from p53 allowing it to get phosphorylated and activated (Moll and Petrenko, 2003). Nutlin-3a, an inhibitor of MDM2 stabilizes p53 activity by blocking the p53:MDM2 interaction (Drakos et al., 2007). In order to test p53 as a potential regulator of *Pcgf6* cells were pretreated with Nutlin-3a for 1 hour and then stimulated with LPS for 2 hours. Nutlin-3a did not have an effect on *Pcgf6* mRNA levels indicating that p53 does not regulate expression of *Pcgf6* **(Figure 12A)**. As an alternative method, we generated DCs from p53 heterozygous and knockout mice and measured *Pcgf6* mRNA levels in unstimulated and stimulated DCs. No difference was seen in *Pcgf6* expression validating the results seen with nutlin-3a treatment **(Figure 12B)**. This data suggests that p53 does not regulate *Pcgf6* transcription in this context.

Another transcription factor we examined was signal transducer and activator of transcription 3 (STAT3). We chose to investigate STAT3 not only because it is predicted to bind to the promoter of *Pcgf6* but also because it is a well-known DC suppressor activated downstream of IL-10 signaling (Yasukawa et al., 2003). Conditional knock out of STAT3 in DCs results in increased production of pro-inflammatory cytokines and enhanced ability to activate T cells (Melillo et al., 2010). This led us to hypothesise that STAT3 may be mediating its negative regulatory effect via induction of *Pcgf6*. In order to test STAT3 as a potential regulator of *Pcgf6* cells were pretreated with STAT3 inhibitor and stimulated with LPS for 6 hours and overnight **(Figure 13)**. In both experiments the STAT3 inhibitor did not have an effect on *Pcgf6* mRNA expression indicating that STAT3 does not regulate *Pcgf6* transcription in this context.

3.8 IL-4 induces *Pcgf6* expression at steady state and in the context of LPS and HDM stimulation.

STAT6 is predicted to bind to the *Pcgf6* gene and it is activated downstream of IL-4 and IL-13 which mediate anti-inflammatory effects by inhibiting IL-12 and TNF-α production by DCs (Levings and Schrader, 1999) (Seegmüller et al., 2003). In order to test STAT6 as a potential regulator of *Pcgf6* cells were treated with IL-4 alone or in the context of Th1-promoting stimulant, LPS as well as Th2-promoting stimulant HDM. IL-4 treatment induced the expression of *Pcgf6* at steady state **(Figure 14A)**. DCs treated with IL-4 and LPS had significantly higher levels of *Pcgf6* mRNA compared to DCs treated with LPS alone. The same trend was seen when IL-4 was added to HDM-stimulated DCs, although this difference was not statistically significant. *IL-12p40* mRNA levels were increased following LPS stimulation but to a lesser

extent in the presence of IL-4 and LPS demonstrating the anti-inflammatory effects of IL-4 **(Figure 14B)**. This result is consistent with previous literature which has shown that IL-4 inhibits IL-12-40 production in DCs (Seegmüller et al., 2003).

In order to investigate the role of STAT6 more directly we measured the expression of *Pcgf6* in DCs generated from STAT6 knockout mice compared to DCs generated from wild type mice. DCs were stimulated with LPS and HDM for 2 hours and 18 hours in the presence or absence of IL-4 and IL-13 to induce STAT6 activation. Our data suggests a trend whereby *Pcgf6* levels in DCs derived from STAT6 KO mice were reduced in all conditions compared to wild type regardless of IL-4 and IL-13 presence **(Figure 15)**. This data suggests that there are other signaling pathways mediating the STAT6 regulatory effect on *Pcgf6*.

3.9 *Pcgf6* is differentially regulated in macrophages compared to DCs.

The effects of IL-4 are more well-defined in macrophages which led us to investigate the regulation of *Pcgf6* in macrophages in comparison to DCs. Activation of STAT6 signaling through treatment with IL-4 skews macrophages towards an alternative activation phenotype which promotes Th2 polarization (Wang et al., 2014). This differs from the classical activation of macrophages which polarizes them towards a Th1 response (Wang et al., 2014). Since IL-4 treatment induces *Pcgf6* expression in DCs we hypothesized that *Pcgf6* may play a role in promoting alternative activation. To examine this possibility macrophages were stimulated with 10 ng/mL of LPS and 50 ng/mL of IFN-γ overnight to promote a classical activation phenotype and 20 ng/mL of IL-4 overnight to induce an alternative activation phenotype. The same conditions were applied to DCs for direct comparison. There was only slight reduction in *Pcgf6* mRNA levels for both IFN-γ-stimulated and IL-4-stimulated macrophages **(Figure 16A)**.

Overnight stimulation with LPS and IFN-γ induced an 80% downregulation of *Pcgf6* in DCs and no change was seen post IL-4 treatment **(Figure 16B)**. This data suggests that it is unlikely that PCGF6 is promoting alternative activation and that *Pcgf6* is regulated differently in macrophages compared to DCs.

3.10 Negative regulators of DCs IL-10, PGE2 and TGF-β do not regulate *Pcgf6* mRNA expression.

Since PCGF6 acts as a suppressor of DC function we hypothesized that other well-known negative regulators of DCs may regulate its expression. First we investigated IL-10, prostaglandin E2 (PGE2) and TGF-β. Previous studies have shown that IL-10 treatment induces a tolerogenic phenotype in DCs (Steinman and Hawiger, 2003). Tolerogenic DCs are characterized by an inability to fully mature following stimulation. They also promote differentiation of T cells towards a Treg phenotype by providing signal 1 without signals 2 and 3 (Steinman and Hawiger, 2003). Previous research has demonstrated that PGE2 promotes the differentiation of DCs to myeloid-derived suppressor cells (MDSC) (Obermajer et al., 2011). In order to test whether PGE2 or IL-10 regulate *Pcgf6*, DCs were pretreated with IL-10 or PGE2 for 1 hour and stimulated with LPS. There was no difference seen in *Pcgf6* mRNA levels at steady state or following activation **(Figure 17A)**. DCs pre-treated with rIL-10 and PGE2 prior to LPS stimulation had lower *IL-12p40* mRNA levels compared to LPS alone, demonstrating that DC activation was inhibited **(Figure 17B)**. We also investigated TGF-β which mediates its regulatory effects by inhibiting the upregulation of costimulatory molecules such as CD80/CD86 upon stimulation (Strobl and Knapp, 1999). This in turn inhibits the DCs ability to promote T cell proliferation and activation. Unstimulated and LPS stimulated DCs were pretreated with

10ng/mL of TGF-β and *Pcgf6* mRNA levels were measured. Our data showed no significant difference between the different conditions **(Figure 17C)**. Together these results suggest that conditioning with TGF-β cannot prevent LPS-mediated *Pcgf6* downregulation.

4. DISCUSSION

The immune system has evolved mechanisms to recognize and respond to a wide variety of pathogens. DCs are innate immune cells that are among the first responders in the presence of infection or injury and are important players in shaping immune responses. Once DCs encounter pathogen in the periphery they migrate to lymph nodes where they are able to interact with and activate other cells of the immune system in order to prime antigen-specific adaptive immunity (Mäkelä et al., 2009) . The ability of DCs to switch from their quiescent state to an active state underlies their ability to exert these activities. DCs interact with pathogens through the engagement of PRRs which trigger a series of downstream signaling cascades including NF-kB, MAPK and PI-3K (Kawai and Akira, 2010) . These pathways lead to the activation of a variety of transcription factors that change the expression of genes responsible for mediating DC maturation. DC activation is followed by an up-regulation of costimulatory molecules, enhanced ability to present antigen and production of pro-inflammatory cytokines such as IFNs, ILs and chemokines (Mäkelä et al., 2009) .

Since their discovery, most research regarding DCs has centered on discovering the mechanisms required to prime their activation/maturation. More recent research has emphasized the importance of negative regulators of DC activation in establishing immune homeostasis. This is particularly important for the prevention of misdirected or defective immune responses which contribute to chronic inflammation and autoimmune diseases. Previous research has demonstrated the role of STAT3 as an important repressor of DC activation (Melillo et al., 2010). STAT3-deficient DCs display a more activated phenotype as demonstrated by an increase in production of proinflammatory cytokines as well as enhanced efficiency in priming antigen

specific T-cell responses (Melillo et al., 2010). The activation of STAT3 via IL-6 signaling suppresses antigen presentation in human DCs (Kitamura et al., 2014). Furthermore, STAT3 conditional knockout mice develop lymphadenopathy and ilecolitis (Melillo et al., 2010). A recent study has demonstrated the role of Blimp-1 as a negative regulator of DC activation (Kim et al., 2011). Blimp-1 is a transcription factor best known for its role as a repressor of IFN-β expression (Keller and Maniatis, 1991). Blimp-1-deficient DCs display an enhanced activation phenotype and female mice with Blimp-1-deficient DCs develop a lupus-like serology (Kim et al., 2011). Previous studies have also emphasized the role of A20 as negative regulator of DC function. A20 is an anti-inflammatory protein known for its role as a negative regulator of NFκB signaling via the disruption of ubiquitin enzyme complexes (Shembade et al., 2010). A20-deficient DCs are spontaneously activated as shown by an increase in ctyokine production and enhanced T-cell activation (Hammer et al., 2011). Furthermore, A20 is important in preventing development of colitis and spondylarthritis (Hammer et al., 2011).

Recent work in our lab has identified PCGF6 as a novel negative regulator of DC activation and function. PCGF6 is a component of Polycomb group (PcG) multiprotein PRC1-like complexes, which play a role in transcriptional repression (Zdzieblo et al., 2014). We have shown that *Pcgf6* is rapidly downregulated upon differential activation of DCs. In addition to this, DCs overexpressing PCGF6 fail to mature as demonstrated by a reduced expression of costimulatory molecules and production of pro-inflammatory cytokines (Boukhaled et al., 2016). Furthermore, their poor efficiency in priming T cell responses demonstrates the functional relevance of PCGF6 (Boukhaled et al., 2016). DCs with reduced expression of PCGF6 have higher levels of costimulatory molecules as well as enhanced production of IL-12p40 both at rest and following stimulation (Boukhaled et al., 2016). *Pcgf6* downregulation is required for the

ability of DCs to become fully activated following stimulation. Our data also demonstrates an inverse correlation between *Pcgf6* expression and extent of activation of the pro-inflammatory phenotype. Stronger activators that induce a more pro-inflammatory response such as Zym and LPS promote the downregulation of *Pcgf6* to a higher extent compared to weak activators such as HDM and ZymD **(Figure 4A,B)**. This suggests that the downregulation of *Pcgf6* is not an all or none response but is a graded response. As such, PCGF6 may be a fine-tuner of DC activation. Further investigation into the kinetics of its downregulation showed that it can be seen as early as 2 hours and is sustained for up to 36 hours even though levels of IL-12p40 recede overtime and DCs begin to quiesce **(Figure 4C)**. This result suggests that downregulation of *Pcgf6* is immediate and maintained following stimulation.

The transcript levels of a gene can be downregulated by a reduction in the transcription activity of the gene or mRNA decay via post-transcriptional mechanisms. To examine whether downregulation of *Pcgf6* following activation is dependent on the transcriptional activity of the gene, unstimulated and LPS-stimulated DCs were treated with actinomycin D an inhibitor of RNA polymerase II mediated global transcription (Cassé et al., 1999). Stimulation of AcD-treated DCs with LPS had a minor but not significant effect on the stability of *Pcgf6* mRNA suggesting that transcriptional regulation plays the most significant role in modulating *Pcgf6* levels post stimulation **(Figure 10)**. In order to examine the factors responsible for transcriptionally regulating *Pcgf6* we began by identifying which molecules downstream of PRR signaling are responsible for regulating the expression of *Pcgf6.* Although there is a wide variety of PRRs, they all initiate downstream signaling cascades that converge in the activation of PI3K, NFkB and MAPKs pathways. Our results showed that *Pcgf6* downregulation is independent of MEK and NFκB signaling pathways **(Figure 5)**. However, *Pcgf6* downregulation was partially

dependent on Syk, PI3K and p38 MAPK signaling pathways with p38 MAPK having the greatest effect **(Figure 6, 7, 8)**.

Syk activity was found to be partially responsible for mediating *Pcgf6* downregulation following Zym activation. Syk is a tyrosine kinase and the first signal transduction molecule recruited and activated downstream of CLRs. A wide variety of intermediate proteins including phospholipase Cγ2 (PLCγ2), PI3K and IκB kinase (IKK) are activated downstream of Syk signaling (Plato et al., 2015) (Whitney et al., 2014). These proteins trigger downstream signaling cascades that result in activation of transcription factors that mediate receptor-specific effects (Kerrigan and Brown, 2011). Upon activation, Syk can activate transcription factors NFκB and nuclear factor of activated T cells (NFAT) to induce the expression of chemokine and pro-inflammatory cytokines (Goodridge et al., 2007). Zymosan is well known to activate NFAT transcription factors upon engagement of Dectin-1 (Goodridge et al., 2007). Furthermore, previous research has shown that NFAT3, one of the members of the NFAT family of transcription factors can act as a transcriptional repressor (Ngyen et al., 2009). Together these data raise the possibility that Zymosan-dependent Syk activation may mediate *Pcgf6* downregulation via NFAT activity. Syk-dependent CLR signaling is widely recognized for its role in anti-fungal immunity (Hardison and Brown, 2012). More recent research however has also indicated the importance of CLR signaling in the recognition of some bacterial and viral pathogens (Hoving et al., 2014). The activation of Syk-dependent CLR signaling triggers many cellular responses including DC activation and cytokine production (Hoving et al., 2014). All together these data suggest that *Pcgf6* downregulation allows DCs to fully mature and elicit DC-driven innate responses against pathogens recognized by Syk- coupled CLRs. Syk can also be activated by TLRs, more specifically TLR4 and TLR2/6 (Miller et al., 2012). TLR4 activation

of Syk enhances cytokine production via activation of transcription factor AP-1 (Miller et al., 2012). This raises the possibility that Syk signaling regulates that transcription activity of *Pcgf6* via AP-1 activity or the activity of AP-1 target genes.

Our data showed that PI3K signaling is also important for LPS-mediated *Pcgf6* downregulation. Evidence on the role of PI3K signaling downstream of TLRs in dendritic cells is currently unclear. Some studies suggest that PI3K signaling has a positive role in the expression of pro-inflammatory cytokines while others suggest the opposite (Hawkins and Stephens, 2015) . Our data complements studies that have demonstrated the importance of PI3K/mTOR signaling in positively regulating many aspects of DC biology such as activation and function. These studies have shown that inhibition of the PI3K pathway blocks the DCs ability to mature as shown by reduced expression of costimulatory molecules and cytokines as well as reduced ability to activate T cells (Mineharu et al., 2014). mTOR activation downstream of PI3K signaling modulates the expression of a series of transcription factors including STAT3, CREB and class II co-activator (CIITA) which in turn alter gene transcription patterns. One of the transcription factors activated downstream of PI3K in dendritic cells upon activation is Twist-related protein 2 (TWIST2) which subsequently activates transcription factor MAF (Weichhart et al., 2015). Transcription factor ChiP-Seq data from ENCODE shows that MAF transcription factors bind to the *Pcgf6* gene suggesting that TLR-dependent PI3K signaling may modulate *Pcgf6* transcription via MAF activity. As discussed, PI3K can also be activated downstream of CLR signaling. Engagement of C-type lectins DC-SIGN and MINCLE can activate PI3K signaling (Caparrós et al., 2006) (Geijtenbeek and Gringhuis, 2016). In the context of MINCLE activation PI3K activates MDM2 which transclocates to the nucleus and targets IRF1 for proteasomal degradation (Geijtenbeek and Gringhuis, 2016). Decreased activity of IRF1 results

in reduced transcription of its target genes, including IL-12p35 which promotes Th2 responses in order to fight fungal infections (Geijtenbeek and Gringhuis, 2016). Transcription factor ChiP-Seq data from ENCODE shows that IRF1 has two binding sites on the *Pcgf6* gene. Together these data suggest an interesting model whereby CLR-dependent PI3K activation may mediate *Pcgf6* downregulation by reducing protein levels of IRF1. As previously discussed, PI3K can also be directly activated downstream of Syk signaling suggesting that the two act sequentially to regulate *Pcgf6* expression. In our experiments so for we have only looked at the role of PI3K signaling in the context of TLR signaling but it would be an interesting future direction to also look at it in the context of CLR signaling.

p38 MAPK signaling was found to have the greatest effect on modulating *Pcgf6* expression. Our data showed that inhibition of p38 MAPK signaling at steady state results in increased levels of *Pcgf6* mRNA suggesting that p38 MAPK is important in regulating *Pcgf6* levels at rest. p38 MAPK activity is also really important in promoting *Pcgf6* downregulation following stimulation. In the presence of p38 MAPK inhibitor *IL-12p40* is upregulated following stimulation even though *Pcgf6* mRNA levels are not downregulated suggesting that *IL12-p40* expression can be modulated through parallel mechanisms and that *Pcgf6* expression may not always be coupled to *IL-12p40* mRNA levels. Likewise, a similar phenotype was seen with rIL10 treatment, whereby *IL-12p40* expression is suppressed even though *Pcgf6* is still downregulated. Overall, our data complements many other studies that have demonstrated the importance of p38 MAPK signaling in modulating DC activation. Activation of p38 MAPK signaling induces the production of various pro-inflammatory cytokines such as IL-1β and IL-6 by DCs (Zarubin and Han, 2005). Furthermore, inhibition of p38 MAPK inhibitor SB203580 prevented LPS and TNFα-mediated upregulation of costimulatory molecules, CD80/86 and

CD40 (Arrighi et al., 2001). Our data also contributes to emerging evidence that p38 MAPK signaling is important in regulating gene expression by modulating the activity of chromatin remodellers. Many genes important for mediating the inflammatory response such as IL-6 and IL-12p40 have an enrichment in H3 phosphorylation mediated by p38 MAPK signaling (Cuadrado and Nebreda, 2010). PCGF6 functions within complexes which also act via chromatin remodelling. Previous research indicates that p38 MAPK overexpression inhibits the activation of SHP-1 following CD-40-induced signaling (Khan et al., 2014). SHP-1, like PCGF6 is a negative regulator of DC function. Deletion of SHP-1 results in spontaneous activation in DCs and enhanced production of proinflammatory cytokines (Ganguly et al., 2013). This data suggests that p38 MAPK signaling promotes DC activation not only by inducing expression of genes important for mediating an inflammatory response but also by inhibiting the expression of negative regulators.

Given that *Pcgf6* downregulation is mostly mediated by changes in the transcriptional activity of the gene, it is more likely that p38 MAPK is mediating its effects by modulating the transcription of *Pcgf6* rather than destabilizing the mRNA. p38 MAPK signaling leads to the activation of many transcription factors that are important in DC biology. One of the targets investigated was GSK3, a serine/threonine protein kinase. The activity of GSK3 is inhibited by p38 MAPK via direct phosphorylation (Thornton et al., 2008). GSK3 plays an important role in inducing IL-12 production in dendritic cells stimulated with LPS (Martin et al., 2005). GSK3 became of particular interest because it is also a downstream target of PI3K, another pathway that regulates *Pcgf6* downregulation. Inhibition of GSK3 did not have a significant effect on *Pcgf6* expression, although a trend was seen. This result suggests that GSK3 plays a minor role in p38 MAPK-mediated *Pcgf6* downregulation.

Another p38 MAPK downstream target of interest is ATF3, a member of the ATF/CREB family of transcription factors which bind to CRE sequences of their target genes. ATF3 can act as a transcriptional repressor or activator depending on what other ATF/CREB proteins it is partnering with (Hai et al., 1999). It can also be alternatively spliced into a shorter isoform which does not contain the leucine zipper used for binding either to DNA or other ATF/CREB proteins (Chen et al., 1994). ATF3 is found in low concentrations at steady state and is induced in response to various stress signals such as DNA damage and cytokines (Hai et al., 1999) (Fan et al., 2002). Previous research has shown that many TLR agonists including LPS induce expression of ATF3 in macrophages and DCs (Whitmore et al., 2007). ATF3 knockout mice have enhanced expression of IL-12 and IL-6 in response to stimulation suggesting a role for ATF3 as a suppressor of TLR signaling (Whitmore et al., 2007). It has also been demonstrated that stress signals induce ATF3 expression specifically via p38 MAPK activation (Lu et al., 2007). In the presence of p38 MAPK inhibitor LPS and Curdlan-mediated ATF3 induction is reduced (data not shown). Furthermore, transcription factor ChiP-Seq data from ENCODE shows that ATF3 binds to the promoter of *Pcgf6*. In order to test the potential modulatory role of ATF3 we designed a hairpin to target and downregulate it. Our hairpin did not however have a substantial effect on ATF3 post LPS stimulation and therefore we cannot make any final conclusions on its effect on *Pcgf6* (data not shown). Our next step will be to examine the expression of *Pcgf6* in DCs generated from ATF3 knockout mice compared to DCs generated from wild type mice. Another p38 target that may regulate *Pcgf6* is CEBPβ. CEBPβ is activated downstream of p38 MAPK and it has multiple binding sites on the *Pcgf6* gene and promoter as shown by ChiP-Seq data from ENCODE. CEBPβ has been shown to act as a chromatin-regulating factor in monocyte derived DCs (Natoli et al., 2011).

In parallel to investigating which signaling molecules downstream of PRR signaling are responsible for mediating *Pcgf6* downregulation we also chose an alternative approach to identify transcription factors that regulate *Pcgf6* expression. We used ECR browser (www.ecrbrowser.dcode.org) to identify transcription predicted to bind to the *Pcgf6* gene. With the help of Vassil Dimitrov from Dr. John White's lab we also used ENCODE, a database of ChiP-seq data to identify transcription factors that bind the *Pcgf6* gene. (www.encodeproject.org) **(Figure 11)**. We found many transcription factors that bind to *Pcgf6* but focused on the ones that are important in DC biology, particularly ones that have a negative effect on DC activation. We examined STAT3, a transcription factor activated by janus kinases (JAK)-mediated phosphorylation in response to IL-10 and IL-6 (Yasukawa et al., 2003). STAT3 is of particular interest because it is predicted to bind to *Pcgf6* on two separate sites, one of which is in the promoter region. As discussed previously, STAT3 is also a well-known DC suppressor. Stimulation of DCs with conditional deletion of STAT3 acquire a more activated phenotype reflected by increased production of pro inflammatory cytokines and enhanced capacity to activate T cells (Melillo et al., 2010). Furthermore, recent work in our lab has shown that DCs overexpressing PCGF6 produce more IL-10 (Boukhaled et al., 2016). This raises the possibility that IL-10 may feedback on the DCs to induce PCGF6 expression in a STAT3-dependent manner. Treatment of DCs with STAT3 inhibitor and IL-10 however did not affect *Pcgf6* mRNA levels suggesting that IL-10-STAT3 signaling does not regulate the expression of *Pcgf6* **(Figure 13, 17)**. Given that STAT3 can inhibit NFκB signaling, this result also complements our previous data showing that NFκB does not regulate *Pcgf6* expression. For further validation we will also treat the cells with IL-6, a STAT3 activator and see its effects on *Pcgf6* expression.

We also examined p53 which is best known for its role as a tumor suppressor mediated by various mechanisms including the control of gene stability, apoptosis as well as inhibition of inflammatory responses (Komarova et al., 2005). Its suppressive roles on inflammation are mediated by inhibiting NFκB activity and cytokine production post stimulation (Liu et al., 2009). We hypothesized that a complementary role for p53 would be to inhibit DC function by inducing *Pcgf6*. We tested its effects by measuring *Pcgf6* mRNA levels in DCs derived from p53 knockout mice as well as DCs treated with nutlin-3a, a p53 stabilizer. There was no change seen in *Pcgf6* mRNA levels suggesting that p53 does not regulate the transcription of *Pcgf6* **(Figure 12)**. Given that p53 can suppress NFκB activity, this result complements our previous data showing that NFκB does not regulate *Pcgf6* expression.

Finally, we investigated the regulatory role of STAT6, a transcription factor activated downstream of IL-4 and IL-13 (Hou et al., 1994). STAT6 is of particular interest because it is predicted to bind *Pcgf6* and IL-4 treatment mediates anti-inflammatory effects by inhibiting IL-12 and TNF-α production (Levings and Schrader, 1999) (Seegmüller et al., 2003). Conditioning DCs derived from C57BL/6 mice with IL-4 induced *Pcgf6* expression at steady state and in the context of LPS and HDM stimulation **(Figure 14)**. In order to test the effects of STAT6 more directly we measured the levels of *Pcgf6* mRNA in DCs generated from BALB/c STAT6 knockout mice compared to DCs generated from BALB/c wild type mice. In this set of experiments IL-4 treatment did not induce *Pcgf6* expression at steady state which contradicts our previous data. This result may be explained by a difference in genetic background of the mice. C57BL/6 mice are more Th1 dominant while BALB/c mice are a prototypical Th2 mouse strain. Since BALB/c mice are more Th2 dominant it is possible that they already have higher levels of *Pcgf6* and conditioning with IL-4 does not induce *Pcgf6* expression further. Our data suggests a

trend whereby DCs derived from STAT6 knockout mice have lower *Pcgf6* mRNA levels compared to wild type control regardless of IL-4 and IL-13 presence suggesting that there are other mechanisms promoting the STAT6 effect **(Figure 15)**. Once possibility is that STAT6 is mediating its effects through interactions with other proteins. Previous data has shown that STAT6 can interact with IRF4, a transcription factor that can be activated downstream of HDM signaling and drives DCs to induce a Th2 response (Lohoff and Mak, 2005) (Williams et al., 2013). IRF4 can bind *Pcgf6* promoter and is known to suppress TLR signaling (Negishi et al., 2005). We will test the potential regulatory role of IRF4 by measuring *Pcgf6* mRNA levels in DCs derived from IRF4 knockout mice compared to wild type mice. We will also investigate the role of PPARγ-coactivator-1β (PGC-1β), one of the transcriptional coactivators induced by STAT6 is. PGC-1β promotes the switch to oxidative metabolism which is important in fueling alternative activation of macrophages (Vats et al., 2006). Preliminary data from our lab shows that PGC-1β is rapidly downregulated upon differential activation of DCs. Taken together, it is possible that PGC-1β or another co-activator and STAT6 are working in concert to induce *Pcgf6* expression in DCs.

We also investigated the role of other DC suppressors PGE2 and TGFβ. PGE2 has been shown to have an inhibitory function on DCs by suppressing their production of CCL3 and CCL4, chemokines important for recruitment of other immune cells (Jing et al., 2003). Other studies have shown that it suppresses DC function by inhibiting MHCII production at the same time as inducing IL-10 production (Harizi et al., 2003). TGF-β mediates its suppressive role by inhibiting the upregulation of costimulatory molecules in the presence of stimuli (Strobl and Knapp, 1999). Conditioning DCs with PGE2 and TGF-β inhibited DC activation as shown by the reduction in *IL-12p40* production but surprisingly it did not change the expression of *Pcgf6*

suggesting that other mechanisms are responsible for modulating *Pcgf6* mRNA levels **(Figure 17)**. Most of the negative regulators we tested did not regulate *Pcgf6* expression suggesting that if the role of PCGF6 is to maintain quiescence then these negative regulators are not important in promoting quiescence but are immunomodulators.

The effects of IL-4 are extensively studied in the context of macrophage activation. Depending on the type of stimulus they encounter, macrophages acquire two main activation phenotypes known as classical activation and alternative activation. Classically activated macrophages are induced by stimulation with LPS and IFN-γ and promote a Th1 response (Cook et al., 2012). As discussed, AAMs are induced by stimulation with IL-4 and induce type 2 immune responses (Cook et al., 2012). We hypothesized that if STAT6 induces *Pcgf6* expression in macrophages, it may play a role in promoting alternative activation. As expected, overnight activation with LPS and IFN-γ resulted in a significant downregulation of *Pcgf6* in DCs. LPS stimulation in macrophages however, only reduced levels of *Pcgf6* mRNA slightly. Overnight IL-4 treatment did not have an effect on *Pcgf6* expression in DCs whereas in macrophages there was a slight downregulation, but not significant **(Figure 16)**. Our results indicate that *Pcgf6* regulation is not the same in DCs and macrophages, suggesting that the role of PCGF6 in DCs may not be translated to macrophages. Furthermore, this data suggests that PCGF6 is regulating a function in DCs that is not shared with macrophages. One such function is the DCs ability to migrate which raises the possibility that one of the functions of PCGF6 may be to suppress DC migration at steady state and following activation PCGF6 is downregulated to allow DCs to migrate.

Our data also suggests that post-transcriptional mechanisms may play a role in destabilizing *Pcgf6* mRNA following LPS activation. Mechanisms responsible for mediating

mRNA turnover include RNA binding proteins and miRNAs. miRNA-mediated post-transcriptional regulation is of particular interest in the lab. miRNAs generally regulate gene expression either via sequence-specific cleavage of the target transcripts, destabilization of mRNA via deadenylation/decapping or translational repression (Beitzinger and Meister, 2011). miR-9, miR-125 and miR-351 are predicted to bind to Pcgf6 via TargetScan. miR-125 is highly expressed in all DC subsets and is an important regulator of macrophage maturation (Banerjee et al., 2013) (Smyth et al., 2015). Previous research has shown that miR-9 is upregulated in differentially activated DCs and it is a negative regulator of inflammatory responses mediated by NFκB signaling (Dueck et al., 2014) (Weber, 2013). Unpublished data from the lab has also shown that miR-9 is upregulated following LPS stimulation and that miR-9 may target PCGF6 to inhibit its expression. Other projects in the lab will further investigate the role of miRNAs in regulating *Pcgf6* expression.

Overall our data suggests that the expression of *Pcgf6* in DCs at steady-state is important in maintaining quiescence. Upon DC activation, *Pcgf6* mRNA is rapidly downregulated to allow full maturation. The engagement of a variety of PRRs that induce a pro-inflammatory phenotype initiate signaling cascades that converge in the activation of Syk, PI3K and p38 MAPK, whose downstream effector targets mediate *Pcgf6* downregulation. STAT6 activation downstream of IL-4, a negative regulator of DC activation promotes *Pcgf6* expression. In contrast to our research and all previous studies, the most recent paper has shown that PCGF6 positively regulates the transcription of various genes important for embryonic stem cell identity (Yang et al., 2016). This data suggests that the function of PCGF6 may change depending on the cell type and the proteins it interacts with. Overall our data demonstrates the importance of inhibitory mechanisms in regulating DC activation and function.

	DC activator	Pattern Recognition Receptor		T helper response
		TLR	CLR	
Highly inflammatory phenotype	Lipopolysaccharide (LPS)	+		Th1
	Zymosan (Zym)	+	+	Th1/Th17
	Curdlan		+	Th17
Less activated phenotype	House Dust Mite (HDM)	+	+	Th2/Th17
	Zymosan-depleted (Zym-D)		+	Th17

Table 1. DC activators and T helper responses induced.

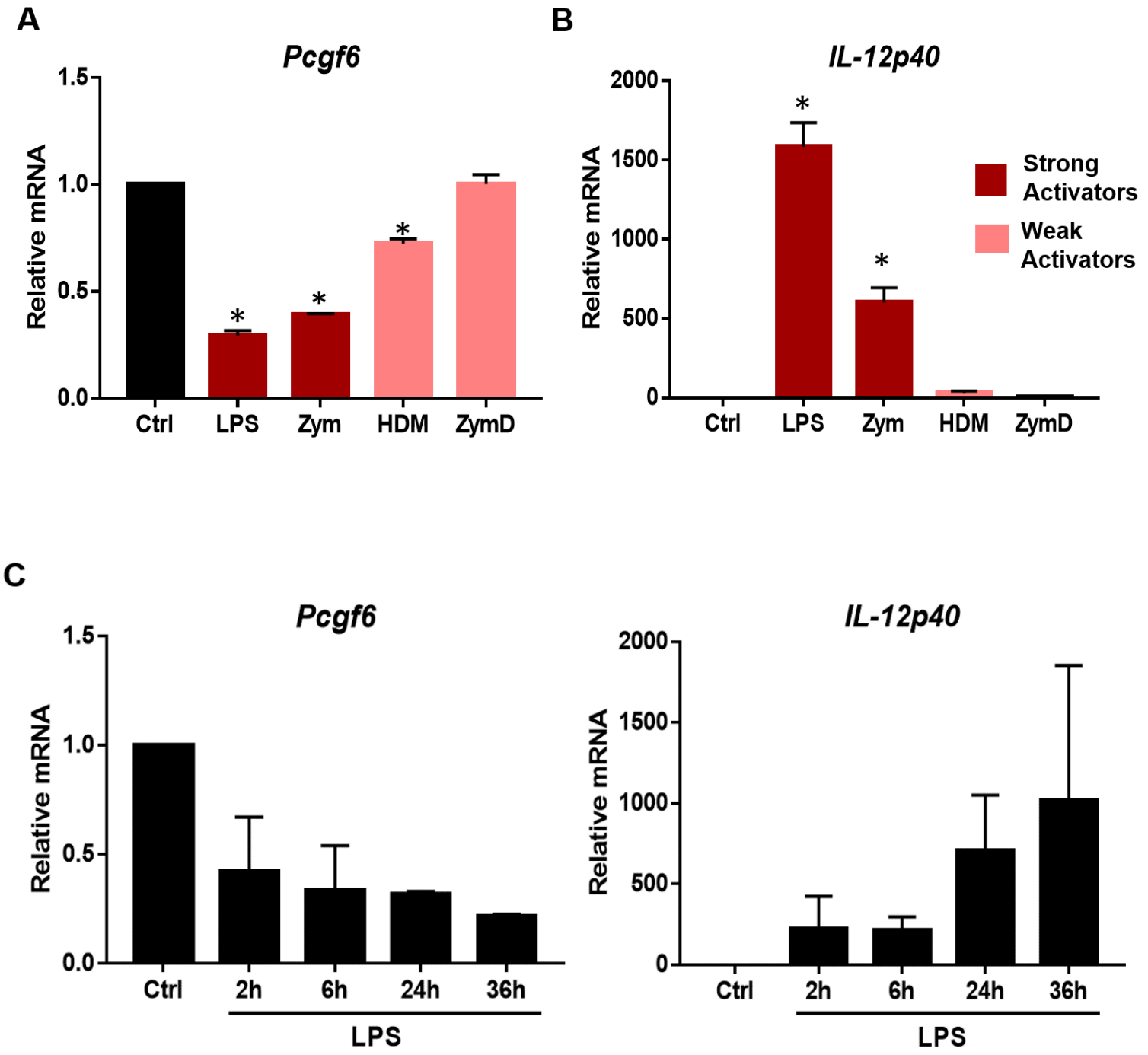


Figure 4. *Pcgf6* expression is downregulated following differential activation of BMDCs. A,B. DCs were stimulated with 10ng/mL of LPS, 50ug/mL of HDM, 10ug/mL of Zym and 10ug/mL of ZymD for 2h. Results are depicted as means ± SE for 3-7 replicates in independent experiments. C. DCs were activated with 10ng/mL of LPS for 2-36h. Results are depicted as means ± SE for 2 independent experiments. A-C. Relative mRNA expression of *Pcgf6* and *IL-12p40* were determined by qRT-PCR. ∗, $p < 0.05$. LPS= lipopolysaccharide; HDM= house dust mite; Zym= Zymosan; ZymD= Zymosan depleted of TLR ligands.

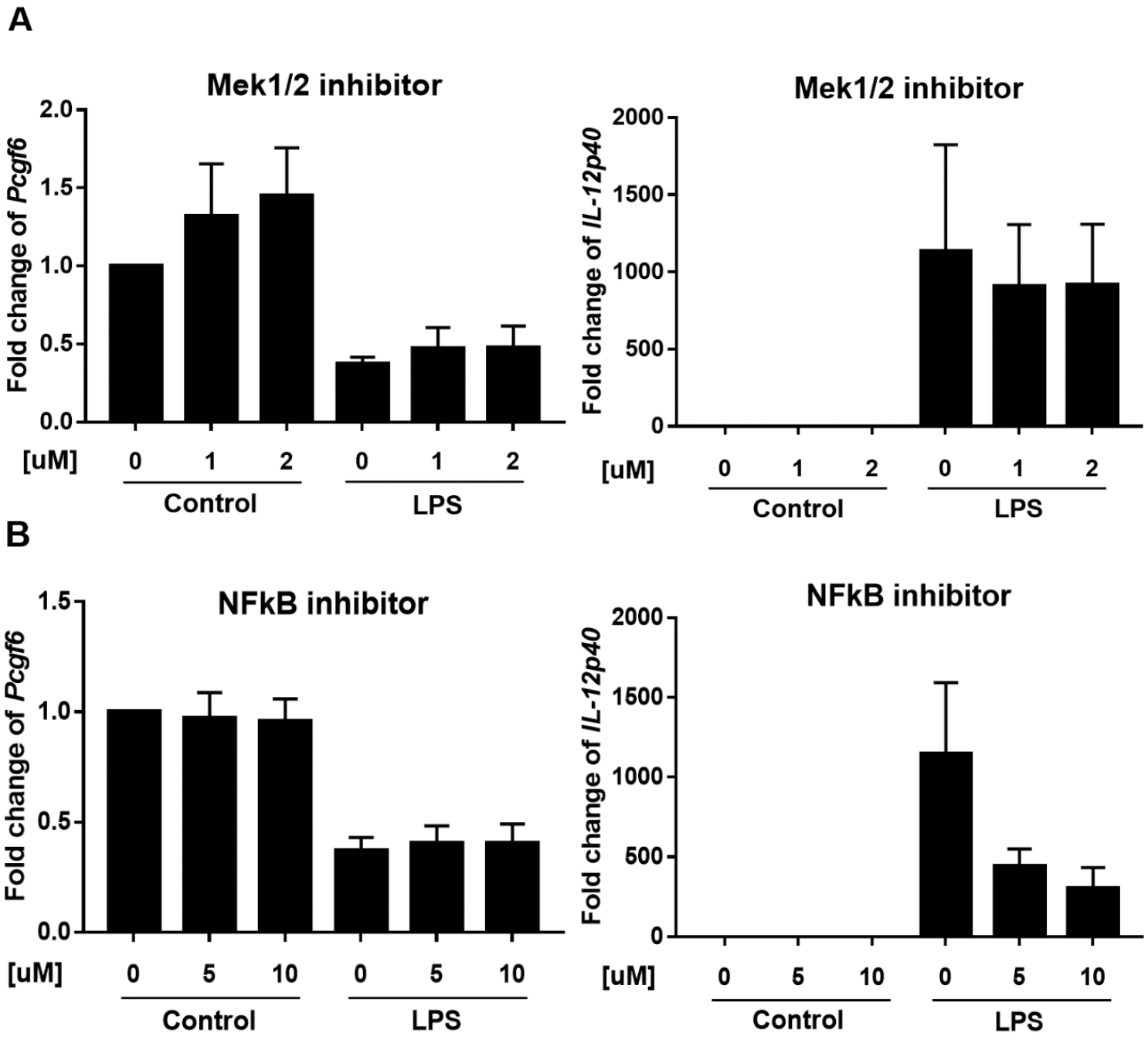


Figure 5. *Pcgf6* downregulation occurs independently of MEK and NFκB signaling. A. DCs were untreated or pretreated with MEK inhibitor PD0325901 for 1h and then unstimulated or stimulated with LPS for 2h. Results are depicted as means ± SE for 3 independent experiments B. DCs were untreated or pretreated with NFκB inhibitor BAY 11-7082 for 1h and then unstimulated or stimulated with LPS for 2h. A+B. Relative mRNA expression of *Pcgf6* and *IL-12p40* were determined via qRT-PCR. Results are depicted as means ± SE for 3 independent experiments.

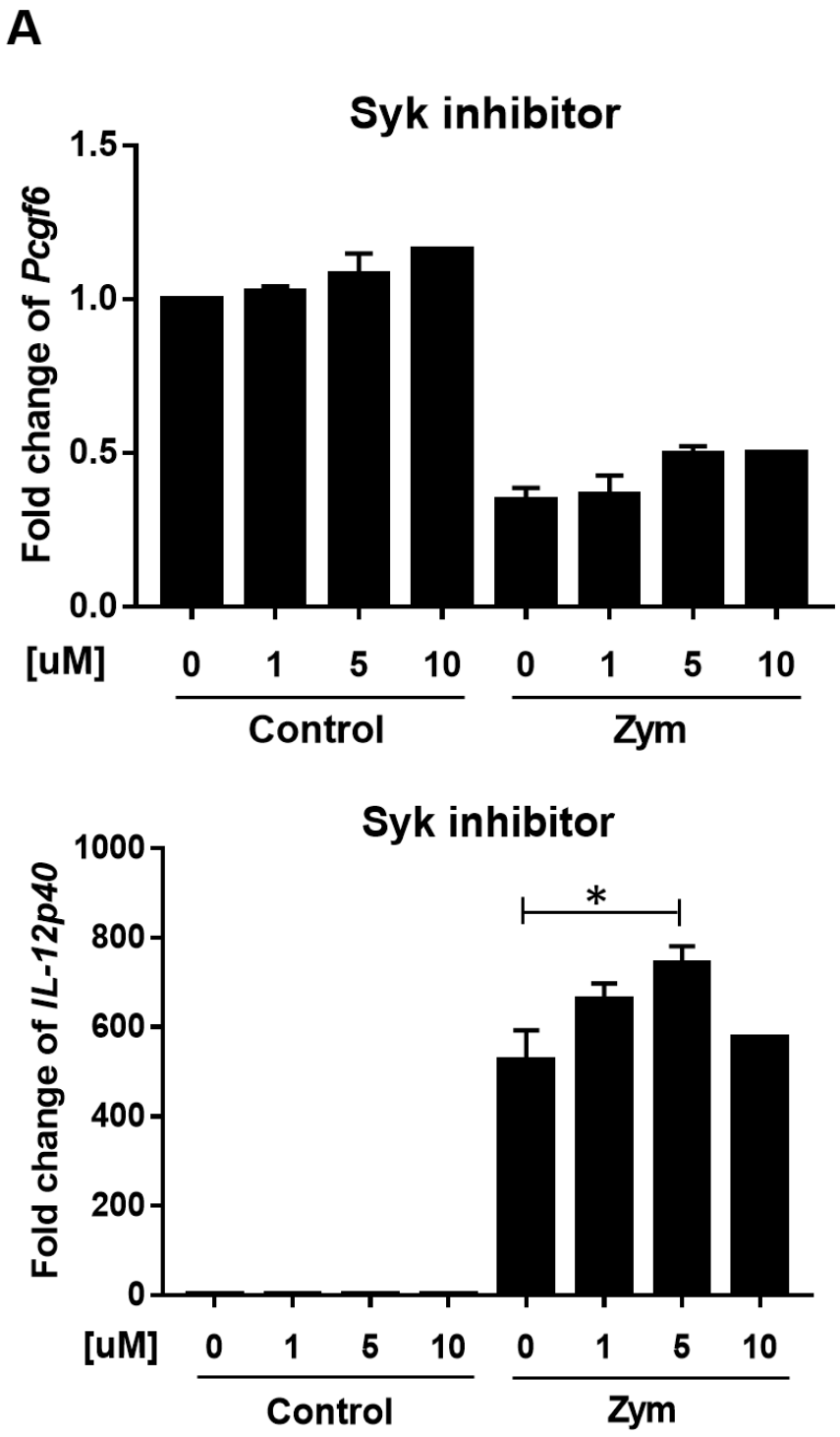


Figure 6. Syk signaling is partially responsible for mediating *Pcgf6* downregulation post Zym stimulation. A. DCs were untreated or pretreated with Syk inhibitor R406 for 1h and then unstimulated or stimulated with Zym for 2h. Relative mRNA expression of *Pcgf6* and *IL-12p40* were determined via qRT-PCR. Results are depicted as means ± SE for1-3 replicates in independent experiments. ∗, $p < 0.05$

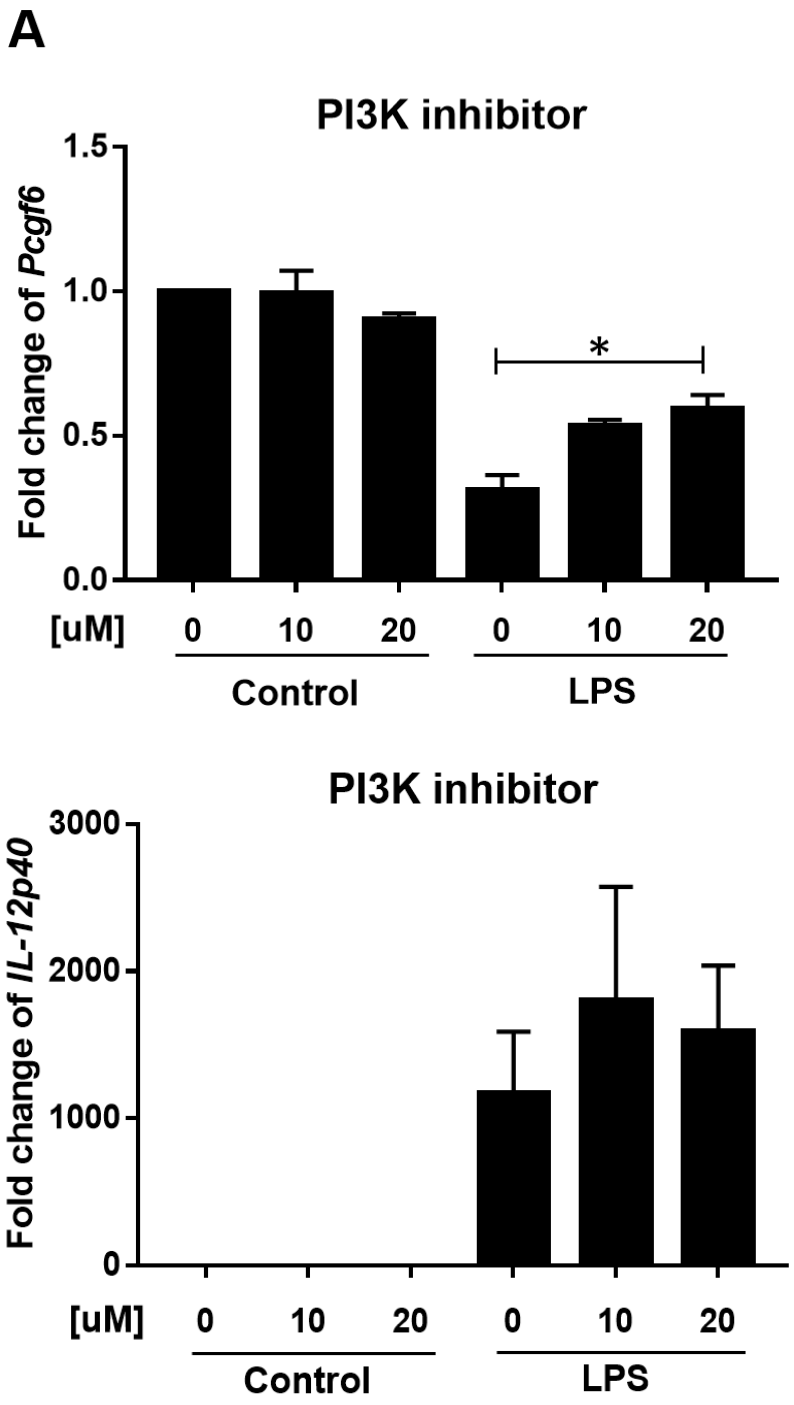


Figure 7. PI3K is partially responsible for mediating *Pcgf6* downregulation post LPS stimulation. A. DCs were untreated or pretreated with PI3K inhibitor Ly294002 for 1h and then stimulated or unstimulated with LPS for 2h. Relative mRNA expression of *Pcgf6* and *IL-12p40* were determined via qRT-PCR. Results are depicted as means ± SE for 2-3 replicates in independent experiments. ∗, $p < 0.05$.

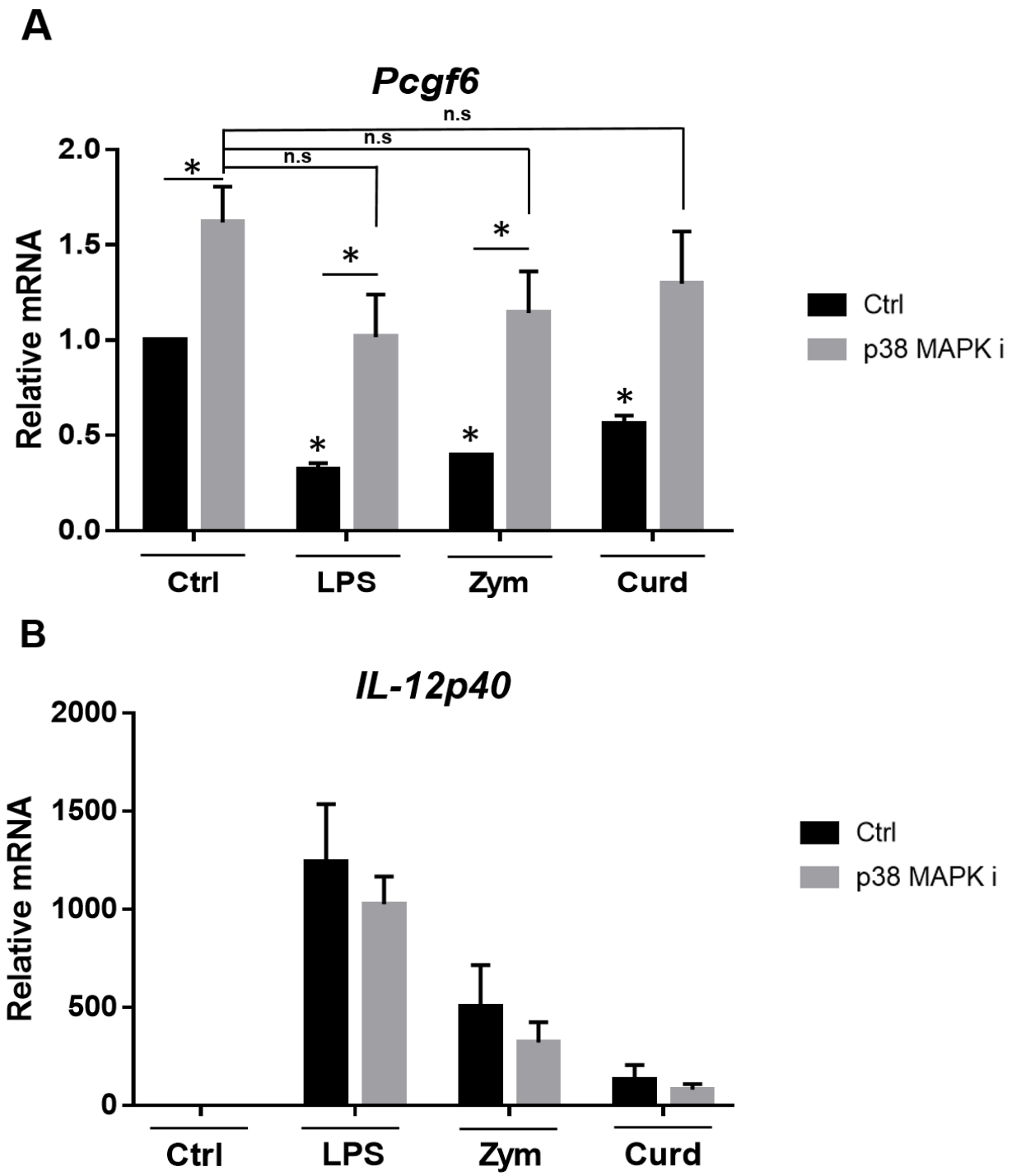


Figure 8. p38 MAPK signaling regulates *Pcgf6* mRNA expression at steady state and following activation. A,B. DCs were untreated or pretreated with 15uM of p38 MAPK inhibitor SB202190 for 1h and then unstimulated or stimulated with LPS, Zym and Curdlan for 2h. Relative mRNA expression of *Pcgf6* and *IL-12p40* were determined via qRT-PCR. Results are depicted as means ± SE for 3-5 replicates of independent experiments. ∗, $p < 0.05$.

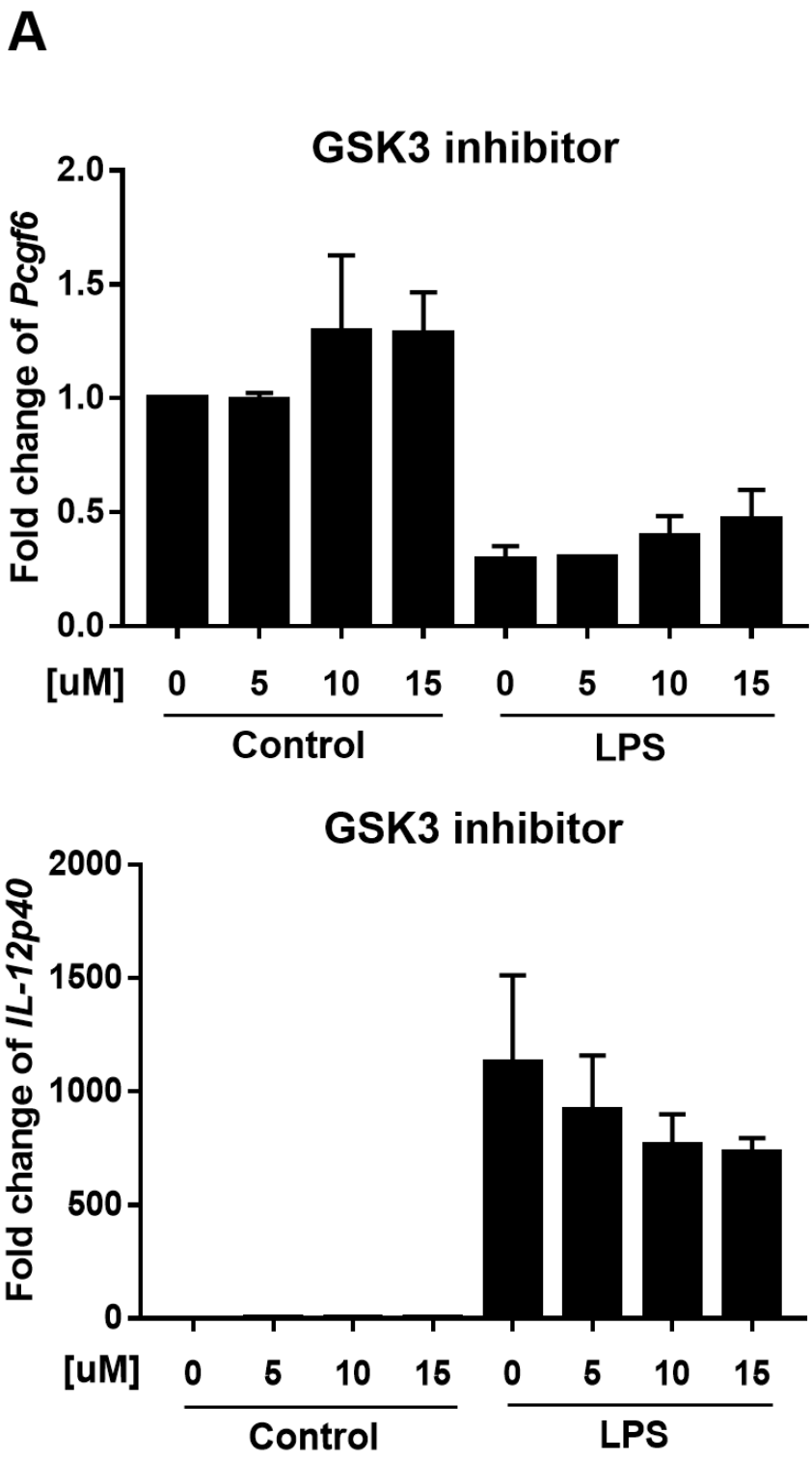


Figure 9. *Pcgf6* downregulation occurs independently of GSK3 signaling. A. DCs were pretreated with GSK3 inhibitor for 1h and then unstimulated or stimulated with LPS for 2h. Relative mRNA expression of *Pcgf6* and *IL-12p40* were determined via qRT-PCR. Results are depicted as means ± SE for 2-3 replicates of independent experiments. $*$, $p < 0.05$.

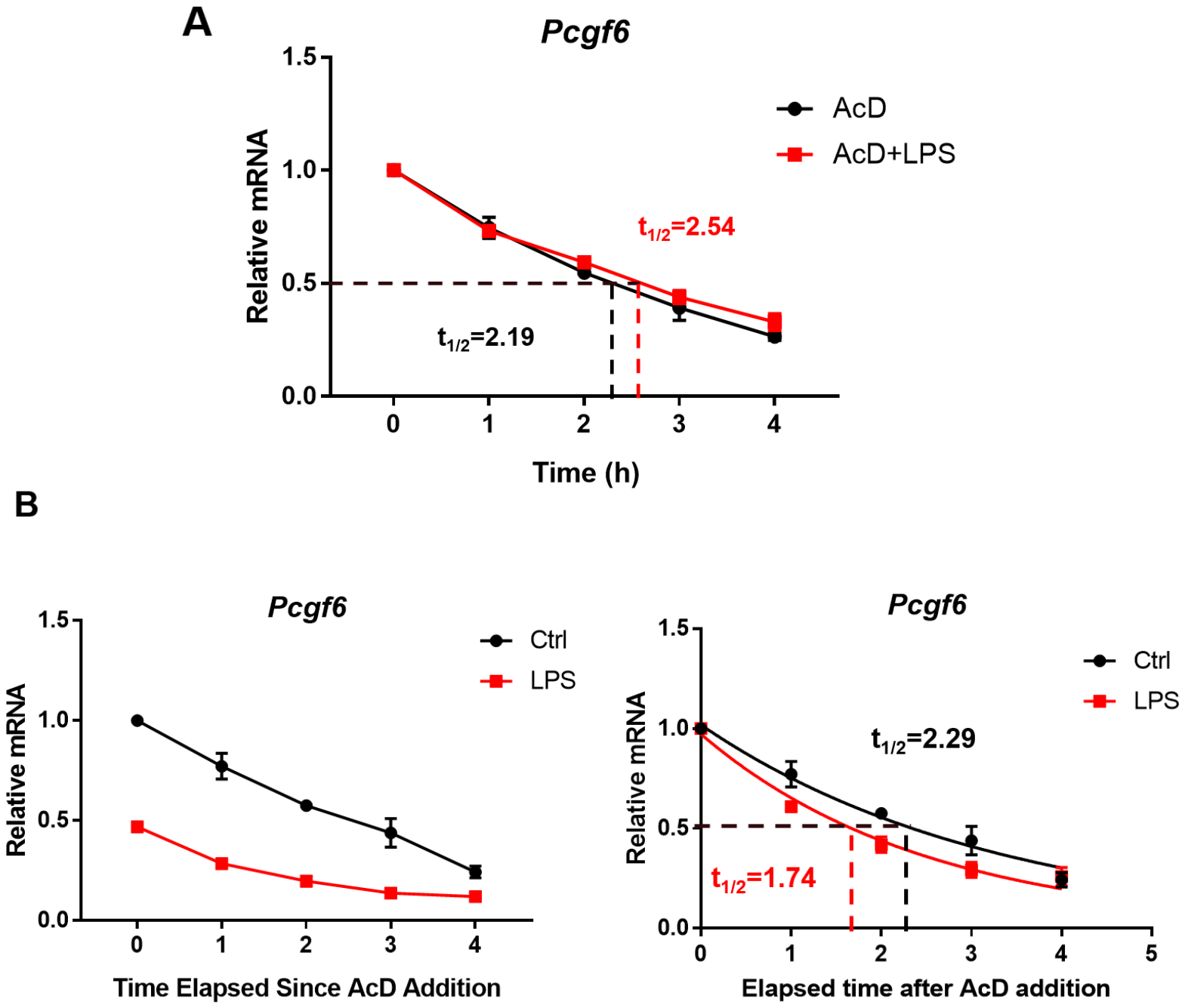
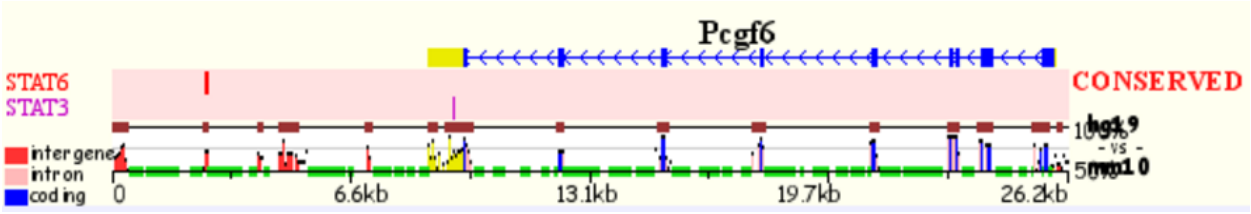


Figure 10. Downregulation of *Pcgf6* post stimulation is mainly dependent on transcriptional activity. A. DCs were treated with 5ug/mL actinomycin D alone or with LPS and of actinomycin D for the indicated time. B. DCs were untreated or pretreated with LPS for 2h and then actinomycin D was added to a final concentration of 5ug/mL for indicated times. On the graph to the left the LPS-treated DCs are normalized to LPS control. On the graph to the right they are normalized to unstimulated control. A+B. Relative mRNA expression of *Pcgf6* was determined by qRT-PCR. Results are depicted as means ± SE for 2-3 replicates of independent experiments.

A



B

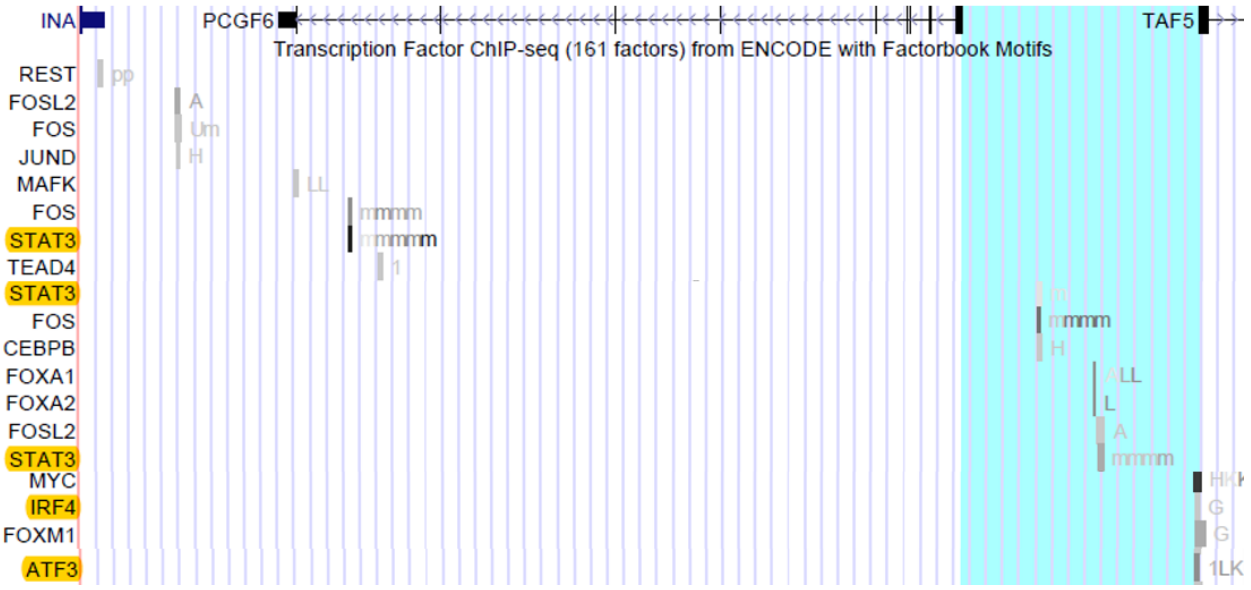


Figure 11. Dynamic visualization of *Pcgf6* transcription factor binding sites. A. Visual representation of select transcription factors predicted to bind the *Pcgf6* gene via ECR browser. B. Visual representation of select transcription factors that bind the *Pcgf6* gene identified via ENCODE. The area highlighted in blue represents the promoter region of the gene. Highlighted in yellow are our transcription factors of interest.

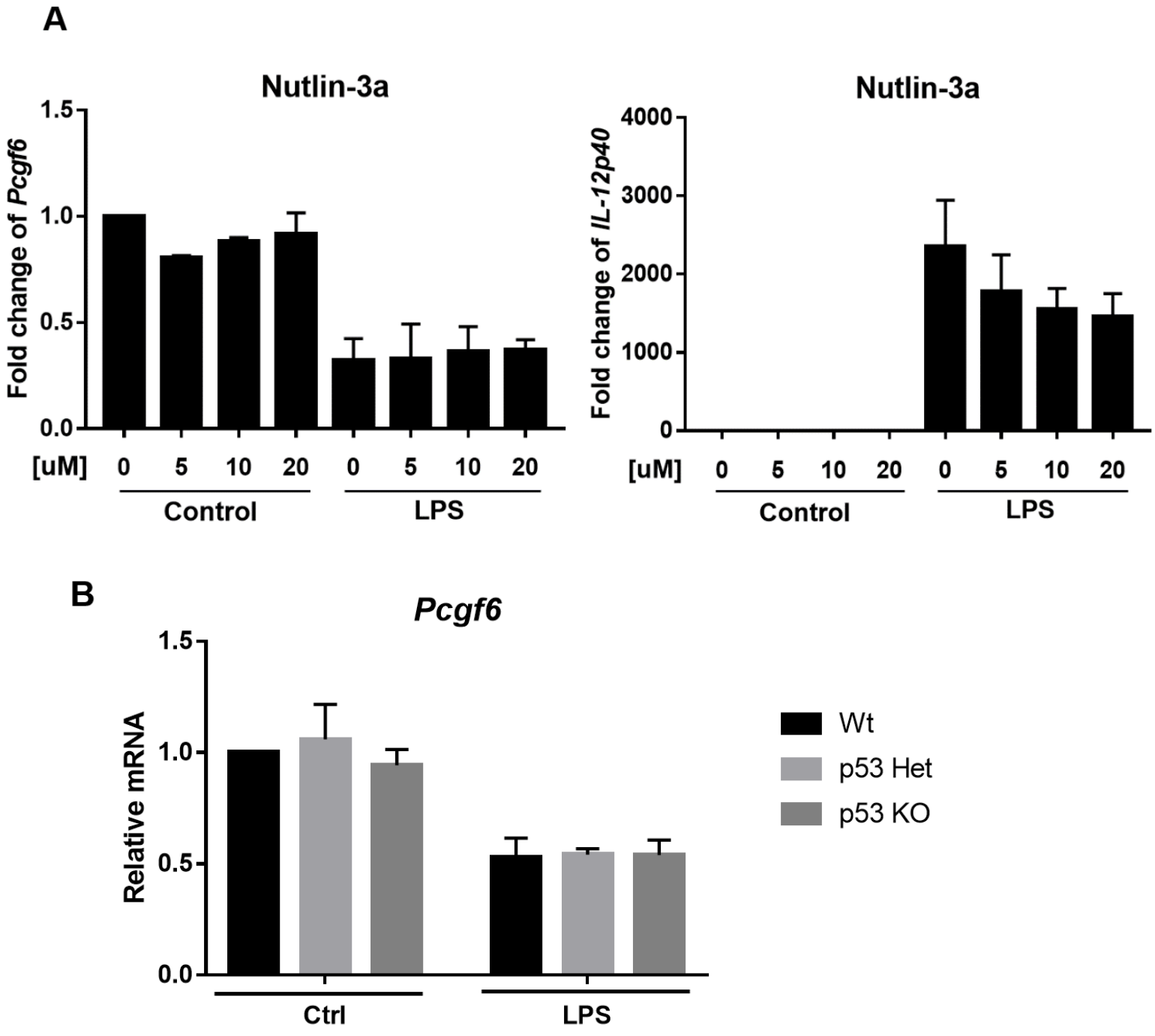


Figure 12. p53 does not regulate mRNA expression of *Pcgf6*. A. DCs were pre-treated with Nutlin-3a for 1h and then unstimulated or stimulated with LPS for 2h. B. DCs generated from wild type, p53 heterozygous and p53 knockout mice were treated with LPS for 2h. A+B. Relative mRNA expression of *Pcgf6* and *IL-12p40* were determined via qRT-PCR. Results are depicted as means ± SE for two independent experiments.

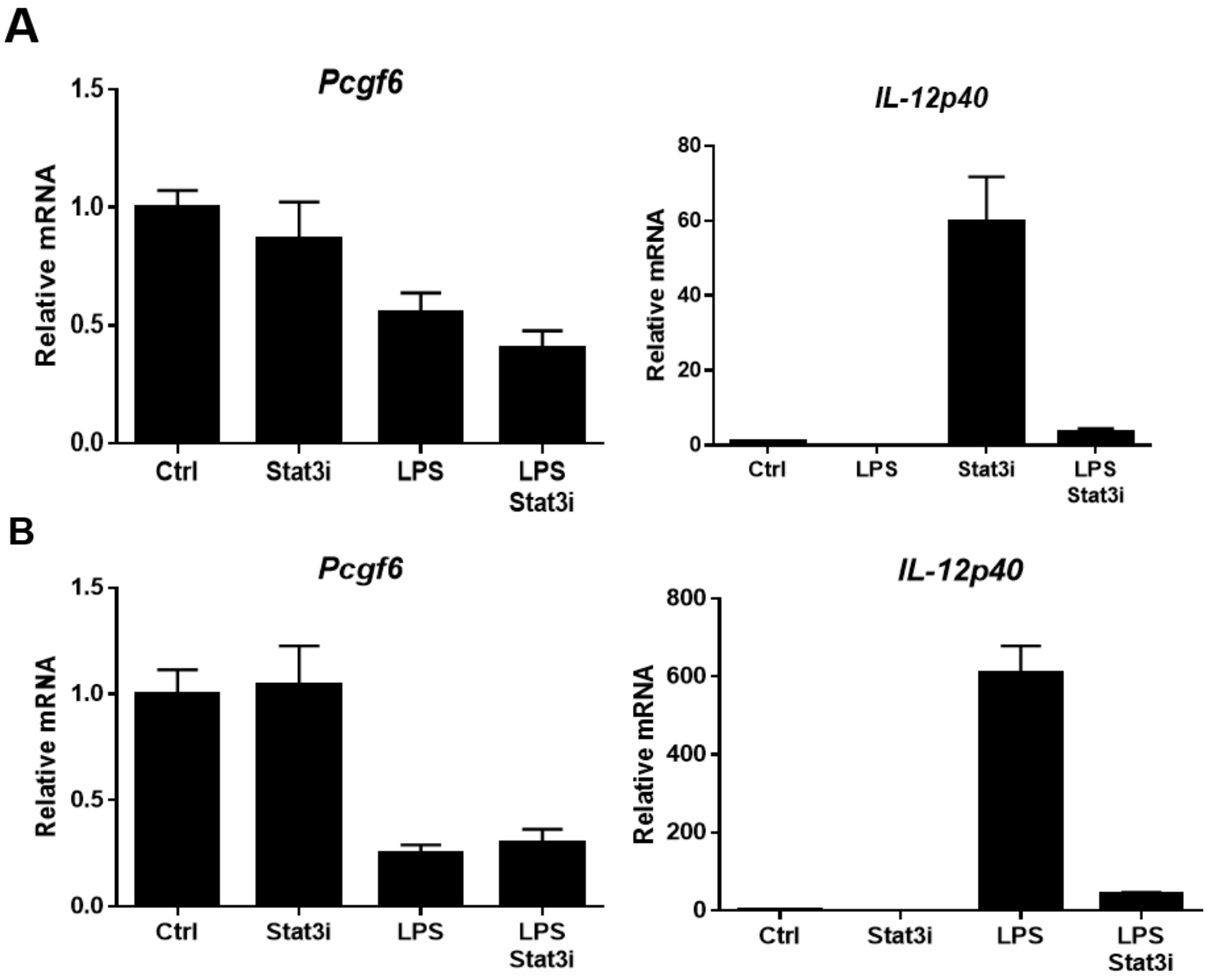


Figure 13. STAT3 does not regulate mRNA expression of *Pcgf6*. A. DCs were pretreated with Stat3 inhibitor for 1h and then unstimulated or stimulated with LPS for 6h. B. DCs were pre-treated with Stat3 inhibitor for 1h and then unstimulated or stimulated with LPS overnight. A+B. Relative mRNA expression of *Pcgf6* and *IL-12p40* were determined via qRT-PCR. Graphs represent one independent experiment for each time point.

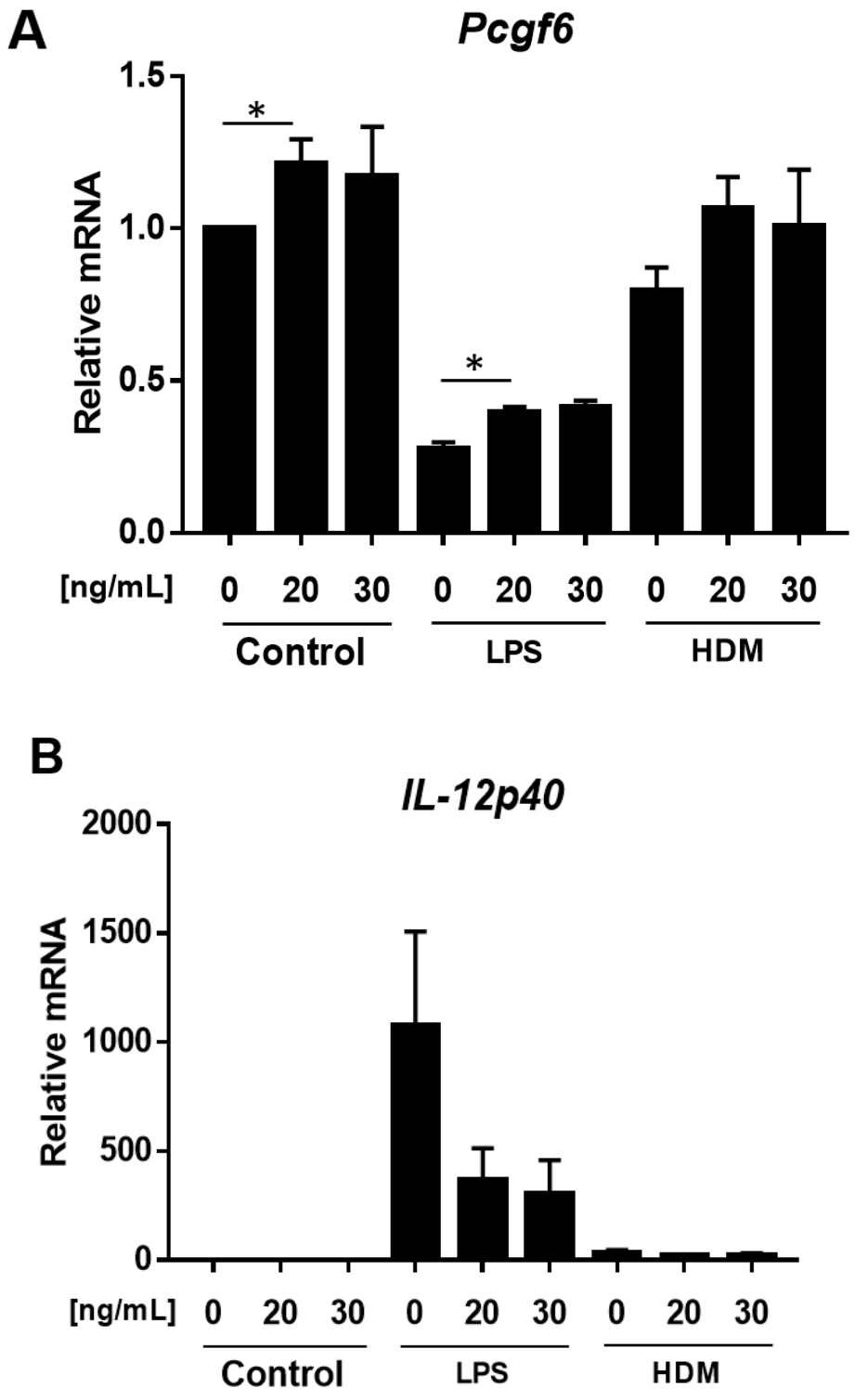


Figure 14. IL-4 induces *Pcgf6* expression at steady state and in the context of LPS and HDM stimulation. A, B. Unstimulated and 2h LPS and HDM stimulated DCs treated with indicated concentrations of IL-4. Relative mRNA expression of *Pcgf6* and *IL-12p40* were determined via qRT-PCR. Results are depicted as means ± SE for 2-5 replicates of independent experiments. ∗, $p < 0.05$.

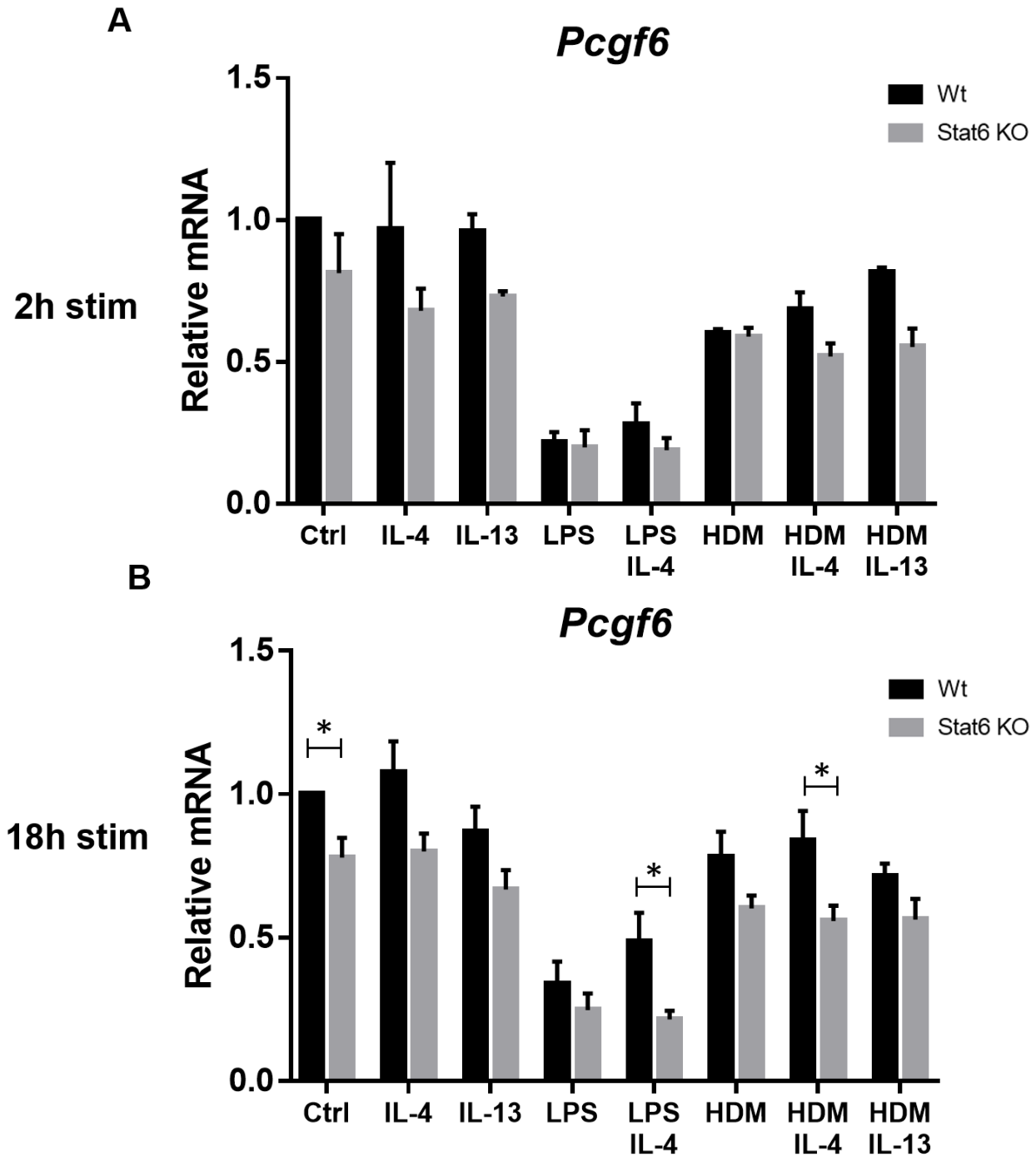


Figure 15. DCs derived from STAT6 KO mice have lower expression of *Pcgf6* mRNA compared to DCs generated from wild type mice. A. DCs generated from STAT6 KO mice and wild type mice were stimulated with LPS and HDM in the presence or absence of IL-4 (30 ng/mL) and IL-13 (30 ng/mL) for 2h.Results are depicted as means ± SE for two independent experiments. B. DCs generated from STAT6 KO mice and wild type mice were stimulated with LPS and HDM in the presence or absence of IL-4 (30 ng/mL) and IL-13 (30 ng/mL) for 18h. Results are depicted as means ± SE for 2-6 replicates of independent experiments. A, B. Relative mRNA expression of *Pcgf6* and *IL-12p40* were determined via qRT-PCR. ∗, $p < 0.05$.

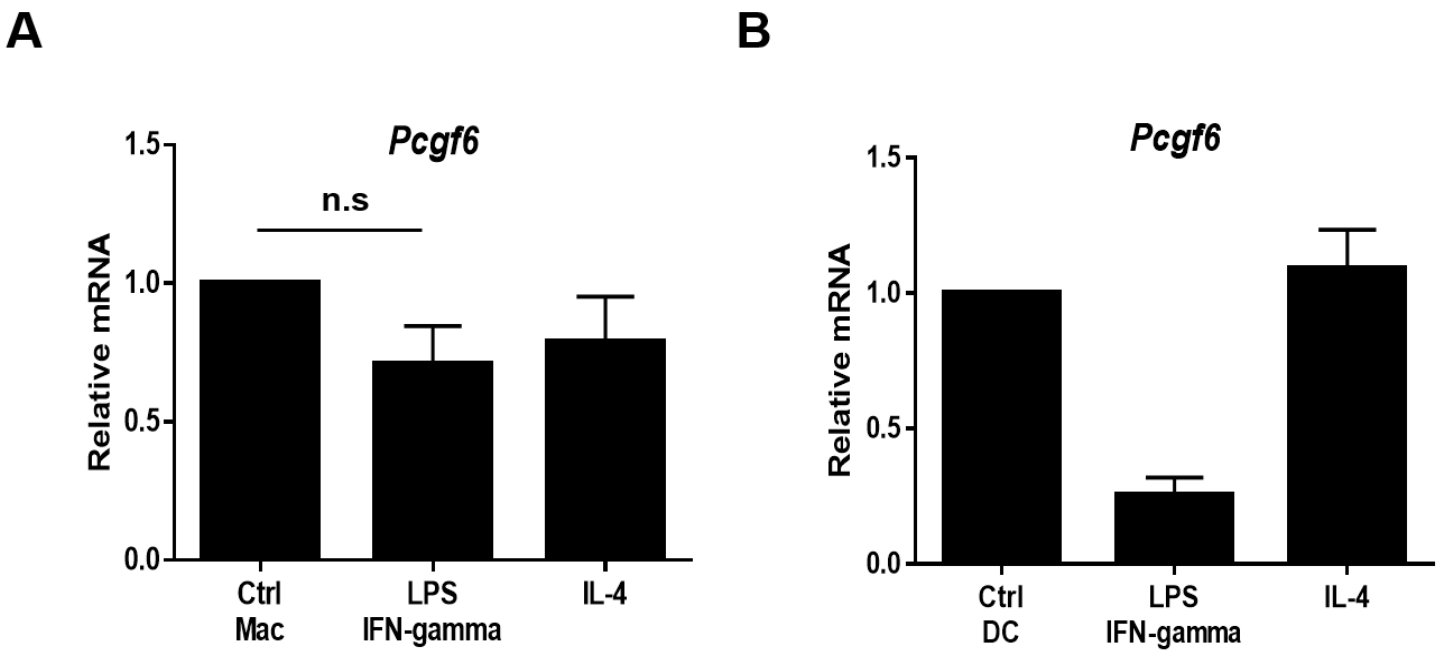


Figure 16. *Pcgf6* is regulated differently in macrophages compared to DCs. A. Macrophages were stimulated with LPS+IFN-γ and IL-4 overnight for differentiation into type 1 and type 2 phenotype, respectively. Results are depicted as means ± SE for 3 independent experiments. B. DCs were stimulated with LPS+IFN-γ and IL-4 overnight. Results are depicted as means ± SE for 2 independent experiments. A, B. Relative mRNA levels of *Pcgf6* were measured via qRT-PCR.

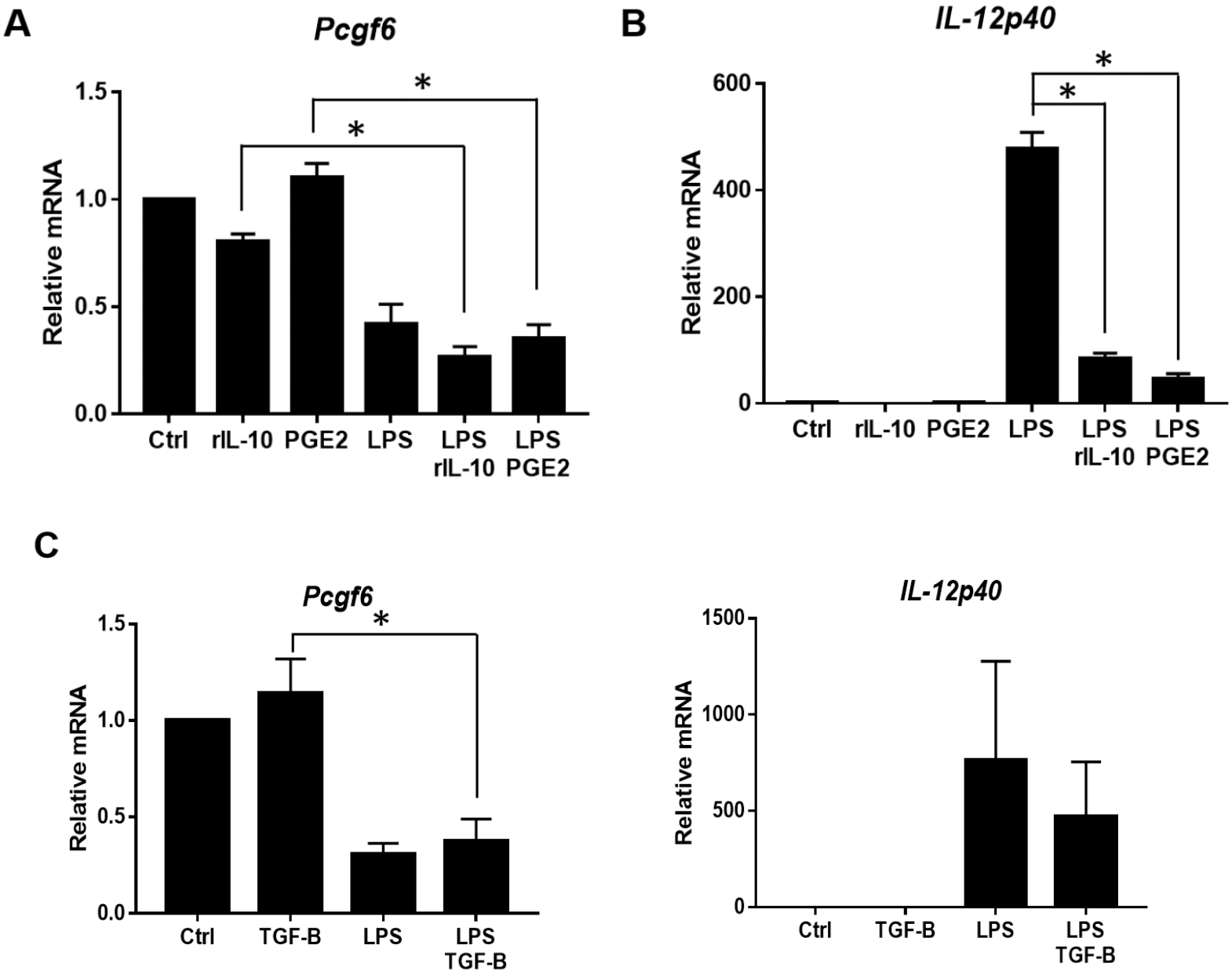


Figure 17. Negative regulators of DCs IL-10, PGE2 and TGFβ do not regulate *Pcgf6* mRNA expression. A, B Unstimulated and 18h LPS stimulated DCs were pre-treated with 20ng/mL rIL-10 and 1uM PGE2. A. Results are depicted as means ± SE for 4 independent experiments. B. Results are depicted as means ± SE for 3 independent experiments. C. Unstimulated and 2h LPS stimulated DCs were pretreated with 10ng/mL of TGFβ for 1h. Results are depicted as means ± SE for 3 independent experiments. A-C Relative mRNA levels of *Pcgf6* and *IL-12p40* were measured via qRT-PCR. $*$, $p < 0.05$.

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