ABSTRACT

Mechanism of C-Type Lectin Receptor Dectin-1 and DC-ASGPR-Mediated Immune Modulation

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Dendritic cells (DCs) are major antigen-presenting cells (APCs) and play a critical role in directing host immune responses towards either immunity or tolerance. C-type lectin receptors (CLRs) expressed on DCs not only facilitate antigen capture and uptake for presentation, but also deliver diverse intracellular signals that modulate DC functions and result in altered immune responses. Among numerous CLRs, Dectin-1 is critical to induce both Th1 and Th17 cell responses that are essential to host immune defense against fungi and mycobacteria whereas DC-ASGPR modulates immune responses by promoting antigen-specific regulatory T cells. Herein, we report that activation of $CD11c^{+}$ myeloid DCs via Dectin-1 significantly downregulates TSLP-induced inflammatory Th2 cell responses by (1) subverting the Th2-permissive microenvironment via IL-10, (2) suppressing OX40L expression by downregulating the transcriptional activity of p50-RelB heterodimer and (3) decreasing Th2 cell-attracting chemokine CCL17 secretion.

In addition, we dissected DC-ASGPR-mediated signaling pathway and found that DC-ASGPR ligation by specific monoclonal antibody (mAb) induces Syk activation. Similar to Dectin-1, engagement of PLCγ2 and PKCδ is conserved downstream of Syk activation upon DC-ASGPR triggering. Unexpectedly, however, DC-ASGPR ligation by mAb does not induce NF-κB activation. Instead, it selectively activates MAPK ERK1/2 and JNK. Rapid and prolonged phosphorylation of ERK1/2 leads to activation of p90RSK and CREB, promoting IL-10 expression in DCs. Moreover, DC-ASGPR ligation activates PI3K-Akt pathway, which differentially regulates the activities of GSK-3α/β and β-Catenin for cytokine expression. Our results provide a molecular explanation for the ability of DC-ASGPR-interacting ligands to preferentially evoke immune modulation. Data from this study support that both Dectin-1 and DC-ASGPR represent promising targets that may allow us to control host immune responses, particularly inflammatory responses.

Mechanism of C-Type Lectin Receptor Dectin-1 and DC-ASGPR-Mediated Immune Modulation

by

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A Dissertation

Approved by the Institute of Biomedical Studies

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Submitted to the Graduate Faculty of
Baylor University in Partial Fulfillment of the
Requirements for the Degree
of

Doctor of Philosophy

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| | |
|---|---|
| AHR | airway hyper-responsiveness |
| AIRE | autoimmune regulator |
| ANOVA | analysis of variance |
| APC | antigen-presenting cell |
| ASGPR | asialoglycoprotein receptor |
| ATP | adenosine triphosphate |
| ATPase | adenosine triphosphatase |
| Bcl10 | B-cell lymphoma/leukemia 10 |
| BIR | baculovirus inhibitor of apoptosis protein repeat |
| CARD9 | caspase recruitment domain family member 9 |
| CCL | chemokine (C-C motif) ligand |
| CCR | chemokine (C-C motif) receptor |
| CLR | C-type lectin receptor |
| CRD | carbohydrate recognition domain |
| CREB | cAMP responsive element binding protein |
| CTL | cytotoxic T lymphocyte |
| CTLD | C-type lectin-like domain |
| CXCL2 | chemokine (C-X-C motif) ligand 2 |
| DAMP | damage-associated molecular pattern |
| DC | dendritic cell |

| | |
|---|---|
| DC-ASGPR | dendritic cell-asialoglycoprotein receptor |
| DCIR | dendritic cell immunoreceptor |
| DC-SIGN | dendritic cell-specific intercellular adhesion molecule-3 grabbing non-integrin |
| Dectin-1 | dendritic cell-associated C-type lectin 1 |
| dsRNA | double-stranded RNA |
| ELISA | enzyme-linked immunosorbent assay |
| ERK | extracellular signal–regulated kinase |
| GM-CSF | granulocyte-macrophage colony-stimulating factor |
| GSK-3 | glycogen synthase kinase 3 |
| HLA | human leukocyte antigen |
| HML | human macrophage lectin |
| HRP | horseradish peroxidase |
| ICAM | intercellular adhesion molecule |
| IDO | indoleamine-pyrrole 2,3-dioxygenase |
| IFN | interferon |
| IL | interleukin |
| IPS-1 | interferon-β-promoter stimulator 1 |
| IRF | interferon regulatory factor |
| ITAM | immunoreceptor tyrosine-based activation motif |
| ITIM | immunoreceptor tyrosine-based inhibition motif |
| JNK | c-Jun N-terminal kinase |
| LC | Langerhans cell |
| Lck | lymphocyte-specific protein tyrosine kinase |

| | |
|---|---|
| LGP2 | laboratory of genetics and physiology 2 |
| LN | lymph node |
| LRR | leucine-rich repeat |
| mAb | monoclonal antibody |
| MAC-1 | macrophage-1 antigen |
| MALT1 | mucosa-associated lymphoid tissue lymphoma translocation 1 |
| MAPK | mitogen-activated protein kinase |
| M-ASGP-BP | macrophage asialoglycoprotein-binding protein |
| MD-2 | myeloid differentiation factor 2 |
| MDA-5 | melanoma differentiation-associated protein 5 |
| MGL | macrophage galactose lectin |
| MHC | major histocompatibility complex |
| MLR | mixed leukocyte reaction |
| MR | mannose receptor |
| mRNA | messenger RNA |
| mTEC | medullary thymic epithelial cell |
| MUC | mucin |
| MyD88 | myeloid differentiation primary response protein 88 |
| NFAT | nuclear factor of activated T-cells |
| NF-κB | nuclear factor kappa-light-chain-enhancer of activated B cells |
| NLR | nucleotide-binding oligomerization domain-like receptor |
| p90RSK | p90 ribosomal S6 kinase |

| | |
|---|---|
| PAMP | pathogen-associated molecular pattern |
| pDC | plasmacytoid dendritic cell |
| PKCδ | protein kinase C delta type |
| PLCγ2 | phospholipase Cγ2 |
| Poly (I:C) | polyinosinic:polycytidylic acid |
| PRR | pattern recognition receptor |
| PYD | pyrin domain |
| RA | retinoic acid |
| RIG-I | retinoic acid-inducible gene 1 |
| RLR | retinoic acid-inducible gene 1-like receptor |
| ROS | reactive oxygen species |
| SD | standard deviation |
| ssRNA | single-stranded RNA |
| STAT | signal transducer and activator of transcription |
| Syk | spleen tyrosine kinase |
| Th1/2/17 | type 1/2/17 helper T cell |
| TIR | Toll/IL-1R homology |
| TLR | Toll-like receptor |
| TNF | tumor necrosis factor |
| TSLP | thymic stromal lymphopoietin |

ACKNOWLEDGMENTS

I would like to express my sincere gratitude first and foremost to my advisor Dr. SangKon Oh, who has provided me the opportunity to work with him and learn from him in the last 5 years. In retrospect, I feel very fortune to have worked on many different projects, including those documented in this very dissertation.

I would also like to thank my committee members, Drs. HyeMee Joo, Venuprasad K. Poojary, Robert R. Kane, Christopher M. Kearney and Cheolho Sim for their sincere guidance and insightful comments and suggestions.

My gratitude also goes to the current and previous members of Dr. Oh's lab for their help in almost every aspect of my graduate study. They are Yaming Xue, Lei Wang, Kuntal Halder, Matthew Wiest, SangMin Ma, Katherine Upchurch, Dorothée Duluc, Dapeng Li, Ling Ni, Wei Zhang, Richard Ouedraogo, Sandra Clayton and Jerome Ellis. Thanks to Wenjie Yin, Trivendra Tripathi, Mahmudul Hasan and LuAnn Thompson-Snipes for their inspiring discussions on many different topics in the Journal Club.

I am grateful to Zhiqing Wang, Sandy Zurawski and Gerard Zurawski for providing recombinant antibodies and fusion proteins. Thanks to Laura Bover and Bob Coffman for providing reagents and thanks to Mark Millard and Shiying Bian for the help in recruiting patients and procuring blood samples. Thanks to Kay Kayembe, Elizabeth M. Lavecchio and Jean P. Blanck of the Flow Cytometry SRL for providing assistance in cell sorting. Thanks to Carson Harrod for taking care of the graduate program. Thanks to Pamela Jennings, Hermila Crear and Annie Mathew of Baylor Institute for Immunology

Research, and Rhonda Bellert of Baylor University for the administrative support throughout.

It has been a fulfilled journey and I am grateful to all the people that have helped me during my days at Baylor.

Last but not least, I would like to thank my parents, my parents-in-law and my wife. I know their support to me is unconditional.

# CHAPTER ONE

# Introduction

## *Overview of Dendritic Cells*

### *Discovery and Recognition of Dendritic Cells as Antigen-Presenting Cells*

Langerhans cells (LCs), which are now recognized as a specialized subset of dendritic cells (DCs) that populate the skin epidermis and mucosal tissues, were first described mistakenly as skin nerve cells by Paul Langerhans in 1868 (Kashem et al., 2017). After more than a century, DCs were re-discovered by Ralph Steinman and Zanvil Cohn (Steinman et al., 1975; Steinman and Cohn, 1973; Steinman and Cohn, 1974; Steinman et al., 1979; Steinman et al., 1974). In 1973, they reported a novel cell type in mouse spleen, which is morphologically distinct from typical macrophages and monocytes, and proven to be about 100 times more potent than non-fractionated mixture of spleen cells in inducing allogeneic mixed leukocyte reaction (MLR) (Steinman and Witmer, 1978). In addition, Steinman also made the leap to suggest that DCs, but not macrophages, likely represent the critical accessory cells required for the induction of lymphocyte responses (Steinman and Witmer, 1978).

The report from Steinman and Cohn was initially received with some skepticism, based on the widely held view at that time that the major antigen presenting cells (APCs) were the far more numerous macrophages and on the uncertainty that many immunologists had about the assay that Steinman and Cohn used to establish the function of DCs (Paul, 2011). In other words, the MLR was not thought to be a typical adaptive

immune response, but more like a spontaneous, innate response as the vigorous *in vitro* responses observed in MLR apparently required no priming. Also, the precise nature of the antigen and the reacting cells in the assay were not well defined.

In 1980, an important breakthrough in the field of DCs was the development of a method to test DCs in inducing antigen-specific adaptive immunity. The system developed by Michel Nussenzweig *et al.* involved modifying DCs with the nitrophenyl moiety and measuring the development of antigen-specific cytotoxic T cell responses *in vitro* (Nussenzweig et al., 1980). Results of these experiments suggested that DCs, in contrast to macrophages and monocytes, functioned as potent accessory cells in eliciting antigen-specific T cell responses (Nussenzweig et al., 1980). Based on this study, Nussenzweig and Steinman established the important principle that DCs efficiently present antigens to T cells to initiate adaptive immunity.

Two years later, the monoclonal antibody (mAb) 33D1 that is specific for the major DC subset in mouse spleen, was developed by Nussenzweig *et al.* (Nussenzweig et al., 1982). The generation of this mAb made it possible to visualize and distinguish DCs in tissue sections. More importantly, depletion of DCs from cell mixtures using this mAb further confirmed the antigen presentation properties of DCs (Inaba et al., 1983; Inaba and Steinman, 1985; Steinman et al., 1983).

Meanwhile, Wes Van Voorhis *et al.* enriched and characterized DCs from human peripheral blood for the first time, which are distinct from blood monocytes, and function as APCs like their counterparts in mouse (Van Voorhis et al., 1982).

Based on the inspiring works by Steinman and his colleagues, DCs are now widely-accepted as the most potent APCs in the immune system and act as the critical

link between innate and adaptive immunity (Banchereau et al., 2000; Banchereau and Steinman, 1998; Steinman, 1991).

*Immunobiology of Dendritic Cells*

Soon after the antibodies specific to DCs became available, anatomic location of DCs throughout the organism was investigated. DCs are found at all the interfaces between body and environment: airway epithelium, skin, and mucosal surface (Merad and Manz, 2009). This perfect positioning allows them to act as sentinels and to capture antigens when and where they enter the organism. In addition, DCs are also found in lymphoid organs including lymph node (LN), spleen, tonsil, and thymus, indicating that DCs are in the right place where they interact with T cells to initiate adaptive immunity (Lindquist et al., 2004).

In order to initiate immune responses, DCs need to be activated by signals from pathogens or other activated immune cells, a process named DC maturation. Immature DCs are found to be either poor stimulators or unable to induce immunity (Schuler and Steinman, 1985). However, once DCs receive activation signals, such as innate signals from Toll-like receptors (TLRs), they mature and become potent APCs. Indeed, activation of DCs stabilizes peptide-MHCII complexes on the cell surface due to reduced MHCII recycling (Cella et al., 1997). Meanwhile, DCs become less efficient in sampling the surrounding environment but upregulate the expression of co-stimulatory molecules including CD80 (Larsen et al., 1992) and CD86 (Lenschow et al., 1993), which are ligands for CD28 expressed on T cells. Additionally, activated DCs become more migratory as they upregulate the expression of chemokine receptor CCR7 (Dieu et al.,

1998), allowing them to enter afferent lymphatics and migrate to the draining LNs following the CCL19/CCL21 chemokine gradient (Forster et al., 1999).

Once DCs migrate to the LNs, they further move towards the T cell area in the paracortex where DCs present antigen to recirculating naive T cells, inducing T cell proliferation and polarization. In addition to presenting antigen to $CD4^+$ T cells on MHCII molecules, DCs can also present antigens to naive $CD8^+$ T cells on MHCI, which is known as cross-presentation. This is crucial for the generation of cytotoxic T lymphocytes (CTLs) against antigens not synthesized by DCs themselves, for example, the viral proteins (Bevan, 1995).

When a naive T cell recognizes a specific peptide displayed on a DC expressing co-stimulatory molecules, it produces IL-2 and enters the cell cycle, a process known as clonal expansion (Guermonprez et al., 2002). After several rounds of cell division, the activated T cells differentiate into specific effector cells needed to eliminate antigens (Figure 1.1). $CD8^+$ T cells differentiate into CTLs, which can kill cells infected with viruses and other intracellular pathogens, while $CD4^+$ T cells differentiate into Th1, Th2, Th9, Th17, Th21, Th22, or regulatory T (Treg) cells, largely classified based on the cytokines they produce (Akdis et al., 2012; Kaplan, 2013; Suto et al., 2008; Zhu et al., 2010). Differentiation of effector T cells is driven by the expression of co-stimulatory molecules on activated DCs such as CD40 and OX40, as well as cytokines, including IL-12, IL-6, IL-1β, TGFβ and IL-23 (De Becker et al., 1998). IL-12 is produced by DCs upon TLR and/or CD40 ligation, leading to the generation of Th1 cells (Athie-Morales et al., 2004; Macatonia et al., 1995). IL-4 production is required to prime Th2 cell response, and is produced by a number of immune cells including T cells, eosinophils, NKT cells

and basophils rather than DCs (O'Garra, 1998). Production of IL-6, TGFβ, IL-1β and IL-23 by DCs results in the induction of Th17 cells (Korn et al., 2009). IL-1β, IL-6 and TGFβ prime Th17 cell differentiation, while IL-23 acts on activated T cells which express the IL-23 receptor (IL-23R) and therefore maintains Th17 cell development (Korn et al., 2009). Different effector T cells play distinct role in host immune response. Th1 cells produce IFNγ that activates macrophages to kill intracellular pathogens (Abbas et al., 1996). Th2 cells produce IL-4 that activates naive B cells and induces class switching for the generation of IgE and IgG1 (Finkelman et al., 1988). The IL-17- and IL-22-producing Th17 cells, function in the control of extracellular pathogens (Korn et al., 2009), but are also implicated in the induction of tissue inflammation in autoimmune disease (Park et al., 2005).

Although DCs are responsible for the initiation of adaptive immunity, they are also essential for the induction of both central and peripheral tolerance. DCs induce central tolerance to self-antigens in the thymus, either by inducing clonal deletion of self-reactive T cells or by generating $FoxP3^+$ natural Treg cells (nTregs) (Hori et al., 2003; McCaughtry and Hogquist, 2008; Proietto et al., 2008). This may occur by antigen-loaded DCs from peripheral tissues migrating to the thymus (Bonasio et al., 2006) or alternatively thymic-resident DCs can acquire self-antigen from medullary thymic epithelial cells (mTECs) expressing the transcription factor autoimmune regulator (AIRE), which drives promiscuous expression of peripheral antigens in the thymus. The antigen acquired in this manner is then cross-presented to T cells (Gallegos and Bevan, 2004; Hubert et al., 2011) inducing negative selection or nTreg polarization. In addition, tolerance can be induced in the peripheral after T cells have left the thymus. Mechanism

of peripheral tolerance involves the generation of suppressive induced regulatory T cells (iTregs) as well as specific immunological hyporesponsiveness or anergy towards antigens. In addition to their role in central tolerance, DCs are also engaged in the peripheral tolerance. Production of TGFβ, IL-10, retinoic acid (RA) and indoleamin-2,3-dioxygenase (IDO) by DCs contributes to the induction of iTregs (Mellor and Munn, 2004; Mucida et al., 2007; Raker et al., 2015). Moreover, T cell anergy occurs when specific antigen is presented to naive T cells by immature or semi-mature DCs with low expression of co-stimulatory molecules. Anergy can also occur when DCs present antigen in the presence of anti-inflammatory cytokines, such as IL-10 (Schwartz, 2003).

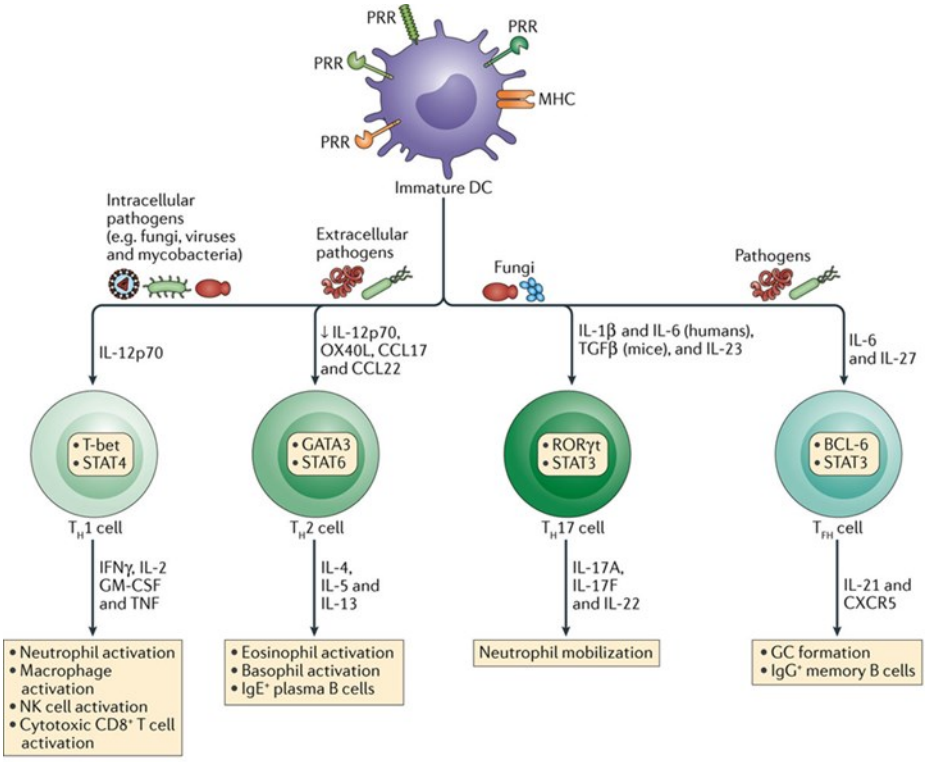


Figure 1.1. Pathogen interactions by DCs dictate T helper cell differentiation. Several different Th cell subsets have been identified that each has specific functions in adaptive immunity. The prime determinant that controls Th cell differentiation is pathogen recognition by DCs, which translates the nature of the invading pathogen into a gene-transcriptional program that drives the appropriate Th cell response. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology (Geijtenbeek and Gringhuis, 2016), copyright 2016.

*Pattern Recognition of Dendritic Cells*

DCs are equipped with a vast array of pattern recognition receptors (PPRs), which are germline-encoded proteins specialized in the recognition of structures conserved among different microbial species, which are called pathogen-associated molecular patterns (PAMPs) (Medzhitov, 2001), and of endogenous molecules released from damaged cells, termed damage-associated molecular patterns (DAMPs) (Kawai and Akira, 2010). So far, four different classes of PRRs have been identified, namely TLRs, C-type lectin receptors (CLRs), retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and NOD-like receptors (NLRs) (Takeuchi and Akira, 2010).

*TLRs*

TLR family is responsible for sensing invading pathogens extracellularly as well as in intracellular endosomes and lysosomes (Akira et al., 2006). Structurally, TLRs are characterized by N-terminal leucine-rich repeats (LRRs) and a transmembrane region followed by a cytoplasmic Toll/IL-1R homology (TIR) domain. Ten TLRs have been identified in human and 12 in mouse. The location of different TLRs is correlated with their ligand recognition (Barton and Kagan, 2009). TLRs 1, 2, 4, 5 and 6 are located primarily in the plasma membrane, where they interact with components of microbial pathogens that come into contact with the cell. TLR2 forms association with TLR1 or TLR6 and recognizes di-acylated, tri-acylated bacterial lipopeptides, lipoteichoic acids from Gram-positive bacteria and lipomannan from mycobacterium. TLR4 requires the association with MD-2 to recognize the lipopolysaccharides (LPS) whereas TLR5 recognizes bacterial flagellin (Akira et al., 2001; O'Neill et al., 2013). In contrast, TLRs 3, 7, 8, and 9 are situated in the membranes of endosomes and lysosomes; the extracellular

domain of the receptor and its ligand-binding site project into the interior of these organelles. After these organelles break down pathogens that have been internalized by endocytosis, TLRs can interact with the now-exposed pathogen DNA and RNA. TLR3 recognizes the double-stranded RNA (dsRNA) formed during the replication of positive stranded RNA virus. TLR9 recognize bacterial DNA with unmethylated CpG motifs while TLR7 and TLR8 recognize viral single-stranded RNA (ssRNA) (Akira et al., 2001; O'Neill et al., 2013). Ligation of the TLRs by their specific ligands results in conformational changes in the receptors, leading to downstream signal transduction that primarily involves MyD88- and TRIF-dependent pathways (Akira and Takeda, 2004). The engagement of TLRs on DCs leads to increased expression of MHC-peptide complexes and co-stimulatory molecules, as well as the production of immunomodulatory cytokines, all of which have profound effects on T cell priming and differentiation (Sousa, 2004).

*RLRs*

Some TLRs can recognize viruses that are present in the endosomal compartments (i.e., ‘‘outside’’ the cell) and elicit antiviral responses via inducing type I interferon (IFN) production. However, viruses usually replicate inside the cell, and therefore intracellular receptors are required for efficient antiviral immunity (Kumagai and Akira, 2010). RLRs are among the intracellular receptors for RNA viruses. They are localized in the cytoplasm and recognize the genomic RNA of dsRNA viruses and dsRNA generated as the replication intermediates of ssRNA viruses. The expression of RLRs is greatly enhanced in response to type I IFN stimulation or virus infection (Akira et al., 2006; Takeuchi and Akira, 2010). The RLR family includes retinoic acid-inducible

gene I (RIG-I), and its homologues melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2) (Takeuchi and Akira, 2009; Yoneyama and Fujita, 2008). Structurally, all three RLRs have a central DExD/H box RNA helicase domain with ATPase activity, and a C-terminal regulatory domain, which is responsible for RNA binding (Takeuchi and Akira, 2010). In addition, both RIG-I and MDA5 have two N-terminal caspase recruitment domains (CARDs), which are responsible for triggering signaling cascades by interacting with the N-terminal CARD-containing adaptor IFN-β-promoter stimulator 1 (IPS-1) (Kawai and Akira, 2006). In terms of ligand specificity, RIG-I recognizes relatively short dsRNA, and the presence of a 5' triphosphate end greatly enhances its type I IFN-inducing activity (Kato et al., 2008; Takahasi et al., 2008). The short dsRNA might be produced during replication of some negative-stranded ssRNA viruses, and therefore RIG-I can sense such viruses. In contrast to RIG-I, MDA5 detects long poly (I:C) and long dsRNA, which is produced during replication of sense-strand ssRNA viruses, such as picornaviruses (Kato et al., 2008; Schlee et al., 2009). When activated during viral infection, RIG-I and MDA5 trigger activation of NF-κB and IRF3/7, which cooperate in the induction of antiviral type I IFN (Andrejeva et al., 2004; Yoneyama et al., 2004). Unlike RIG-I and MDA5, LGP2 has no CARD in the N-terminal region and is therefore unable to interact with IPS-1. However, it has been reported that LGP1 positively regulates production of type I IFNs in response to RNA viruses recognized by both RIG-I and MDA5 (Satoh et al., 2010). In contrast to the roles of RLRs in the innate immune response, their role in the adaptive immune response still remains to be clarified.

*NLRs*

NLRs are a specialized group of intracellular proteins that play a critical role in the regulation of host innate immune response. Structurally, NLRs share a common domain organization with C-terminal leucine-rich repeats (LRRs), a central nucleotide-binding oligomerization domain (NOD), and a N-terminal effector domain (Franchi et al., 2009; Inohara et al., 2005b). The C-terminal LRR domain is involved in ligand binding. The NOD in NLRs is also known as NACHT domain, which has been named after NAIP, CIITA, HET-E, and TP1 proteins. The NACHT domain, which consists of seven distinct conserved motifs including the ATP/GTPase specific loop, the $Mg^{2+}$-binding site, and five more specific motifs, is involved in dNTPase activity and oligomerization (Koonin and Aravind, 2000). The N-terminal effector domain mediates signaling through its interaction with downstream factors. NLR family is classified into five subfamilies: NLRA, NLRB, NLRC, NLRP and NLRX based on the different N-terminal effector domains including acidic transactivation domain, baculovirus inhibitor of apoptosis protein repeat (BIR) domain, CARD, pyrin domain (PYD) and N-terminal domain without significant homology to any known domains (Inohara et al., 2005a; Inohara and Nunez, 2003; Moore et al., 2008; Tattoli et al., 2008; Ting et al., 2008). NLRs are involved in the recognition of conserved microbial components including bacterial peptidoglycan, flagellin, viral RNA, as well as non-microbial signals including uric acid crystals and silica. NLR signaling results in the activation of NF-κB, MAPK, and caspase-1 that induce the production of proinflammatory cytokines, chemokines, and antimicrobial molecules (Krishnaswamy et al., 2013).

*CLRs*

The classical C-type lectin receptors (CLRs) recognize carbohydrates in a $Ca^{2+}$-dependent manner. Loss of the $Ca^{2+}$ ion, which is coordinated by the primary binding site within the carbohydrate recognition domain (CRD), results in conformational change and loss of binding function (Weis et al., 1998). This primary binding site is selective for single monosaccharide residue. A secondary binding site exists adjacent to the primary site that interacts with neighboring monosaccharide residues present in the interacting carbohydrate structure. This secondary binding site strongly enhances binding affinity and fine-tunes binding specificity for certain types of linkages and substitutions (Zelensky and Gready, 2005).

Many other proteins contain large parts of the CRD fold, but usually lack the coordinated $Ca^{2+}$ ions and therefore generally do not have the carbohydrate binding property. This common fold is therefore referred to as the C-type lectin-like domain (CTLD) (Drickamer, 1999). Although this rule applies to most proteins containing CTLD, some of them display $Ca^{2+}$-independent carbohydrate recognition, such as Dectin-1 (Brown et al., 2003), and other classical CLRs, which possess the structurally classical CRD fold, like DCIR, have so far not been shown to recognize any glycan structure (Bates et al., 1999). Based on these observations, the term CLR is now used to nominate all the CTLD-containing proteins, regardless of their carbohydrate binding capacity or dependence of $Ca^{2+}$ for ligand binding.

Classical CLRs can be divided into two categories based upon the amino acid motif involved in carbohydrate recognition and coordination of the $Ca^{2+}$-ion. In mannose-type CLRs, this is facilitated by the amino acid sequence Glu-Pro-Asn (EPN) within the

CRD and in galactose-type CLRs by the sequence Gln-Pro-Asp (QPD) (Kobata, 2003). Based upon this sequence, predictions can be made regarding the carbohydrate recognition profile of a certain CLR. Mannose-type CLRs (containing the EPN-sequence) possess a basic specificity for mannose and/or fucose terminated glycans (Appelmelk et al., 2003; Stambach and Taylor, 2003; Taylor et al., 2005), whereas galactose-type CLRs (containing QPD-sequence) recognize galactose or GalNAc terminated glycan structures (van Vliet et al., 2005a). The secondary binding site fine-tunes this specificity so that each CLR has its own unique glycan specificity. Furthermore, CLRs form oligomers within the cell membrane. Oligomerization not only strengthens binding to a certain structure, it also limits binding to ligands with a complementary carbohydrate density and spacing (Weis et al., 1998). Thus, carbohydrate recognition is primarily determined by the amino acid sequence of the CRD fold. However, it is strongly influenced by the oligomerization of the receptor and the spacing of carbohydrates on the ligand. So, CLRs with similar basic specificities for mannose, can still interact with a very diverse set of ligands.

In the past 20 years, much progress has been made in understanding the role of CLRs in host immunity and tolerance. Remarkably, several different CLRs share common characteristics, including cell-cell and cell-matrix interactions, pattern recognition and internalization of antigens for presentation onto MHC molecules. Furthermore, some CLRs possess signaling properties and are capable of modulating immune responses. These properties of CLRs are important for vaccine design (Figure 1.2) (Geijtenbeek and Gringhuis, 2016; Steinman and Banchereau, 2007).

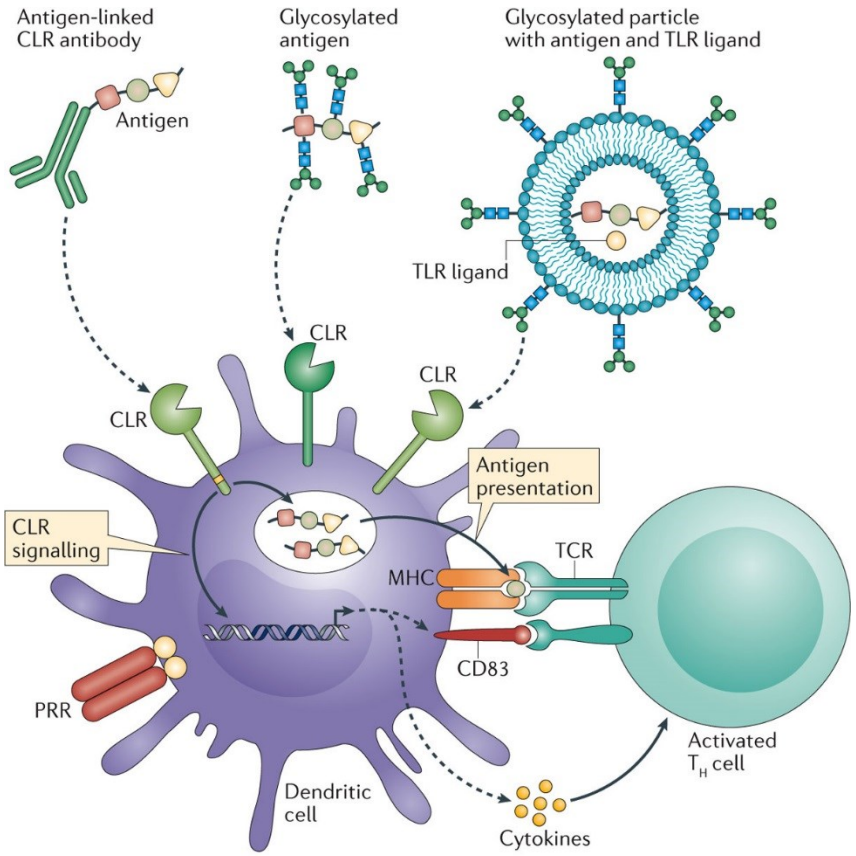


Figure 1.2. CLR signaling in vaccine development. CLR targeting not only enhances antigen presentation but also may offer an approach to target specific Th cell-mediated responses to vaccines against a pathogen or disease of choice. Different methods of targeting CLRs have been studied, including antigen-linked antibodies, glycosylated antigens, and glycosylated particles containing both antigens and possible adjuvants such as TLR ligands. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology (Geijtenbeek and Gringhuis, 2016), copyright 2016.

Many CLRs recognize self-ligands including soluble or cellular glycosylated molecules, and components of the extracellular matrix. For example, mannose receptor (MR) participates in the homeostatic clearance of glycosylated proteins from circulation or body fluids (Lee et al., 2002). DC-SIGN mediates cell-cell adhesion between DCs and other cell types, including naive T cells via interaction with ICAM-3, allowing T cells to quickly scan the peptide repertoire presented on MHC molecules (Geijtenbeek et al., 2000b). In addition, interaction between DC-SIGN and ICAM-2 is found to facilitate DC

migration and homing of DC precursors to the peripheral tissues (Geijtenbeek et al., 2000a). Furthermore, engagement of MAC-1 and CD66a on neutrophils by DC-SIGN induces DC maturation, thereby establishing a molecular bridge between the innate and adaptive immune system (van Gisbergen et al., 2005a; van Gisbergen et al., 2005b).

Engagement of CLRs generally results in internalization of the ligands (Engering et al., 2002; Engering et al., 1997). The internalized lectins travel through the endocytic pathway, releasing the cargo at low pH environment in either endosomal or lysosomal compartments. Some CLRs, such as MR and DEC-205, recycle back to the cell surface, whereas other CLRs are degraded together with their cargo (Mahnke et al., 2000). Although targeting protein antigens to CLRs could result in enhanced presentation of antigenic peptides on MHC molecules, this strategy does not necessarily lead to activation of adaptive immunity (Burgdorf et al., 2006; Carter et al., 2006a; Carter et al., 2006b; Ramakrishna et al., 2004; Tacken et al., 2005). For example, targeting antigens via the murine DEC-205 *in vivo* enhances antigen presentation on MHC class I up to 400-fold, however, in the absence of a danger signal, responder $CD8^+$ T cells are deleted from the repertoire (Bonifaz et al., 2002; Engering et al., 1997).

The absence of immune activation after antigen binding to CLRs, led to the hypothesis that their primary function is to internalize antigen in the steady state for homeostasis control and that pathogens specifically target CLRs to evade specific immune responses in favor of pathogen survival (Geijtenbeek et al., 2004). In addition, CLRs are capable of modulating and fine-tuning immune responses. Combined triggering of Dectin-1 and TLR2 by fungal pathogens leads to the synergistic production of TNFα, IL-12 and reactive oxygen species (ROS) (Brown et al., 2003; Gantner et al., 2003). In

contrast, collaborative recognition of *Mycobacterium tuberculosis* by DC-SIGN and TLR4 results in enhanced IL-10 production and inhibition of DC maturation (Geijtenbeek et al., 2003a). DC-SIGN binding to bacteria *Helicobacter pylori* does not alter DC maturation; instead, immune responses are skewed towards a Th2 phenotype (Bergman et al., 2004). Strikingly, triggering of DC-SIGN with a lipopolysaccharide mutant of *Neisseria meningitidis* results in Th1-mediated immunity (Steeghs et al., 2006). Thus, the balance between TLR and CLR activation in DCs may be instrumental in inducing either tolerance or immune activation.

*Dectin-1 (CLEC7A, CD369).* Dectin-1 is a glycosylated type II transmembrane CLR with a single extracellular CTLD and a cytoplasmic immunoreceptor tyrosine-based activation (ITAM)-like motif (also known as hemITAM) (Brown, 2006). Dectin-1 is known to have a number of isoforms due to alternative splicing in both human and mouse (Brown, 2006; Heinsbroek et al., 2006). Dectin-1 is expressed by LCs and different myeloid DCs, including inflammatory DCs, $CD1c^+$ DCs and $CD141^+$ DCs, which places Dectin-1 in an ideal position to detect invasive fungi (Geijtenbeek and Gringhuis, 2009). In addition, expression of Dectin-1 has been reported on other cell types, including various populations of lymphocytes (Brown, 2006; Joo et al., 2015).

Dectin-1 recognizes β-glucan that are exposed at the budding scars of pathogenic and opportunistic fungi (Tsoni and Brown, 2008). Engagement of Dectin-1 on DCs by β-glucan clusters the receptor in synapse-like structures (Goodridge et al., 2011) and triggers a variety of cellular responses including DC maturation, ligand uptake by endocytosis and phagocytosis, the respiratory burst, the production of arachidonic acid

metabolites and numerous cytokines and chemokines, including IL-1β, TNFα, CXCL2, IL-23, IL-6 and IL-10 (Brown, 2006; Gringhuis et al., 2009).

Indeed, Dectin-1 is the first PRR outside of the TLR family found to be capable of inducing its own intracellular signals (Figure 1.3) (Brown, 2006; Geijtenbeek and Gringhuis, 2016). Dectin-1 clustering upon ligation triggers phosphorylation of hemITAM in the cytoplasmic region, leading to recruitment and activation of tyrosine kinase Syk, followed by the formation of CARD9-Bcl10-MALT1 (CBM) scaffold complex. This CBM complex initiates a signaling cascade that leads to the activation of canonical NF-κB pathway (Gross et al., 2006), which triggers the expression of the genes encoding IL-6 and IL-23p19 for the induction of Th17 cells (Gringhuis et al., 2009). Non-canonical NF-κB is also activated in a Syk-dependent way, although the detailed mechanism remains largely unknown. In addition, Dectin-1 ligation activates a second, Syk-independent pathway via the serine/threonine kinase Raf-1, leading to the expression of IL-1β and IL-12p40 that are equally essential to the induction of Th17 cells (Gringhuis et al., 2009). The crosstalk between Syk- and Raf-1-dependent signaling pathways further fine-tunes the gene expression, resulting in a cytokine environment that directs both Th1 and Th17 cell differentiation. Dectin-1 can also activate NFAT, implicating these transcription factors in innate anti-microbial immunity, although the involvement of Syk in this response has not been established (Goodridge et al., 2007). There is also report of Syk-dependent but CARD9-independent pathway, which induces the activation of ERK and regulates Dectin-1-mediated cytokine production, particularly IL-10 and IL-2 (Slack et al., 2007).

Furthermore, Dectin-1 is found to interact with other MyD88-coupled TLRs. Dectin-1-mediated Raf-1 activation leads to the phosphorylation as well as acetylation of TLR-induced p65, which increases TLR-induced cytokine expression, including IL-6, IL-10 and IL-12p35 (Gringhuis et al., 2009). On the other hand, the Syk pathway modulates TLR signaling by inducing the NF-κB subunits c-Rel and RelB, which increase IL-23p19 and decrease IL-12p40 expression, respectively (Gringhuis et al., 2009). Because Raf-1 signaling counteracts RelB activation by sequestering RelB in inactive dimers, the crosstalk between the Raf-1 and Syk pathways increases the production of TLR-induced IL-6, IL-10 and IL-12 (Gringhuis et al., 2009). Therefore, the crosstalk between TLR and Dectin-1 signaling cascade is complex and involves both the Syk and Raf-1 signaling pathways, which integrate at the level of NF-κB to shape adaptive immune responses (Geijtenbeek and Gringhuis, 2016).

*DC-ASGPR (MGL, CLEC10A, HML, CD301).* Three CLR genes have been found within a single cluster on human chromosome 17p13, namely, DC-ASGPR, ASGPR H1 and ASGPR H2. Their close location and high sequence similarity suggest that they might originate from a common ancestor; however, proteins encoded by these genes have distinct cellular distribution and carbohydrate specificity, indicating that these CLRs perform different biological functions *in vivo* (van Vliet et al., 2008b). The liver-specific ASGPR was the first CLR discovered and it is expressed in all mammalian species examined so far (Ashwell and Harford, 1982; Park and Baenziger, 2004). Human hepatic ASGPR consists of hetero-oligomers of H1 and H2 subunits (Weigel and Yik, 2002). In contrast, human DC-ASGPR (hDC-ASGPR) is expressed as a homo-oligomer

on myeloid DCs, macrophages, monocytes and B cells that are devoid of hepatic ASPGR expression (van Vliet et al., 2008b).

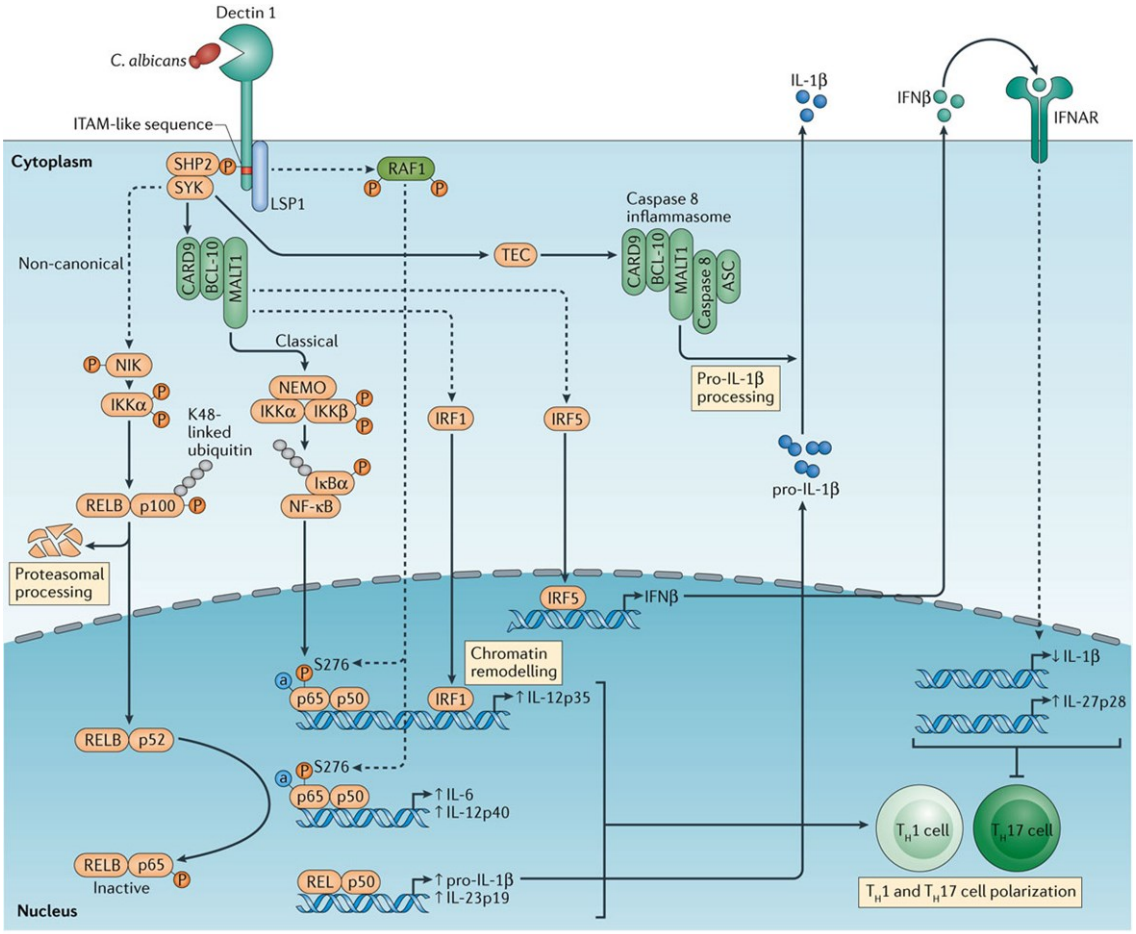


Figure 1.3. Dectin-1 signaling pathway. Ligation of Dectin-1 by fungal β-glucan activates Syk, which triggers both canonical and non-canonical NF-κB pathway. Dectin-1 activates a second pathway, which depends on Raf-1 but not Syk. The cooperation between Syk- and Raf-1-mediated signaling pathways leads to expression of a cytokine profile that promotes Th1 and Th17 cell differentiation for antifungal immunity. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology (Geijtenbeek and Gringhuis, 2016), copyright 2016.

In history, hDC-ASGPR was independently identified and cloned by two groups (Suzuki et al., 1996; Valladeau et al., 2001). Initially, it was cloned from macrophages and was therefore named macrophage galactose lectin (MGL). Valladeau *et al.* cloned a lectin from DCs, which was named DC-asialoglycoprotein receptor (DC-ASGPR) after

considerable homology to hepatic ASGPR. In addition to MGL, DC-ASGPR is also known as CLEC10A, human macrophage lectin (HML) and CD301. Herein, we choose to use the term DC-ASGPR for consistency with our previous reports (Li et al., 2012; Valladeau et al., 2001).

The mRNA of hDC-ASGPR is subject to alternative splicing, which gives rise to several isoforms resulting from deletions at three potential sites (Valladeau et al., 2001). It is currently unknown whether the mAbs generated by different groups could recognize all the isoforms or whether the alternative splicing has any functional consequence, such as glycan specificity and ligand recognition.

In addition to human, orthologues of DC-ASGPR have been found in rat and mouse (Valladeau et al., 2001). In the 1980s, Kawasaki *et al.* discovered that rat peritoneal macrophages express a $Ca^{2+}$-dependent lectin, which is distinct from the hepatic ASGPR, with specificity for galactose and GalNAc (Kawasaki et al., 1986). Based on the binding property, this lectin was isolated and named rat MGL (rMGL) or macrophage asialoglycoprotein-binding protein (M-ASGP-BP) (Ii et al., 1990). Two years later, another group independently identified and characterized the rMGL as well (Kelm and Schauer, 1988). Mouse has two distinct MGL genes, namely *mgl1* and *mgl2*, whereas in rat and human, only one copy is found (Higashi et al., 2002; Tsuiji et al., 2002; Valladeau et al., 2001). The mouse *mgl* genes are located in chromosome 11 and are also closely clustered to gene encoding mouse hepatic ASGPR. Mouse MGL1 (mMGL1) and mMGL2 are 79% identical on the nucleotide level and 91.5% identical in amino acid sequence. Compared with mMGL1, mMGL2 contains an insertion of 14 amino acids in

its cytoplasmic region and a few amino acid changes in the CRD that affect the secondary binding site, leading to a distinct carbohydrate recognition profile.

Whereas ASGPR is only expressed on liver parenchymal cells, hDC-ASGPR is expressed by many different cell types including myeloid DCs, monocytes, macrophages and B cells. In contrast, T cells and pDCs do not express hDC-ASGPR (Li et al., 2012). In skin, hDC-ASGPR is expressed by dermal $CD1a^{+}$ APCs that are distinct from migrating LCs (Angel et al., 2006; Li et al., 2012). In addition, APCs that express hDC-ASGPR have been found in small intestine, thymus and LN (van Vliet et al., 2006a). Monocyte-derived DCs express moderate level of hDC-ASGPR, which becomes negative after DC maturation. Moreover, hDC-ASGPR expression is upregulated on tolerogenic DCs generated in the presence of glucocorticoids and during chronic inflammatory conditions such as rheumatoid arthritis, suggesting the role of hDC-ASGPR in immune regulation (van Vliet et al., 2006b).

The mouse homologues of hDC-ASGPR, namely mMGL1 and mMGL2, have similar expression patterns (Tsuiji et al., 2002). Initially, mMGL expression was only found on APCs in the connective tissue near epithelial surfaces (Dupasquier et al., 2006). Later, mMGL was also detected on DCs and macrophages in skin dermis and in subcapsular and intrafollicular sinuses and T cell areas of mouse LNs (Dupasquier et al., 2004). In addition, Raes *et al.* identified mMGL1 and mMGL2 as surface markers for alternatively activated macrophages (Raes et al., 2005).

In rat, thioglycolate-elicited peritoneal macrophages, but not the resting counterparts, express high level of rMGL (Kawasaki et al., 1986). Moreover, expression

of rMGL was found upregulated on macrophages that accumulated at chronic rejection sites in rat cardiac allografts (Russell et al., 1994).

Yvette van Kooyk *et al.* reported the carbohydrate specificity of hDC-ASGPR analyzed by glycan microarray profiling. According to their report, hDC-ASGPR expressed on either transfectants or primary DCs, displays an exclusive specificity for terminal α- and β-linked GalNAc residues that naturally occur on glycoproteins and glycosphingolipids (van Vliet et al., 2008b). Specific glycans expressed by human helminth parasite *Schistosoma mansoni*, tumor-specific MUC1 and gangliosides GM2 and GD2 were identified as ligands for hDC-ASGPR (Saeland et al., 2007; van Vliet et al., 2005a). In addition, CD45, which contains GalNAc epitopes, has been identified as a natural cellular ligand of hDC-ASGPR (van Vliet et al., 2006a). Human CD45 mRNA undergoes alternative splicing in the A, B, and C domains, and gives rise to five different isoforms on human leukocytes (ABC, AB, BC, B, and RO). Indeed, hDC-AGPR recognizes all CD45 isoforms, except CD45RO. CD45RO has only two *O*-linked glycans, which are either not properly glycosylated or insufficient for sustained hDC-ASGPR binding. In addition, hDC-ASGR preferentially binds to activated effector T cells, which highly express the CD45B isoform. More importantly, the interaction of hDC-ASGPR with CD45 on effector T cells negatively regulates T cell receptor-mediated signaling, suggesting that cell-specific glycosylation of CD45 could provide a mechanism for regulating various immunological pathways, including TCR signaling (van Vliet et al., 2006a).

In contrast, hepatic ASGPR preferentially interacts with tri- or tetra-antennary glycans containing both galactose or GalNAc residues (Ozaki et al., 1995; Park and

Baenziger, 2004). Thus, the carbohydrate specificities of hDC-ASGPR and hepatic ASGPR are significantly different.

It has been reported that mMGL1 and mMGL2 have distinct carbohydrate-recognition profiles. Recombinant mMGL1 specifically recognized Lewis X structures, whereas mMGL2 showed the highest affinity for α- and β-GalNAc and didn't interact with Lewis X structures (Tsuiji et al., 2002; Yamamoto et al., 1994). However, the different carbohydrate specificities of mouse MGLs were not verified using transfectants or cells that naturally express the CLRs. Therefore, detailed carbohydrate specificities of mMGLs remain to be clarified.

The carbohydrate specificity of rMGL was investigated using recombinant rMGL, which bound a restricted set of glycans including those with Lewis A and Lewis X structures. In addition, rMGL also displayed the highest affinity for bi-antennary galactose and GalNAc-terminated structures (Coombs et al., 2006).

Taken together, MGL orthologues in human, mouse and rat show distinct carbohydrate specificities, although they share high similarity in terms of amino acid sequence.

DC-ASGPR, like many other CLRs, mediate rapid internalization upon ligation (Higashi et al., 2002; Valladeau et al., 2001). All DC-ASGPR orthologues contain a conserved YXXØ motif in their cytoplasmic region, where X denotes any amino acid and Ø denotes any hydrophobic amino acid. To be specific, hDC-ASGPR, mMGL2 and rMGL have a YENF motif, whereas mMGL1 has a YENL motif (Higashi et al., 2002; Valladeau et al., 2001). Disruption of the YENF motif in hDC-ASGPR by substituting the tyrosine residue with alanine completely abrogated hDC-ASGPR-mediated

internalization (van Vliet et al., 2007) whereas mutation of the same motif in rMGL resulted in a ligand-induced internalization rate of about one-fourth of the wild-type counterpart (Ozaki et al., 1993). The YXXØ motif is found in other CLRs, including ASPGR H1 and DC-SIGN. In addition, DC-ASGPR orthologues have a partial dileucine motif (LL) in the cytoplasmic region, which is also involved in the receptor-mediated internalization (Bonifacino and Traub, 2003). Similarly, this partial dileucine motif can be also found in ASGPR H1 and DC-SIGN.

Antigens internalized via hDC-ASGPR are transported along the endosomal-lysosomal pathway and then presented on the MHCII molecules (van Vliet et al., 2007). It has been observed that under steady-status, a large proportion of hDC-ASGPR molecules reside intracellularly in early endosomes and small vesicles, suggesting that hDC-ASGPR might continuously recycles between the cell surface and intracellular compartments (Valladeau et al., 2001).

Mouse MGL1 was originally isolated from tumoricidal macrophages that are located in the lung metastases in a mouse model of ovarian cancer (Oda et al., 1988). Surprisingly, injection of mMGL1-transfected T cell line resulted in selective homing of these cells to metastatic tumor nodules, probably through the recognition of tumor-associated glycans. Administration of anti-mMGL blocking antibodies significantly increased tumor loads at metastatic sites, suggesting that $mMGL^+$ APCs could contribute to the host defense against tumor metastasis (Ichii et al., 2000).

Human DC-ASGPR might participate in the immune response to human adenocarcinomas through the preferential recognition of the tumor-associated MUC1 (Napoletano et al., 2007; Saeland et al., 2007). Indeed. hDC-AGPR is capable of

distinguishing MUC1 derived from healthy tissue and the tumor-associated MUC1 based on their different glycosylation pattern (Saeland et al., 2007). As hDC-ASGPR is preferentially expressed by tolerogenic APCs (van Vliet et al., 2006b), binding of tumor-associated antigen MUC1 to hDC-ASGPR could promote tumor progression instead of tumor rejection.

Previous study reported that binding of hDC-ASGPR to CD45 on effector T cells negatively regulates their function. The hDC-ASGPR-CD45 interaction suppresses TCR-mediated signaling, resulting in decreased phosphatase activity of CD45 and inhibition of Lck activation and calcium mobilization (van Vliet et al., 2006a). Via this mechanism, hDC-ASGPR$^+$ tolerogenic APCs can downregulate effector T cell activation, decrease cytokine and proliferative responses, and even induce T cell apoptosis. Because memory T cells retain their CD45RB expression, hDC-ASGPR binding to these cells could also potentially dampen memory T cell responses. The observed upregulation of hDC-ASGPR expression in chronic inflammatory diseases (van Vliet et al., 2006a) could thus be a self-protecting mechanism initiated to prevent excessive inflammation and tissue damage.

However, this report mainly focused on the signaling events in T cells upon CD45 and hDC-ASGPR interaction while leaves DC-ASGPR-mediated signaling pathway in DCs unresolved.

Indeed, some CLRs such as Dectin-1 can serve as signaling receptors capable of modulating innate and adaptive immune responses (Geijtenbeek and Gringhuis, 2009; Geijtenbeek and Gringhuis, 2016). For instance, DCs activated via Dectin-1 produces Th1- and Th17-polarizing cytokines (Gringhuis et al., 2009; LeibundGut-Landmann et al.,

2007). Binding of pathogens to DC-SIGN modulates TLR signaling via Raf-1, leading to enhanced IL-10 production of DCs (Gringhuis et al., 2007; Hodges et al., 2007).

The tyrosine of the YXXØ motif and some serine residues in the hepatic ASGPR are found phosphorylated for regulation of receptor distribution. Given the similarity in the amino acid sequence, it is highly possible that hDC-ASGPR could be phosphorylated and further initiate intrinsic intracellular signaling cascade. Although we have previously reported that antigen targeting to hDC-ASGPR induces IL-10 expression by DCs, which promotes antigen-specific Treg responses (Li et al., 2012), detailed molecular mechanism for the DC-ASGPR-induced immune tolerance is still unclear.

# CHAPTER TWO

## Objectives

CLRs expressed on DCs not only facilitate antigen capture and uptake for presentation, but also deliver diverse intracellular signals that modulate DC functions and result in altered immune responses. Based on these special properties of CLRs, targeting of the signaling pathways downstream of CLRs could be a promising strategy to manipulate host immune responses.

Among numerous CLRs, Dectin-1 ligation triggers a complex signaling cascade, resulting in activation of both canonical and non-canonical NF-κB pathway and subsequent expression of Th1- and Th17-polarizing cytokines (Gringhuis et al., 2009). Preliminary studies suggest that the presence of Dectin-1 agonistic ligand downregulates antigen-specific Th2 responses whereas thymic stromal lymphopoietin (TSLP) is known to induce OX40L expression and trigger DC-mediated inflammatory Th2 cell responses (Ito et al., 2005; Soumelis et al., 2002). We therefore propose to test whether Dectin-1-activated DCs could regulate TSLP-induced inflammatory Th2 cell responses.

In addition, DC-ASGPR shows a unique ability to modulate immune responses by promoting antigen-specific regulatory T cells (Tregs) (Li et al., 2012). Signals via DC-ASGPR are known to induce IL-10 expression in DCs, which promotes the generation of antigen-specific Tregs (Li et al., 2012). Given the unique property of DC-ASGPR in immune modulation, we propose to investigate the detailed signaling pathway mediated by DC-ASGPR.

*Aim1: Investigate the Role of Dectin-1in Regulating Inflammatory Th2 Cell Responses*

a) Investigate the potency and mechanism of Dectin-1 in regulating TSLP-induced OX40L expression

b) Investigate whether Dectin-1 signaling in DCs could repress allergen-specific Th2 cell responses *in vitro*

In Chapter Three, TSLP was used to induce OX40L expression on DCs and subsequent inflammatory Th2 cell responses *in vitro*. The molecular mechanism of Dectin-1 signaling in regulating TSLP-induced OX40L expression was investigated. The potency of Dectin-1-activated DCs in regulating allergen-specific Th2 cell responses was also explored in the presence of TSLP.

*Aim2: Investigate DC-ASGPR-Mediated Signaling Pathway in Human DCs*

a) Investigate the phenotype and cytokine profile of DCs upon DC-ASGPR ligation

b) Identify the signaling intermediates engaged in DC-ASGPR signaling pathway and their roles in regulating cell activation and cytokine expression

c) Investigate the relationship of different kinases involved in DC-ASGPR-mediated signaling cascade

In Chapter Four, a monoclonal antibody (mAb) (clone 49C11) was used as specific ligand of DC-ASGPR. Phenotype and cytokine profile of DCs treated with 49C11 were investigated by surface staining and Luminex assay, respectively. The signaling intermediates including kinases and transcription factors inducibly activated in DCs by 49C11 treatment was investigated by western blot. In addition, different kinase

inhibitors were used to confirm their engagement and their role in regulating subsequent cell activation and cytokine production.

# CHAPTER THREE

## Cellular and Molecular Mechanisms for Dectin-1-Mediated Suppression of Th2-Type Immune Response


*Abstract*

Thymic stromal lymphopoietin (TSLP) can induce OX40 ligand (OX40L) expression on myeloid dendritic cells (mDCs) that promote inflammatory type 2 T helper (Th2) cell responses. Herein, we report that activation of human mDCs via Dectin-1 can effectively suppress TSLP-induced inflammatory Th2 cell response. Mechanistically, we have demonstrated that in the presence of TSLP, activation of mDCs via Dectin-1 induces IL-10 production, which contributes to downregulation of OX40L expression. In addition, Dectin-1- and TSLP receptor-mediated signaling pathways crosstalk at NF-κB level, leading to decreased OX40L expression by suppressing the transcriptional activity of p50-RelB heterodimer. We have also found that Dectin-1 ligation inhibits TSLP-induced phosphorylation of STAT6 in mDCs, which results in a decreased expression of Th2 cell-attracting chemokine CCL17. Moreover, Dectin-1-activated mDCs are able to suppress Th2 cytokine expression by $CRTH2^+$ memory $CD4^+$ T cells, along with a significant decrease of surface CRTH2 expression on T cells. Finally, Dectin-1 ligation can effectively suppress Th2-type cytokine expression by T cells from patients with allergic diseases in an antigen-specific manner. Therefore, Dectin-1 expressed on mDCs represents one of the promising targets for the treatment of Th2 cell-mediated inflammatory diseases, including allergic asthma.

*Introduction*

Human thymic stromal lymphopoietin (TSLP), an IL-7-like cytokine, is mainly produced by skin keratinocytes and mucosal epithelial cells upon allergic stimulations (Soumelis et al., 2002). TSLP has been well known for its capacity to potently activate $CD11c^+$ mDCs and induce robust Th2 allergic inflammation featuring large amounts of IL-4, IL-5, IL-13 in the presence of TNFα, and in the absence of IL-10 and IFNγ, which are two physiological inhibitors of Th2 inflammation (Soumelis et al., 2002). More importantly, these $TNF\alpha^+IL\text{-}10^-$ Th2 cells, in contrast to the conventional IL-10-producing Th2 cells, are recognized as the pathogenic Th2 cells that can cause allergic inflammation (Ito et al., 2005).

Previous studies reported that TSLP does not stimulate mDCs to produce Th1, Th2 or Th17-polarizing cytokines, including IL-4, IL-12, IL-23, IL-27, IFNα, IFNβ, IFNγ or anti-inflammatory cytokine IL-10 (Ito et al., 2005). However, TSLP preferentially induces mDCs to express OX40L, which serves as the positive Th2 cell-polarizing signal that operates under a permissive condition for Th2 development (Ito et al., 2005). More importantly, OX40L expressed by TSLP-activated mDCs (referred to hereafter as TSLP-mDCs) also acts as a switch that inhibits IL-10 production, but promotes TNFα expression in Th2 cells, thus endows the Th2 cells with a $TNF\alpha^+IL\text{-}10^-$ inflammatory phenotype (Ito et al., 2005). Recent studies suggested that TSLP induces the nuclear translocation of p50, which forms a transcriptionally active complex with RelB to induce the expression of OX40L on mDCs (Arima et al., 2010). In addition, TSLP-mDCs can further enhance Th2-mediated inflammation by producing chemokines, including CCL17 and CCL22, which preferentially recruit Th2 cells into the original inflamed tissues

(Soumelis et al., 2002). Indeed, TSLP induces robust and sustained activation of JAK-STAT signaling pathway (Arima et al., 2010). STAT6, which is preferentially activated by TSLP, binds to the *CCL17* promoter to initiate its expression (Arima et al., 2010).

The unique inflammatory Th2-inducing signal initiated in DCs upon TSLP stimulation exemplifies the functional plasticity of DCs in inducing distinct T helper cell responses and also emphasizes that DCs play a critical role in the pathogenesis of allergic diseases (Soumelis et al., 2002). Therefore, modulation of DCs to regulate the undesired inflammatory Th2 cell responses could be a promising strategy to control TSLP-induced allergic inflammation.

DCs are specialized in the recognition of self- and foreign antigens and play a pivotal role in the control of host immune responses (Banchereau et al., 2000; Banchereau and Steinman, 1998). The nature of the antigen recognized by DCs tilts the balance towards either immunity or tolerance (Banchereau et al., 2000; Banchereau and Steinman, 1998; Steinman et al., 2003). C-type lectin receptors (CLRs) expressed by DCs are involved in the recognition of glycosylated self-antigens and pathogens (Janeway and Medzhitov, 2002; van Vliet et al., 2008a). It is now becoming clear that CLRs not only serve as antigen receptors allowing efficient internalization and antigen presentation, but also function in determining and regulating T-cell polarization (Geijtenbeek and Gringhuis, 2009; Geijtenbeek and Gringhuis, 2016). For example, DC-SIGN ligation by different pathogens leads to promotion of Th2 responses and induction of regulatory T cell differentiation (Geijtenbeek et al., 2003b; Gringhuis et al., 2007). Targeting self- and foreign antigens to DCs via DC-ASGPR generates IL-10-producing suppressive $CD4^+$ T cells (Napoletano et al., 2012). Furthermore, we and other groups have reported that DCs

activated via Dectin-1 result in polarized Th17 cell responses (Duluc et al., 2014; Gringhuis et al., 2009; LeibundGut-Landmann et al., 2007) and signals via Dectin-1 induce IL-10 expression in DCs (Ni et al., 2010; Rogers et al., 2005), and activation of DCs via Dectin-1 and TLR2 results in regulatory T cell responses (Dillon et al., 2006).

Dectin-1 is a unique CLR that induces NF-κB activation upon ligation (Gringhuis et al., 2009). DCs activated via Dectin-1 can induce both Th1 and Th17 cell responses that are essential to the host defense against fungi and mycobacteria (Gringhuis et al., 2009). Recent studies have reported the role of Dectin-1 in allergic asthma. It has been shown that IL-17 and Dectin-1 protected against airway hyper-responsiveness (AHR) and eosinophil inflammation following *A. versicolor* exposure (Mintz-Cole et al., 2012). Dectin-1 agonists have been shown to suppress the epicutaneously introduced Th2 responses by modulating cytokine expression profiles in draining lymph nodes (LNs) and the migration of epidermal Langerhans cells (Lin et al., 2013). Thus, Dectin-1 plays an important role in the control of allergic asthma, but little is known about the mechanism by which Dectin-1 suppresses inflammatory Th2 cell responses.

In this study, we report that activation of $CD11c^+$ mDCs via Dectin-1 by agonistic ligand curdlan, can suppress the generation and maintenance of inflammatory Th2 cells by repressing TSLP-induced OX40L expression on mDCs through inhibiting the transcriptional activity of p50-RelB. We also demonstrate that TSLP-mDCs upon curdlan treatment decrease the production of Th2-attracting chemokine CCL17 and downregulate the expression of chemokine-related CRTH2 receptor on T cells. Taken together, we have identified a new innate signaling pathway mediated by Dectin-1 that serves to control TSLP-mDC-mediated inflammatory Th2 immune responses.

## *Materials and Methods*

### *Purification and Culture of DCs and T Cells*

All blood donors provided a written informed consent prior to inclusion in the study in accordance with the approval by the Institutional Review Boards at Baylor Research Institute. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using FicollPaque PLUS (GE Healthcare). Blood myeloid DCs (mDCs, Lin-1$^{-}$HLA-DR$^{+}$CD11c$^{+}$CD123$^{-}$) were pre-enriched from PBMCs using a pan-DC enrichment kit (Stemcell Technologies) and then sorted by FACSAria (BD Biosciences). Sorted mDCs with purity > 99% were cultured in RPMI 1640 (GIBCO) supplemented with 25 mM HEPES (Life Technologies), 2 mM L-glutamine, 100 μM nonessential amino acids, 1 mM sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin (Sigma) and 10% heat-inactivated normal FCS (Invitrogen). CD4$^{+}$ T cells were purified from PBMCs using EasySep Human CD4$^{+}$ T Cell Enrichment Kit according to manufacturer's instructions (Stemcell Technologies). Naive (CD45RA$^{+}$CD45RO$^{-}$CCR7$^{+}$) and CRTH2$^{+}$ memory (CD45RO$^{+}$CRTH2$^{+}$CCR7$^{+}$) T cells were sorted by FACSAria (purity>99.0%).

### *Antibodies, Peptides and Other Reagents*

Fluorescence-conjugated antibodies including anti-CD4, anti-IL-4, anti-IL-5, anti-IL-13, anti-IFNγ and anti-CCR7 were purchased from Biolegend; anti-CD45RA, anti-CD45RO, anti-CRTH2 (BM16) and anti-OX40L (IK-1) were purchased from BD Biosciences; anti-IL-17 and anti-CD86 were purchased from eBioscience. Recombinant human TNFα and purified antibodies including anti-IL-10, anti-IL-10R, anti-TNFα and

isotype controls were purchased from R&D. Recombinant human IL-10 (PeproTech) and purified anti-TNFαR (US Biologicals) were used in this study. GolgiPlug was purchased from BD Biosciences. Curdlan were purchased from Sigma-Aldrich.

*DC Activation*

Sorted mDCs were left untreated in medium or activated with 20 ng/ml TSLP (a generous gift from Dr. Laura Bover at MD Anderson Cancer Center, Houston), 100 μg/ml curdlan or a combination of TSLP and curdlan. In some experiments, anti-IL-10, anti-IL-10R, anti-TNFα, anti-TNFαR (10 μg/ml for each), TNFα or IL-10 (1 μg/ml), was added into the cell culture. After 48h, mDCs were stained with 7-AAD, CD86 and OX40L. Cytokines in the supernatants were assessed by the BeadLyte cytokine assay kit (Upstate, MA) as per the manufacturer's protocol. Fluorescence was analyzed with a Bio-Plex Luminex 100 XYP instrument (Bio-Rad), with the Bio-Plex Manager 4.1 software, with a 5-parameter curve-fitting algorithm applied for standard curve calculations. Concentration of chemokine CCL17 in the culture supernatants after 15h or 40h incubation was measured using a Human CCL17/TARC DuoSet ELISA kit according to manufacturer's instructions (R&D systems).

*NF-κB DNA Binding*

Nuclear extracts of mDCs were prepared after 24h of stimulation. NF-κB DNA binding was determined with a TransAM NF-κB family kit (Active Motif) according to the manufacturer's instructions.

*DC/CD4$^+$ T Cell Co-cultures and Allergen-specific CD4$^+$ T Cell Responses*

After 24h of stimulation, mDCs were co-cultured with 1-2×10$^5$ autologous CRTH2$^+$ memory, total, or allogeneic naive CD4$^+$ T cells at a DC:T cell ratio of 1:20. After 7 days, CD4$^+$ T cells were restimulated with PMA/ionomycin for 6h in the presence of Brafeldin A, followed by intracellular staining for flow cytometry analysis (FACS CantoII, BD). In parallel experiments, CD4$^+$ T cells were restimulated with immobilized anti-CD3 (10 μg/mL) and soluble anti-CD28 (1 μg/mL) for 48h. Culture supernatants were assessed by the BeadLyte cytokine assay kit (Upstate, MA). For assessing allergen-specific CD4$^+$ T cell responses, patient PBMCs were left untreated or incubated with TSLP, curdlan, or both for 3h before loaded with 2 μg/mL Amb a1 and cultured for 7d. Cells were then re-stimulated with autologous PBMCs loaded with 2ug μg/mL Amb a1 and Amb a1-derived peptide for 48h, cytokines in the supernatants were assessed by the BeadLyte cytokine assay kit.

*Western Blot*

After activation, mDCs were collected and lysed with PhosphoSafe Extraction Buffer (EMD Biosciences) supplemented with Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific) according to the manufacturer's instructions. Cell lysates were mixed with SDS sample buffer and boiled at 90°C for 5 min. Samples were separated using SDS-PAGE, transferred to a PVDF membrane and probed with the indicated antibodies against P-STAT5 (Tyr694), P-STAT6 (Tyr641), or β-actin (Sigma-Aldrich).

*Co-Immunoprecipitation (Co-IP)*

Nuclear extraction and Co-IP assay were conducted using Nuclear Complex Co-IP Kit (Active Motif) according to the manufacturer's instructions. Antibody against RelB was used for immunoprecipitation.

*ChIP*

ChIP assay was performed using the ChIP-IT Express Chromatin Immunoprecipitation Kits (Active Motif) according to the manufacturer's instructions. Antibodies against p50, RelA, and RelB were used for immunoprecipitations. Primers for the detection of precipitated DNA in polymerase chain reaction (PCR) assay were as follows: *OX40L* surrounding region of κB-like sequences. The detailed sequences are 5'-CCTGTTAGCCCAGAGGAAAA-3' and 5'-CCAGGGCCAGAGATAAAAGG-3'.

*Statistics*

Statistical significance was determined using the Student's *t*-test and significance was set at $p<0.05$. Spearman's rho statistics were used.

## *Results*

### *Dectin-1 Ligation Downregulates TSLP-induced OX40L Expression on mDCs via IL-10 and TNFα*

TSLP preferentially induces OX40L expression on mDCs, which polarizes naive $CD4^+$ T cells into Th2 cells with a $TNF\alpha^+IL\text{-}10^-$ inflammatory phenotype (Ito et al., 2005). Previously, we reported that activation of mDCs via Dectin-1 downregulates OX40L expression on cell surface under homeostatic condition (Joo et al., 2015). Herein, we investigated whether engagement of Dectin-1 by its agonistic ligand curdlan could

further suppress high level of OX40L on TSLP-mDCs. Consistent with previous studies, curdlan or TSLP alone, activated mDCs meanwhile differentially regulated OX40L expression (Figure 3.1A & 3.1B). In contrast, the combination of TSLP and curdlan, when compared with TSLP alone, further activated mDCs as evidenced by higher CD86 expression, but significantly inhibited surface OX40L expression (Figure 3.1A & 3.1B), suggesting that Dectin-1 signaling is dominant over TSLP in controlling OX40L expression on mDCs.

Unlike Toll-like receptor (TLR) ligands and CD40 ligand (CD40L), TSLP promotes mDCs maturation without inducing the production of Th1-polarizing cytokines or pro-inflammatory cytokines, such as IL-1β, IL-6 and TNFα (Ito et al., 2005). Besides, TSLP does not stimulate mDCs to produce the anti-inflammatory cytokine IL-10 (Ito et al., 2005). This unique property of TSLP can thus modulate mDC functions to create a Th2-permissive microenvironment. On the other hand, Dectin-1 signaling is known to induce a complex cytokine profile, including both pro- and anti-inflammatory cytokines, in addition to the Th1 and Th17-polarizing cytokines (Arthur and Elcombe, 2012; Gringhuis et al., 2009; Ni et al., 2010). We thus hypothesized that activation of TSLP-mDCs via Dectin-1 could induce a different pattern of cytokine production, which might subvert the Th2-permissive microenvironment and work in an autocrine way to suppress the TSLP-induced OX40L expression. Indeed, mDCs activated via Dectin-1 produced high levels of IL-10, TNFα and a low but substantial level of IL-1β while TSLP-mDCs produced these cytokines only at background level (Figure 3.1C). Interestingly, a synergy was observed between curdlan and TSLP in inducing the production of IL-10 and TNFα, but not IL-1β (Figure 3.1C). To better dissect the biological consequence of elevated IL-

10 and TNFα production, exogenous recombinant IL-10 or TNFα was added to TSLP-mDCs, which significantly suppressed OX40L expression on TSLP-mDCs (Figure 3.2A & 3.2B), suggesting that upon Dectin-1 ligation, IL-10 and TNFα produced by TSLP-mDCs could be the soluble mediators responsible for the decreased OX40L expression. To confirm this, we blocked IL-10, TNFα or both using neutralizing Abs and found that blocking IL-10 or TNFα alone partially recovered the OX40L expression on TSLP-mDCs upon Dectin-l ligation, however, blocking both of them led to nearly full restoration of OX40L expression (Figure 3.2C & 3.2D). In parallel experiment, blocking IL-6 or IL-1β did not significantly alter surface OX40L expression on TSLP-mDCs upon Dectin-1 ligation (date not shown). Taken together, Dectin-1 signaling can indirectly suppress OX40L expression on TSLP-mDCs through inducing IL-10 and TNFα secretion.

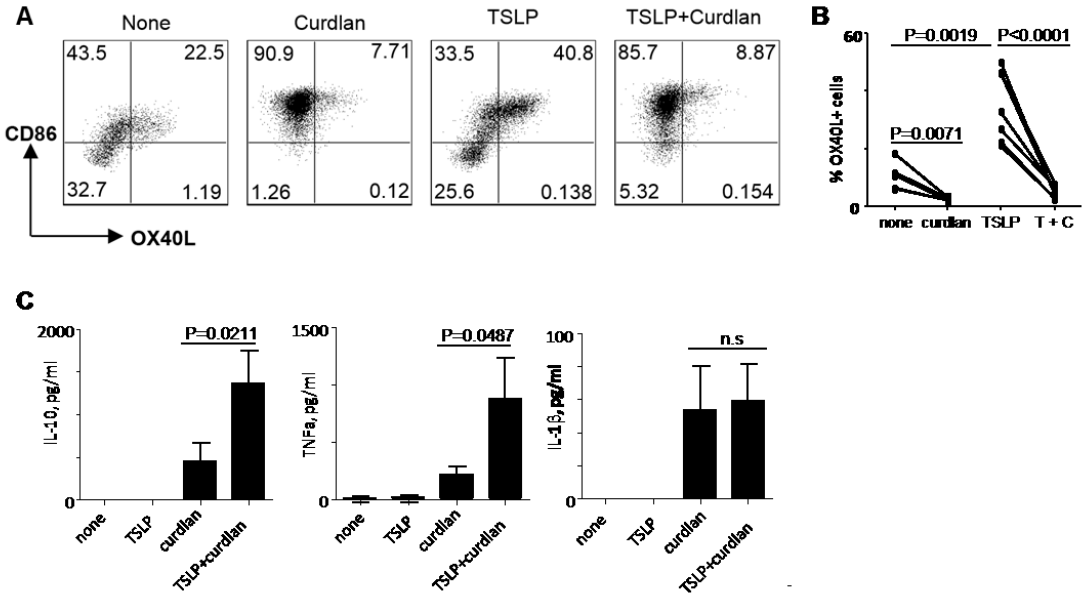


Figure 3.1. Dectin-1 ligation on mDCs suppresses TSLP-induced OX40L expression while increases IL-10 and TNFα production. (A–C) Sorted mDCs were left untreated in culture medium (none) or incubated with TSLP, curdlan or both for 48h. (A & B) Expression of CD86 and OX40L was measured by FACS staining. Gating was based on isotype staining. Representative data (A) and summarized data from different donors (B) (n=6) are shown. (C) Cytokine production of mDCs from different donors (n=6) was measured by Luminex.

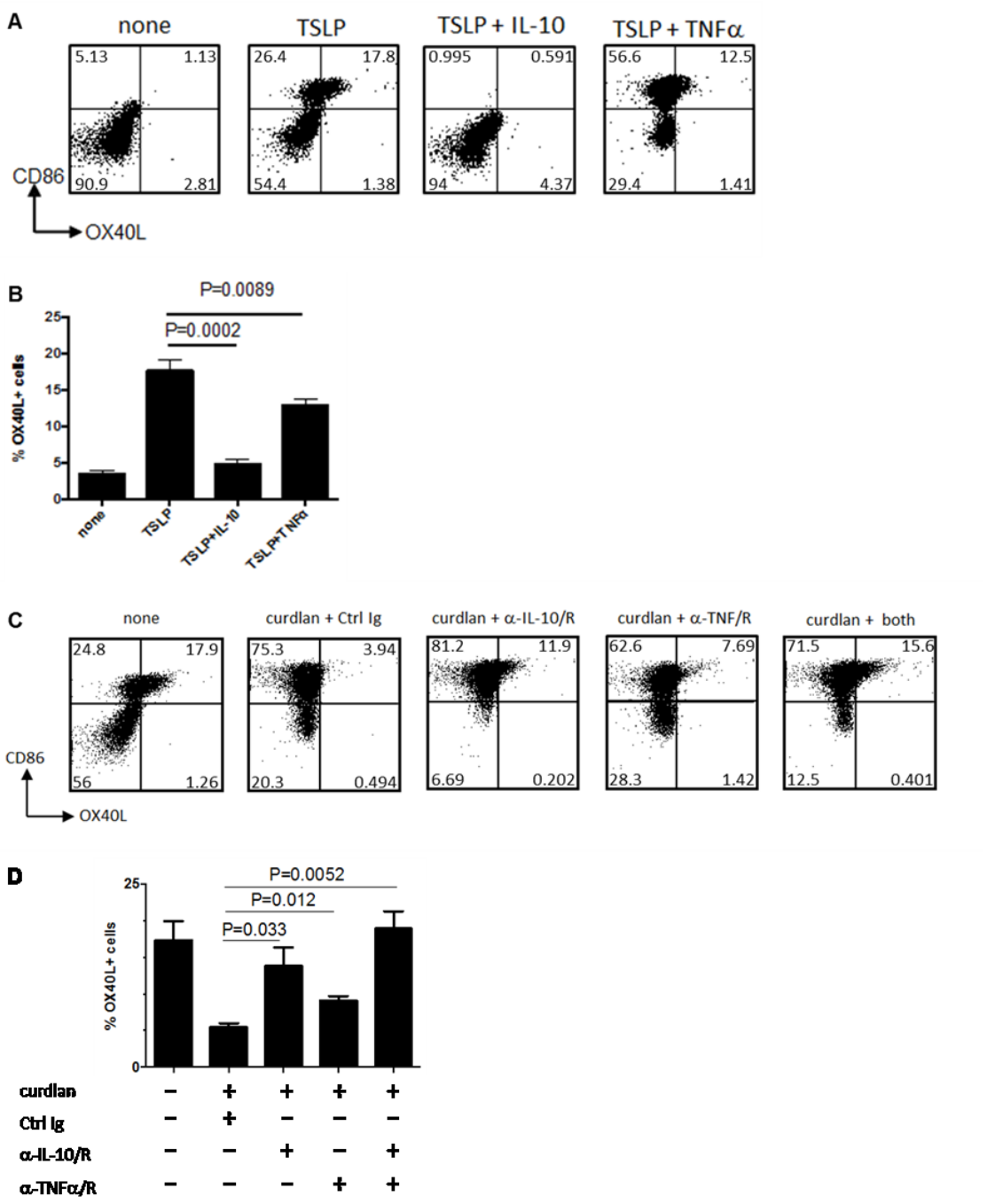


Figure 3.2. Curdlan-induced IL-10 and TNFα production inhibits OX40L expression on TSLP-mDCs. (A–B) Sorted mDCs were incubated with or without TSLP for 48h in the absence or presence of exogenous IL-10 or TNFα. Expression of CD86 and OX40L was measured by FACS staining. Representative data (A) and summarized data from different donors (B) (n=6) are shown. (C–D) Sorted mDCs were incubated with TSLP for 48h in the presence or absence of curdlan or blocking antibodies against IL-10, IL-10R, TNFα, TNFαR. Isotype-matched antibodies of irrelevant specificity (Ctrl Ig) were used as control. Expression of CD86 and OX40L was measured by FACS staining. Representative data (C) and summarized data from different donors (D) (n=6) are shown.

*Dectin-1 Ligation Downregulates OX40L Expression on TSLP-mDCs by Repressing p50-RelB Transcriptional Activity*

Both Dectin-1 and TSLP are known to activate NF-κB pathway. TSLP is reported to induce sustained activation of p50-RelB heterodimer, which binds to the promoter of *OX40L* to initiate its expression (Arima et al., 2010). Dectin-1 ligation activates Syk, which triggers both canonical and non-canonical NF-κB pathways (Gringhuis et al., 2009). In addition, Dectin-1 ligation induces a second signaling pathway through Raf-1, which induces p65 phosphorylation at Ser276 and represses Syk-induced RelB activity by forming $p65^{Ser276}$-RelB inactive dimer that cannot bind to DNA (Gringhuis et al., 2009). Therefore, it is possible that signaling pathways induced by Dectin-1 and TSLP could crosstalk at NF-κB level, leading to decreased abundance and/or transcriptional activity of p50-RelB complex for OX40L expression. To test this hypothesis, we first investigated the phosphorylation status of p65 in mDCs upon TSLP and/or curdlan stimulation. As shown in Figure 3.3A, in contrast to TSLP, curdlan alone, or in combination with TSLP, preferentially induced p65 phosphorylation at Ser276, suggesting that even in the presence of TSLP, Dectin-1 signaling could still induce the formation of inactive $p65^{Ser276}$-RelB complex. Moreover, to investigate the composition of NF-κB complexes induced by TSLP and curdlan stimulation, we performed co-immunoprecipitation (Co-IP) assay on mDCs nuclear extracts using a RelB-specific antibody. As shown in Figure 3.3B, in TSLP-mDCs, RelB bound to p50 and p52 but not p65, p100 or p105, forming two different heterodimers. RelB-p50 dimer is known induce OX40L expression by binding to its promoter whereas RelB-p52 dimer cannot bind to *OX40L* promoter (Arima et al., 2010). Surprisingly, we found that RelB formed complex with p50 in mDCs treated with curdlan alone or both curdlan and TSLP, even to a higher

extent when compared with those treated with TSLP alone, suggesting that Dectin-1 ligation may not reduce the abundance of p50-RelB to downregulate OX40L expression on TSLP-mDCs. We therefore hypothesized that Dectin-1 ligation could inhibit transcriptional activity of p50-RelB to downregulate OX40L expression. The DNA binding activity of different NF-κB subunits was therefore examined. As shown in Figure 3.4A, Dectin-1 ligation by curdlan, even in the presence of TSLP, significantly decreased RelB binding activity to DNA probe. Using ChIP assay, we further investigated the specific binding of RelB and p50 to the *OX40L* promoter in mDCs upon TSLP and/or curdlan treatment Indeed, recruitment of both RelB and p50 to the *OX40L* promoter was decreased in TSLP-mDCs upon curdlan stimulation (Figure 3.4B), which is in accordance with that curdlan inhibited OX40L expression at mRNA level (Figure 3.4C). These findings suggest that Dectin-1 ligation preferentially inhibits binding of RelB and p50 to *OX40L* promoter, which results in downregulated OX40L transcription.

*Dectin-1 Signaling in mDCs Suppresses CRTH2$^+$ Memory CD4$^+$ T cell Responses*

CRTH2 is known as one of the most specific markers for circulating Th2 and type-2 cytotoxic T (Tc2) cells in human beings (Cosmi et al., 2001; Cosmi et al., 2000; Nagata et al., 1999). Consistent with previous reports, a large fraction of sorted CRTH2$^+$ memory CD4$^+$ T cells expressed at least one of the Th2-type cytokines including IL-4, IL-5 and IL-13 while an extremely small portion of them expressed IL-17 or IFNγ (Figure 3.5A). To investigate the effects of Dectin-1 signaling in mDCs on memory Th2 cell responses, we co-cultured sorted CRTH2$^+$ memory CD4$^+$ T cells with allogeneic mDCs in the presence or absence of curdlan. Figure 3.5B showed that the presence of curdlan decreased the frequencies of Th2 cells producing IL-4, IL-5 or IL-13.

Furthermore, CRTH2$^+$ CD4$^+$ cells co-cultured with allogeneic mDCs in the presence of curdlan secreted significantly lower levels of Th2-type cytokines but higher level of IL-17 (Figure 3.5C). Taken together, mDCs activated via Dectin-1 exert strong capacity to downregulate CRTH2$^+$ memory Th2 cell responses.

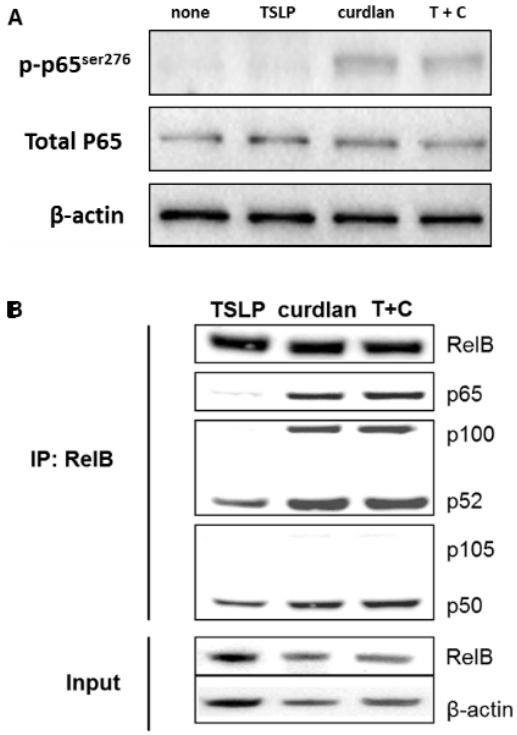


Figure 3.3. Dectin-1 ligation induces p65 phosphorylation at Ser276 whereas it does not decrease the abundance of p50-RelB in TSLP-mDCs. (A & B) Sorted mDCs were left untreated in culture medium (none) or incubated with TSLP, curdlan or both for 48h. (A) Whole cell lysates were used for western blot. Curdlan alone or curdlan plus TSLP, induced the phosphorylation of p65 at Serine 276. (B) Nuclear extracts were used to perform Co-IP using RelB antibody. Immunoprecipitants were subjected to western blot analysis with the indicated antibodies.

*Dectin-1 Signaling Decreases the Capacity of TSLP-mDCs in Maintaining Th2 Cell Commitment*

TSLP-mDCs are reported to not only maintain the central memory phenotype but also further strengthen the Th2 properties of CRTH2$^+$ memory CD4$^+$ cells (Wang et al.,

2006). To investigate the effect of curdlan on strengthened Th2 cells elicited by TSLP-mDCs, we treated TSLP-mDCs with curdlan and co-cultured them with sorted autologous CRTH2$^+$ memory CD4$^+$ cells. In the end of the culture, CRTH2 expression on T cells was measured (Figure 3.6). It has been known that T cell activation signals lead to significant loss of CRTH2 (Annunziato et al., 2001). Consistently, mDCs-mediated T cell activation reduced the frequency of CRTH2$^+$ CD4$^+$ T cells from 99% to 26% after 4 days of culture. In addition, TSLP-mDCs induced a similar loss of CRTH2 expression when compared with mDCs. However, only about 4% of CRTH2$^+$ CD4$^+$ T cells were detected in the co-cultures in which DCs were treated with curdlan, in the presence of absence of TSLP. Similar results were observed after 7 days of co-culture. These data suggest that Dectin-1 signaling in TSLP-mDCs can reverse Th2 commitment and stability of CRTH2$^+$ memory CD4$^+$ T cells.

*Activation of mDCs via Dectin-1 Inhibits Allergen-specific Th2 Cell Response*

Allergic asthma is the most common type of asthma. It is a chronic inflammatory disease with a pathogenesis progress of strong Th2 type cell response (Galli et al., 2008). Repetitive exposure to allergens activates allergen-specific resident Th2 memory cells to trigger production of chemokines and pro-inflammatory cytokines (Liu, 2006). Ragweed is the second most frequent cause of respiratory allergy next to grass pollen. Amb a1, the major allergen in ragweed pollen, is a highly allergenic molecule that is recognized by above 90% of ragweed-sensitized individuals and accounts for above 90% of the allergenic activity in ragweed pollen (King et al., 1967; Wopfner et al., 2005). PBMCs from ragweed-allergic patient were isolated and treated with TSLP, curdlan or a combination of both in the presence of a low concentration of Amb a1 allergen for 7 days.

The Amb a1-specific T cell responses were measured by re-stimulating the cells with autologous PBMCs pre-loaded with the Amb a1 as well as Amb a1-derived peptides. As is shown in Figure 3.7, upon Amb a1 re-stimulation, the presence of TSLP induced higher levels of Th2-type cytokines including IL-4, IL-5 and IL-13, while curdlan inhibited this allergen-specific Th2 cell response. These findings suggest that the Dectin-1 signaling can inhibit the common allergen-specific Th2 response, even in the presence of TSLP.

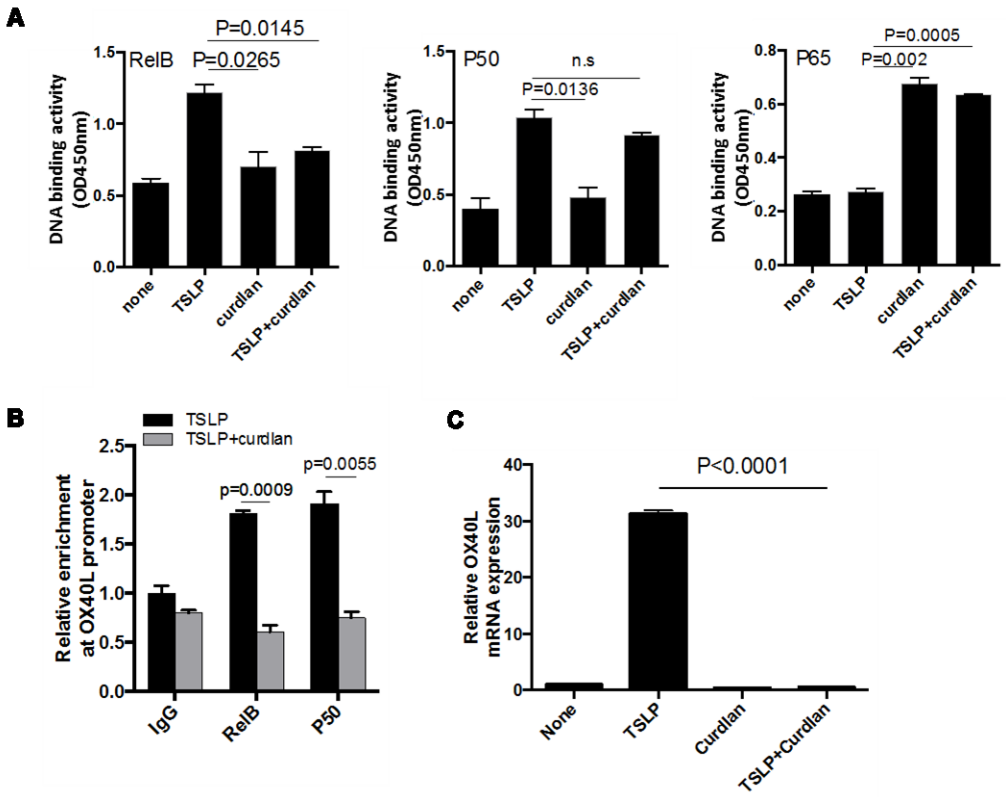


Figure 3.4. Dectin-1 ligation decreases transcriptional activity of RelB and p50 in TSLP-mDCs. (A & B) Nuclear proteins were extracted after mDCs were left untreated in culture medium (none) or incubated with TSLP, curdlan or both for 24h. (A) DNA binding activity of indicated NF-κB subunits was measured by ELISA. Summarized data from different donors are shown (n=5). (B) Binding of RelB and p50 to *OX40L* promoter was measured by ChIP assay. Summarized data from different donors are shown (n=5). (C) mRNAs were isolated after mDCs were left untreated in culture medium (none) or incubated with TSLP, curdlan or both for 24h. Relative expression of OX40L at mRNA level (normalized to GAPDH) was measured by qPCR. Summarized data from different donors are shown (n=4).

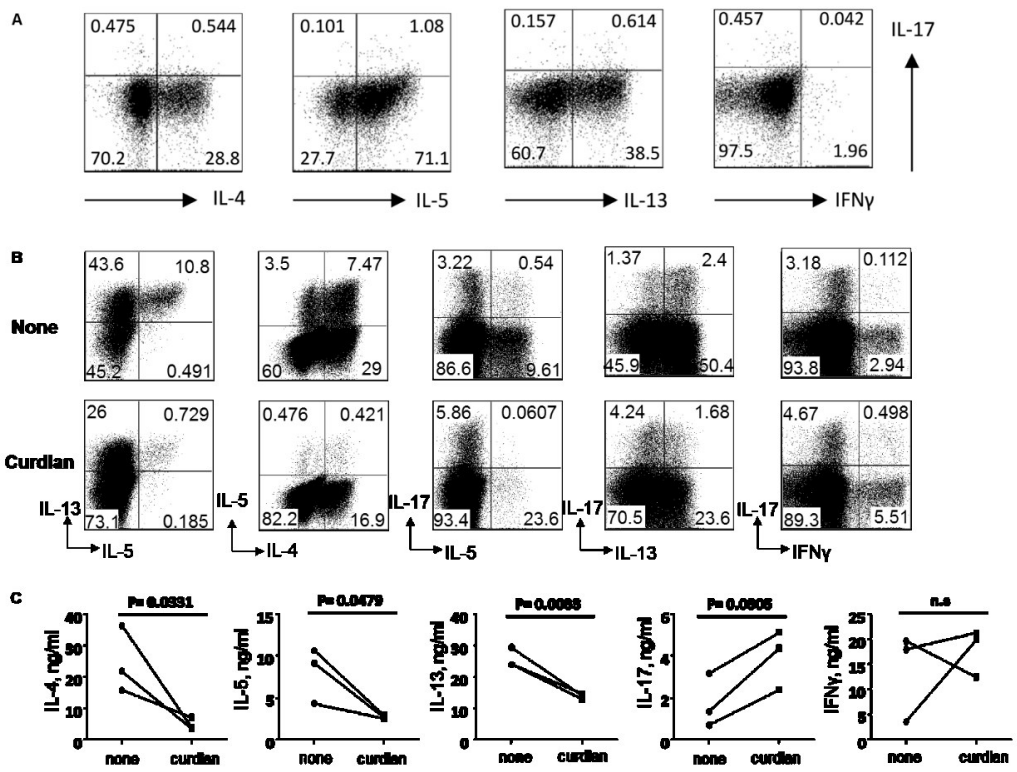


Figure 3.5. Dectin-1-activated mDCs suppress CRTH2$^+$ memory CD4$^+$ T cell response. (A) Phenotype of sorted CRTH2$^+$ memory CD4$^+$ T cells by intracellular staining. (B & C) Sorted mDCs were left untreated (none) or incubated with curdlan for 24h and then co-cultured with sorted allogeneic CRTH2$^+$ memory CD4$^+$ T cells for 7d. (B) Upon stimulation with PMA/Ionomycin, cytokine expression by CRTH2$^+$ memory CD4$^+$ T cells was measured by intracellular staining. (C) After 7d co-culture, cells were further stimulated with anti-CD3/CD28 for 2d. Concentration of indicated cytokines in the culture supernatants was measured by Luminex. Summarized data from different donors are shown (n=3).

*Dectin-1 Signaling in mDCs Decreases TSLP-mediated CCL17 Production*

TSLP is known to induce robust and sustained activation of JAK-STAT signaling pathway (Arima et al., 2010). STAT6, which is preferentially activated by TSLP, binds to the *CCL17* promoter to initiate its production (Arima et al., 2010). Besides, the expression of CCL17 is also regulated by the transcriptional activity of RelB-p52 dimer (Gringhuis et al., 2009). So far, Dectin-1 is the only CLR known to activate the non-canonical NF-κB pathway and induce a limited expression of CCL17 (Geijtenbeek and

Gringhuis, 2009). Consistent with these reports, mDCs treated with TSLP secreted high levels of CCL17 whereas curdlan induced a lower but substantial level of CCL17 only at late time point (Figure 3.8A & 3.8B). Surprisingly, the combination of TSLP and curdlan induced a limited amount of CCL17 at both early and late time points (Figure 3.8A & 3.8B), suggesting that Dectin-1 and TSLP signaling antagonized with each other in inducing CCL17 instead of acting in a synergistic way.

To investigate the mechanism by which curdlan inhibited TSLP-mediated CCL17 production, we performed western blot to assess the phosphorylation status of STAT5 and STAT6 upon Dectin-1 triggering (Figure 3.8C). We found in TSLP-mDCs, there was much weaker detection of P-STAT6 following exposure to curdlan, suggesting that Dectin-1 signaling inhibits TSLP-induced CCL17 by inactivating P-STAT6.

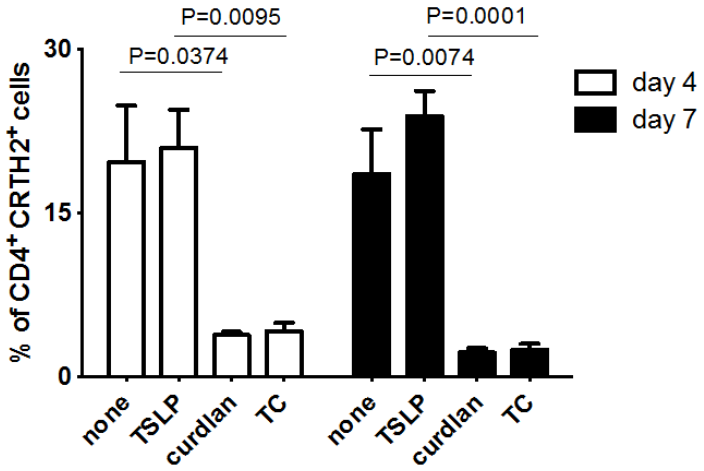


Figure 3.6. Dectin-1-activated mDCs suppress Th2 commitment and stability of CRTH2$^+$ memory CD4$^+$ T cells. Sorted mDCs were left untreated in medium (none) or incubated with TSLP, curdlan or both for 24h and then co-cultured with sorted autologous CRTH2$^+$ memory CD4$^+$ T cells up to 7d. Frequencies of CRTH2$^+$ T cells were measured by FACS staining on d4 and d7. Summarized data from different donors are shown (n=4).

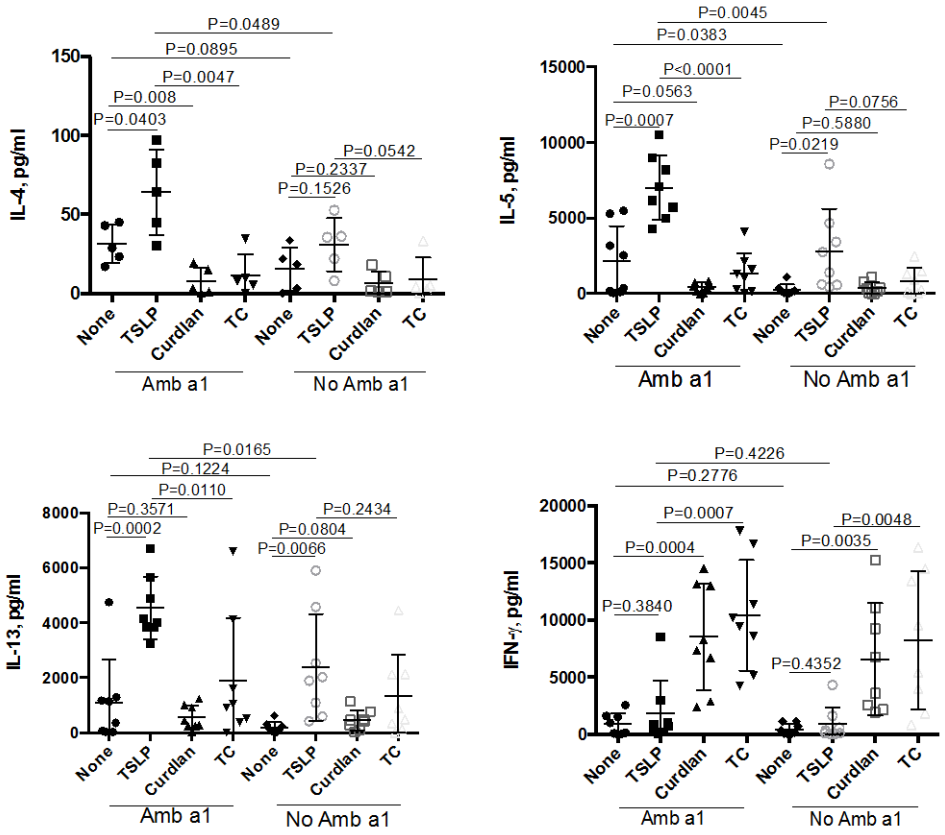


Figure 3.7. Dectin-1 ligation downregulates Amb a 1 allergen-specific Th2 cell response. Patient PBMCs were left untreated in culture medium or treated TSLP, curdlan or both for 3h and then loaded with Amb a 1 at 2 μg/ml and incubated for 7d. Cells were then either untreated or restimulated with autologous PBMCs loaded with Amb a1 and Amb a1-deirved peptide for 2d, concentration of indicated cytokines was measured by Luminex. Summarized data from different donors are shown (n=8).

## *Discussion*

Dectin-1 is an important CLR on DCs that dictates antifungal immunity by inducing Th1- and Th17-polarizing cytokines. Here we show that NF-κB signaling pathway activated by Dectin-1 agonist can not only direct Th1 and Th17 differentiation but also suppress the generation and maintenance of Th2 cells by repression of OX40L expression on mDCs. We also found that Dectin-1 ligation inhibited the recruitment of Th2 cells into inflammatory sites by downregulating the secretion of Th2 cell-attracting chemokine CCL17 by mDCs and the expression of chemokine-related CRTH2 receptor

on T cells. Thus, Dectin-1 ligation induces downregulation of Th2 cell response as well as suppression of the recruitment of Th2 cells to the sites of inflammation.

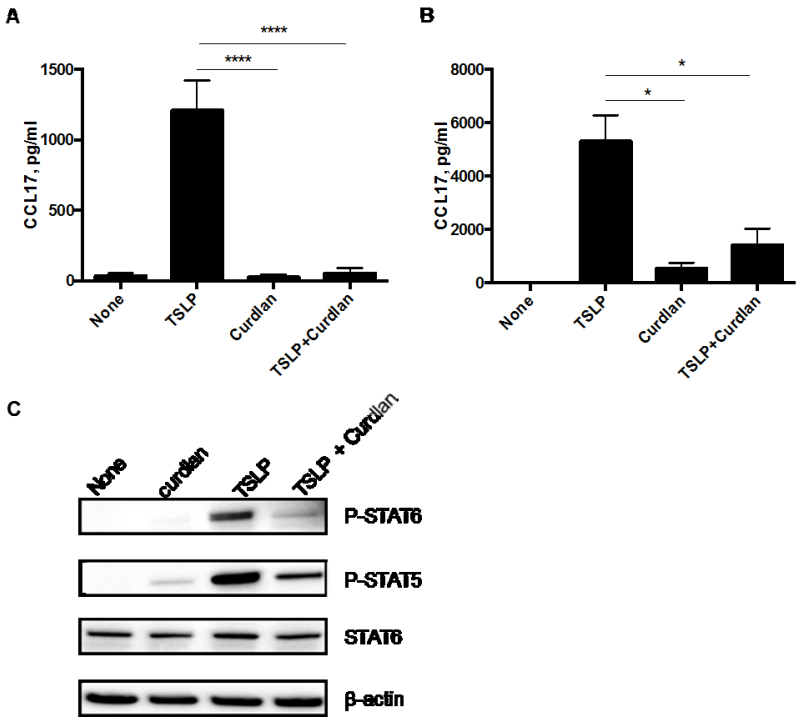


Figure 3.8. Dectin-1 ligation decreases Th2 cell-attracting chemokine CCL17 production by TSLP-mDCs. (A & B) Sorted mDCs were untreated or incubated with TSLP, curdlan or both for 15h (A) and 40h (B). Concentration of CCL17 in culture supernatants was measurement by ELISA. Summarized data from different donors are shown (n=4). (B) Whole cell lysates were extracted from mDCs treated as in (B) for detection of phosphorylated STAT5 and STAT6. Total STAT6 and β-Actin were used as loading control.

TSLP strongly activates DCs and plays an important role in the generation and maintenance of Th2 cells by inducing the formation of p50-RelB followed by the expression of OX40L (Ito et al., 2005; Soumelis et al., 2002). *OX40L* is distinct from typical NF-κB-regulated genes, whose promoter regions contain the well-conserved consensus NF-κB binding sites (κB sites). The *OX40L* promoter contains two atypical NF-κB binding sites that consist of 11 base pairs (bp), which may have relatively low binding affinity for canonical NF-κB dimers as it takes 48-72h for TSLP to induce

OX40L expression in mDCs. This unique feature suggests that downregulation of TSLP-induced OX40L expression could be achieved by interfering the p50-RelB-mediated *OX40L* transcription.

Dectin-1 ligation activates not only the Syk-dependent canonical NF-κB subunits RelA and c-Rel, but also the noncanonical NF-κB subunit RelB. In addition, Dectin-1 induces a second signaling pathway mediated by the serine-threonine kinase Raf-1, which is integrated with the Syk pathway at the level of NF-κB. Raf-1 signaling acetylates RelA, which results in increased RelA transcriptional activity to induce IL-10, IL-6 and IL-12p40. As shown in this study, DCs secreted high amounts of IL-10 and TNFα following exposure to curdlan in 24 hours. The autocrine IL-10 can induce p50 homodimer and repress OX40L expression through inhibition of RelB activity (Chen et al., 1998). On the other hand, Dectin-1-induced Raf-1 signaling pathway represses DNA binding activity of most RelB in the nucleus. Consistently, in this study, we found that the binding of RelB and p50 to *OX40L* promoter is significantly suppressed in DCs upon Dectin-1 ligation, even in the presence of TSLP.

It is well known that Dectin-1 signaling induces Th17 differentiation and expansion. Experimental studies have not adequately clarified the role of IL-17 in the experimental asthmatic responses, since this cytokine can negatively regulate or exacerbate features of this disease. The severity of asthma is found to be correlated with the level of IL-17 in the lung, sputum, BALF, or serum of patients (Chakir et al., 2003; Molet et al., 2001). Wang *et al.* has found that IL-17-producing Th2 cells may represent the key pathogenic Th2 cells promoting the exacerbation of allergic asthma (Wang et al., 2010). In contrast, IL-17 plays a protective role in fungus-induced asthma (Moreira et al.,

2011). Thus, further studies are important to dissect the role of IL-17-producing T cells in allergic asthma by detailed phenotyping and classification of them.

The correlation between Prostaglandin D2 (PGD2) and asthma is well established. PGD2 is the major proteinoid produced by mast cells, a key cell type involved in the pathogenesis of allergic inflammatory diseases. PGD2 can bind to either the prostaglandin D2 receptor DP1 or CRTH2 to induce multiple proinflammatory sequelae. CRTH2 is a rather promiscuous receptor, which is expressed on Th2 cells, eosinophils, basophils, and monocytes. It was reported that PGD2 could selectively attract peripheral blood-derived $CRTH2^+$ Th2 cells at nanomolar concentration. PGD2 produced at the sites of allergen challenge may recruit circulating $CRTH2^+$ Th2 cells. Therefore, several companies have taken CRTH2 antagonists into clinical trials for treatment of allergic rhinitis, asthma, and chronic obstructive pulmonary disease (COPD). In this study, we found that Dectin-1 signaling markedly reduced the expression of CRTH2 on T cells. Therefore, it is desirable to test whether administration of Dectin-1 agonist could decrease the recruitment of $CRTH2^+$ Th2 cells into the sites of allergen challenge and reduce the disease severity.

Allergenic asthma is a typical Th2-type inflammatory disease. We tested the effects of Dectin-1 signaling in regulating the ragweed allergen-specific Th2 cell response *in vitro* using blood samples from asthma patients and found that TSLP-induced allergen-specific Th2 cell response was significantly suppressed by Dectin-1 agonist, indicating that targeting Dectin-1 signaling pathway could be innovative therapy for allergic asthma.

# CHAPTER FOUR

## DC-ASGPR-Mediated Signaling Pathway in Human Dendritic Cells


### *Abstract*

C-type lectin receptors (CLRs) expressed on dendritic cells (DCs) are originally perceived as constituents of the powerful antigen capture and uptake system. It is now becoming clear that CLRs not only serve as antigen receptors, but also function as input transducers which are capable of delivering intracellular signals, either by themselves or in cooperation with other pattern recognition receptors (PRRs), that modulate DC functions and result in altered immune responses.

Among CLRs, human DC-ASGPR (also known as CLEC10A, MGL, HML or CD301) expressed on DCs is known to play an immunoregulatory role via modulation of effector T cell responses. Here, we investigated the signaling properties of DC-ASGPR in human DCs and report that ligation of DC-ASGPR by specific monoclonal antibody (clone 49C11) activates spleen tyrosine kinase (Syk). In addition, similar to the signaling cascades initiated by other Syk-coupled CLRs, engagement of PLCγ2 and PKCδ is conserved downstream of Syk activation upon DC-ASGPR triggering. Unexpectedly, DC-ASGPR ligation by 49C11 does not induce NF-κB activation. Instead, it selectively activates MAPK ERK1/2 and JNK. Rapid and prolonged phosphorylation of ERK1/2 leads to activation of p90RSK and CREB, which promote IL-10 expression in DCs. Moreover, upon DC-ASGPR ligation, activities of GSK-3α/β and β-Catenin are

differentially regulated downstream of Akt activation, which fine-tunes the expression of cytokines.

Our results demonstrate that DC-ASGPR triggers a complicated intracellular signaling cascade and provide a molecular explanation for the ability of DC-ASGPR-interacting ligands to preferentially evoke immune modulation.

## *Introduction*

DCs are major antigen-presenting cells (APCs) and play a critical role in directing host immune responses towards either immunity or tolerance (Banchereau et al., 2000; Banchereau and Steinman, 1998; Pulendran et al., 2001; Steinman et al., 2003). A large repertoire of receptors expressed by DCs enable them to perform various functions. In particular, pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), C-type lectin receptors (CLRs), nucleotide-oligomerization domain (Nod)-like receptors (NLRs) and retinoic acid-inducible gene 1 (RIG-I)-like receptors (RLRs), are fundamental to DC biology (Akira et al., 2001; Geijtenbeek and Gringhuis, 2009; Geijtenbeek and Gringhuis, 2016; Janeway and Medzhitov, 2002; Kato et al., 2005; Krishnaswamy et al., 2013). Among them, CLRs are especially important for recognition and internalization of glycosylated antigens into intracellular compartments in DCs, leading to processing and presentation of antigens on MHC class I and II molecules (Figdor et al., 2002). These properties of CLRs are important for vaccine design (Steinman and Banchereau, 2007). In addition, CLRs function as signaling receptors, which upon ligation, initiate diverse intracellular signaling cascades that modulate DCs function and can thus induce or regulate host immune responses (Dambuza and Brown,

2015; Geijtenbeek and Gringhuis, 2009; Geijtenbeek and Gringhuis, 2016; van Vliet et al., 2008a).

Dectin-1 is the first CLR (also the first PRR of non-TLR family) that is found capable of inducing intracellular signaling cascade (Brown, 2006). Indeed, signaling events downstream of Dectin-1 ligation are often regarded as paradigm for CLR signaling (Robinson et al., 2006). Dectin-1 recognizes β-glucans in fungal and mycobacterial cell walls in a $Ca^{2+}$-independent manner (Brown and Gordon, 2001; Willment et al., 2001). Upon ligation, Dectin-1 signals via its internal hemITAM (single YxxL/I motif), which is inducibly phosphorylated by member(s) of the Src family kinases (SFKs) (Fuller et al., 2007). Recruitment of Syk to the phosphorylated hemITAM of Dectin-1 results in Syk activation, which triggers downstream cascade including mitogen-activated protein kinase (MAPK) and NF-κB signaling (Mocsai et al., 2010; Rogers et al., 2005). Syk-mediated canonical NF-κB signaling is dependent on the activation of PLCγ2 and PKCδ (Elsori et al., 2011; Tassi et al., 2009; Xu et al., 2009), formation of the CARD9-Bcl10-MALT1 (CBM) complex (Gross et al., 2006; Roth and Ruland, 2013) and subsequent TAK1 activation (Strasser et al., 2012; Wang et al., 2001). Non-canonical NF-κB pathway is also activated upon Dectin-1 triggering via a Syk and NIK-dependent manner (Gringhuis et al., 2009). However, the intricacies of the pathway which relays signals from Syk to the activation of RelB-p52 dimers remain undefined at present. In addition, Dectin-1 is reported to induce a second signaling pathway through the kinase Raf-1 (also known as c-Raf), which is independent of the Syk pathway but integrated with it at the level of NF-κB activation (Gringhuis et al., 2009). Consequently, Dectin-1 signaling pathway in DCs can direct T helper cell (Th) differentiation by inducing Th1- and Th17-

polarizing cytokines, including IL-12p70, IL-1β, IL-6 and IL-23, which are crucial for anti-fungal immunity (Gringhuis et al., 2009).

In human, DC-ASGPR is a type II transmembrane glycoprotein. Expression of this CLR has been found on DCs, monocytes, macrophages and B cells *in vivo* and monocyte-derived DCs *in vitro* (Li et al., 2012). Intriguingly, expression of DC-ASGPR is upregulated on tolerogenic DCs generated in the presence of glucocorticoids and during chronic inflammatory conditions such as rheumatoid arthritis, implicating DC-ASGPR in immune regulation (van Vliet et al., 2006b).

The conserved Gln-Pro-Asp (QPD) motif in the carbohydrate recognition domain (CRD) renders DC-ASGPR unique specificity for terminal *N*-acetylgalactosamine (GalNAc or Tn) residues of *N*- and *O*-glycans carried by foreign, altered-self- and self-glycoproteins and glycosphingolipids (Mortezai et al., 2013; van Vliet et al., 2008b; van Vliet et al., 2005b). Indeed, the rare terminal Tn antigens are found on glycans expressed by a limited array of pathogens including bacteria *Neisseria gonorrhoeae* and *Campylobacter jejuni* (van Sorge et al., 2009; van Vliet et al., 2009), parasite *Trichuris suis* (Klaver et al., 2013) and filovirus Ebola (Takada et al., 2004). Pathogens may target DC-ASGPR to evade immunity, as the interaction between DC-ASGPR and *C. jejuni* specifically inhibits DC maturation and limits production of pro-inflammatory cytokine (van Sorge et al., 2009). During oncogenic transformation and progression to cancer, Tn antigens are unveiled and therefore highly abundant on cancers, especially of epithelial origin. DC-ASGPR selectively recognizes tumor-derived mucin proteins, such as tumor-associated MUC1 and MUC2 (Nollau et al., 2013; Saeland et al., 2007). Given that tumor-specific Tn expression not only promotes tumor invasiveness, but also alters the

immunogenicity of tumor antigens, DC-ASGPR could be involved in tumor immune evasion (Freire et al., 2011; Gill et al., 2013). In addition, the appearance of Tn antigens is found on CD45 of human effector T cells and the interaction between CD45-Tn antigens on effector T cells and DC-ASGPR on DCs decreases the phosphatase activity of CD45, thereby reducing T cell proliferation and inflammatory cytokine production that ultimately lead to T cell apoptosis (van Vliet et al., 2006a; van Vliet et al., 2013b).

Previously, we reported that ligation of DC-ASGPR by specific mAb (clone 49C11) induces IL-10 production by DCs. Accordingly, DC-ASGPR-activated DCs adopt a tolerogenic program and instruct the differentiation of Type-1 regulatory T (Tr1) cells in an IL-10-dependent manner (Li et al., 2012). Other groups reported that DC-ASGPR engagement using mAb or synthesized carbohydrate ligand induces ERK activation independently and modifies intracellular signals originating from TLRs, which leads to enhanced IL-10 and TNFα expression (Napoletano et al., 2012; van Vliet et al., 2013a). Nevertheless, the current understanding of human DC-ASGPR signaling property is still fairly limited.

In this study, we further dissected DC-ASGPR-mediated signaling pathway in human DCs and report that, ligation of DC-ASGPR by 49C11 results in Syk activation, which is dependent on the activity of Src-family kinase (SFK). In addition, PLCγ2 and PKCδ are activated downstream of Syk. However, DC-ASGPR-medicated Syk-PLCγ2-PKCδ cascade does not lead to NF-κB activation. Consistent with previous studies (Li et al., 2012; Napoletano et al., 2012; van Vliet et al., 2013a), DC-ASGPR ligation induces MAPK activation. Rapid and prolonged ERK1/2 phosphorylation activates p90RSK and CREB, giving rise to enhanced IL-10 expression. Additionally, DC-ASGPR ligation

activates Akt in a PI3K-dependdent manner, which differentially regulates the activities of GSK-3α/β and β-Catenin to fine-tune cytokine expression.

## *Materials and Methods*

### *DC Generation and Cell Culture Medium*

All healthy blood donors provided a written informed consent prior to inclusion in the study in accordance with the approval by the Institutional Review Boards at Baylor Research Institute. IL-4-DCs were generated by culturing purified blood monocytes from healthy donors in CellGro serum-free Dendritic Cell Medium (CellGenix) supplemented with 100 ng/ml GM-CSF (Peprotech) and 50 ng/ml IL-4 (Peprotech) for 5 days. On day 3, the medium was replenished with cytokines. IL-4-DCs were then harvested from DC generation medium on day 5 and washed intensively in cRPMI medium before further procedure.

Complete RPMI (cRPMI) medium consisted of RPMI 1640 (Gibco) supplemented with 25 mM HEPES (Life Technologies), 2 mM L-glutamine, 100 μM nonessential amino acids, 1 mM sodium pyruvate, 100 units/ml penicillin and 100 μg/ml streptomycin (Sigma). Cell culture medium consisted of cRPMI and 10% heat-inactivated GemCell normal human serum of AB serotype (Gemini).

### *Anti-DC-ASGPR Monoclonal Ab and Isotype Control*

Mouse mAb (clone 49C11) specific for human DC-ASGPR was generated as previously described (Valladeau et al., 2001). Mouse IgG2a of irrelevant specificity (clone MG2a-53, Biolegend) was used as isotype-matched control antibody.

*DC Activation and Kinase Inhibition*

Anti-DC-ASGPR or mouse IgG2a isotype control was pre-coated at 5 μg/well in Falcon 96 well U-bottom tissue culture plate (Corning). Dectin-1 specific ligand Curdlan (Wako Chemicals) or Zymosan Depleted (InvivoGen) was used at 100 μg/ml.

IL-4-DCs were suspended in cell culture medium and pre-incubated with kinase inhibitor or vehicle control DMSO (0.1%) for 1h at 37°C. The cells were then plated at $2\times10^5$/well for activation by plate-bound mAb or indicated stimuli overnight at 37°C. The supernatants were harvested for cytokine measurement and cells were stained for phenotyping.

The following kinase inhibitors were used: Syk inhibitor R406 at 10 μM (Invivogen), Src inhibitor PP2 at 10 μM, PI3K inhibitor LY294002 at 10 μM, PLC inhibitor U-73122 at 1 μM (EMD Millipore), p90RSK inhibitor BI-D1870 at 10 μM (Axon), MEK1/2 inhibitor U0126 at 5 μM (EMD Millipore).

*Cell Staining and Flow Cytometry Analysis*

Fluorescence-conjugated antibodies including anti-CD86 and anti-HLA-DR (Biolegend), Live/Dead Aqua (Invitrogen) were used for surfacing staining of DCs. FACS data were acquired on a BD FACSCanto II system (BD) and analyzed using FlowJo software.

*Protein Immunoblots*

IL-4-DCs were rested in cRPMI medium up to overnight before activation with plate-bound mAb or other stimuli. By the end of activation, DCs were washed in ice-cold PBS and lyzed in RIPA lysis and extraction buffer (Thermo Fisher Scientific)

supplemented with 1× Halt protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific) according to the manufacturer's instruction. The protein concentration of cell lysates was measured by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) before cell lysates were reduced and denatured by heating at 90c with 6x SDS sample buffer (Boston BioProducts). Cell extracts were separated by SDS-PAGE using Tris-glycine gel (Invitrogen) and transferred to a PVDF membrane (Bio-Rad). Immunodetection was performed by incubation with anti-primary antibodies followed by HRP-conjugated secondary antibodies. The bands were then visualized using the ECL chemiluminescence immunoblotting detection system (Bio-Rad). The antibodies used in western blot assay are listed in Table 1.

*Cytokine Measurement*

The concentration of cytokines in the supernatants of cell culture was assessed with the BeadLyte Cytokine Assay Kit (Upstate), as per the manufacturer's protocol. Fluorescence was analyzed with a Bio-Plex Luminex 100 XYP instrument (Bio-Rad), with the Bio-Plex Manager 4.1 software, with a 5-parameter curve-fitting algorithm applied for standard curve calculations.

*Statistical Analysis*

All bar graphs represent mean ± SD. Statistical significance was determined using the analysis of variance (ANOVA) and Student's *t* test with Prism 7 software (GraphPad Software, CA). Significance was set at $p < 0.05$.

Table 1. Antibodies used in the western blot assay for DC-ASGPR signaling study

| Cat # | Target | Host | Dilution | Vendor |
|---|---|---|---|---|
| 2710S | Phospho-Syk (Tyr525/526) | Rabbit | 1:1000 | CST |
| 13198S | Syk | Rabbit | 1:1000 | CST |
| 3871S | Phospho-PLCγ2 (Tyr1217) | Rabbit | 1:1000 | CST |
| 3872S | PLCγ2 | Rabbit | 1:1000 | CST |
| 2055S | Phospho-PKCδ (Tyr311) | Rabbit | 1:1000 | CST |
| 2697S | Phospho-IKKα/β (Ser176/180) | Rabbit | 1:1000 | CST |
| 4812S | IκBα | Rabbit | 1:1000 | CST |
| 2859S | Phospho-IκBα (Ser32) | Rabbit | 1:1000 | CST |
| 3033S | Phospho-NF-κB p65 (Ser536) | Rabbit | 1:1000 | CST |
| 4531S | Phospho-TAK1 (Thr184/187) | Rabbit | 1:1000 | CST |
| 4536S | Phospho-TAK1 (Thr187) | Rabbit | 1:1000 | CST |
| 4370S | Phospho-p44/42 MAPK (Erk1/2) | Rabbit | 1:2000 | CST |
| 4668S | Phospho-SAPK/JNK (Thr183/Tyr185) | Rabbit | 1:1000 | CST |
| 9215S | Phospho-p38 MAPK (Thr180/Tyr182) | Rabbit | 1:1000 | CST |
| 9154S | Phospho-MEK1/2 (Ser217/221) | Rabbit | 1:1000 | CST |
| 8753S | Phospho-p90RSK (Thr359) | Rabbit | 1:1000 | CST |
| 11989S | Phospho-p90RSK (Ser380) | Rabbit | 1:1000 | CST |
| 9355S | RSK1/RSK2/RSK3 | Rabbit | 1:1000 | CST |
| 9595S | Phospho-MSK1 (Thr581) | Rabbit | 1:1000 | CST |
| 13038S | Phospho-Akt (Thr308) | Rabbit | 1:1000 | CST |
| 5651S | Phospho-β-Catenin (Ser552) | Rabbit | 1:1000 | CST |
| 8566S | Phospho-GSK-3α/β (Ser21/9) | Rabbit | 1:1000 | CST |
| 05-807 | Phospho-CREB (Ser133) | Mouse | 1:1000 | Millipore |
| 5125S | β-Actin-HRP | Rabbit | 1:1000 | CST |
| 7074S | Rabbit IgG, HRP conjugated | Goat | 1:1000 | CST |
| 7076S | Mouse IgG, HRP conjugated | Horse | 1:1000 | CST |

## *Results*

### *DC-ASGPR Ligation by Ab (49C11) Induces DC Activation and Cytokine Production*

Previously, we reported that ligation of human DC-ASGPR by mAb (clone 49C11) induces IL-10 expression in DCs, which contributes to the generation of Ag-specific Tr1 cells (Li et al., 2012). In this study, using the same mAb, we further investigated the activation status and cytokine profile of DCs upon DC-ASGPR engagement. Indeed, DCs

treated with 49C11, when compared with those treated with isotype control antibody, significantly upregulated CD86 expression on the cell surface, whereas HLA-DR, CD80 (data not shown) and CD83 (data not shown) remained unaffected (Figure 4.1A & 4.1B), suggesting that ligation of DC-ASGPR by 49C11 can partially activate DCs.

Along with cell activation, higher levels of IL-6, IL-10 and TNFα (Figure 4.1C), but not IL-1β or IL-12p70 (data not shown) were detected in the supernatants of DCs treated with 49C11 when compared with those treated with isotype control mAb. However, the absolute levels of cytokine secretion upon DC-ASGPR ligation varied extensively among different donors tested.

*DC-ASGPR Ligation by 49C11 Induces Syk Activation*

A large number of CLRs, including CLEC2 (CLEC1B) (Hughes et al., 2010; Suzuki-Inoue et al., 2006), BDCA-2 (CLEC4C) (Cao et al., 2007), Mcl (CLEC4D) (Graham et al., 2012), Mincle (CLEC4E) (Ostrop et al., 2015), Dectin-2 (CLEC6A) (Robinson et al., 2009), Dectin-1 (CLEC7A) (Gringhuis et al., 2009; Rogers et al., 2005) and DNGR-1 (CLEC9A) (Huysamen et al., 2008b), have been found coupled to Syk for downstream signaling cascade. We therefore investigated whether Syk is also engaged in DC-ASGPR signaling pathway. Indeed, upon DC-ASGPR crosslinking by 49C11, rapid phosphorylation of Syk at Tyr525 and Tyr526 was observed in DCs (Figure 4.2A & 4.2B). In addition, the presence of Syk inhibitor R406 blocked this phosphorylation (Figure 4.2C & 4.2D). In contrast, IgG2a isotype control failed to phosphorylate Syk.

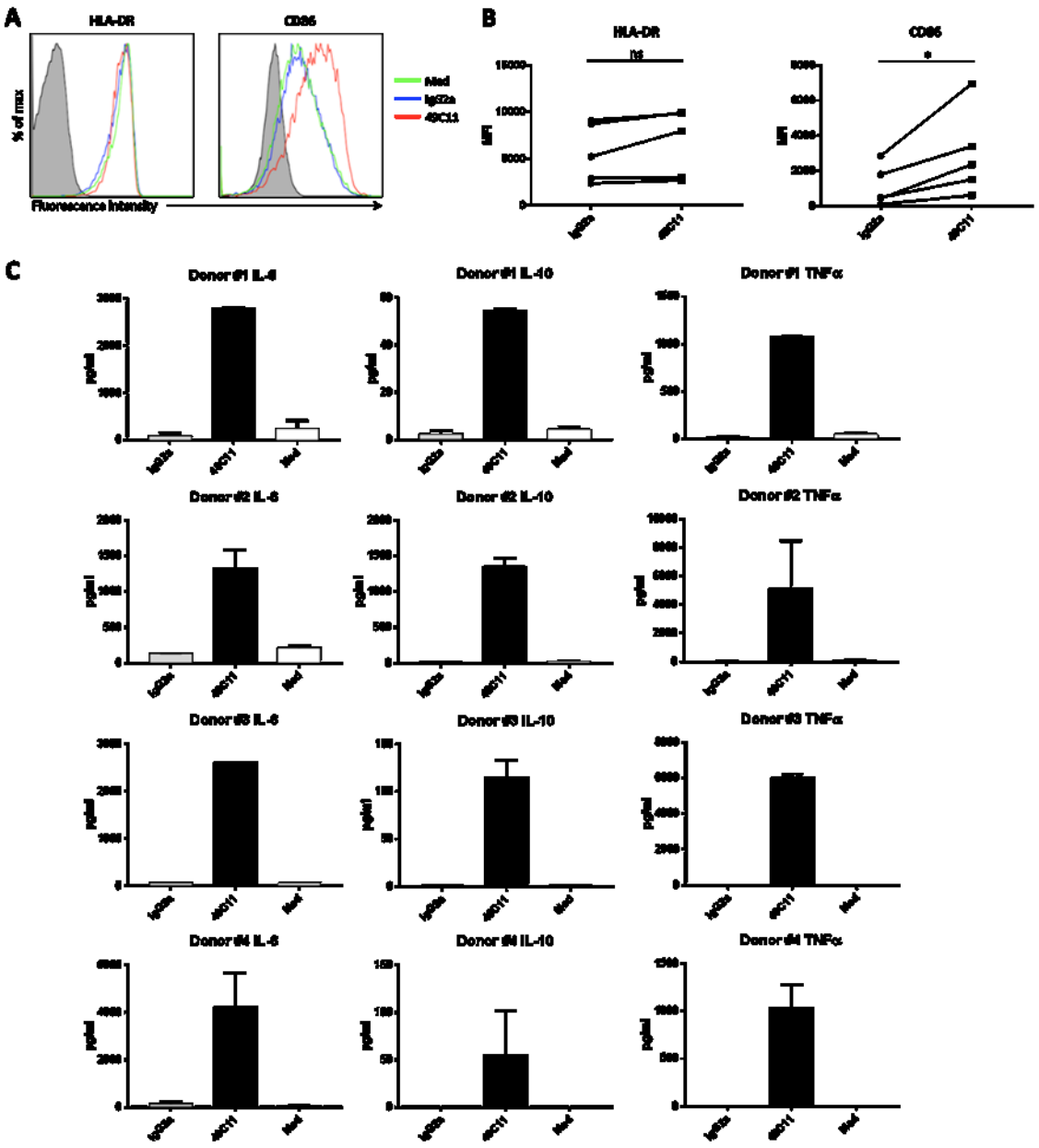


Figure 4.1. DC-ASGPR ligation activates DCs and induces cytokine expression. (A and B) IL-4-DCs were stained with fluorescence-labeled mAbs specific to HLA-DR and CD86 or with their isotype-matched controls. (A) Representative data of HLA-DR and CD86 staining. Tinted histogram denotes the isotype staining. Colored histograms denote staining of indicated surface marker on DCs after different treatment. (B) Summarized data of mean fluorescence intensities (MFIs) after isotype subtraction of indicated surface marker on DCs after indicated treatment. Dots represent data generated from cells of different donors (n=5). (C) Cytokine levels in the supernatant harvested from IL-4-DCs after indicated treatment were analyzed by Luminex. Significance was determined using a paired *t* test. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$; ****, $P < 0.0001$.

Previous studies reported that in DCs, phosphorylation of Syk upon CLR triggering is mediated by member(s) of Src family kinases (SFKs) (Cao et al., 2007; Severin et al., 2011; Underhill et al., 2005). Indeed, SFK inhibitor PP2 completely abolished Syk phosphorylation induced by DC-ASGPR ligation (Figure 4.2C & 4.2D), suggesting that DC-ASGPR, similar to other CLRs (Cao et al., 2007; Severin et al., 2011; Underhill et al., 2005), activates Syk in a SFK-dependent manner. In support of these observations (Figure 4.2C & 4.2D), inhibition of Syk or SFK suppressed DC activation (Figure 4.2E & 4.2F) and cytokine production upon DC-ASGPR ligation (Figure 4.2G).

*DC-ASGPR Ligation by 49C11 Induces PLCγ2 and PKCδ Activation Downstream of Syk*

In CLR signaling, Syk is known to activate signaling intermediates that trigger downstream pathways, such as MAPKs and NF-κB (Mocsai et al., 2010; Rogers et al., 2005). Previous studies reported that Dectin-1-induced activation of canonical NF-κB pathway is dependent on a series of sequential events downstream of Syk activation, including activation of PLCγ2 and PKCδ, formation of the CARD9-Bcl10-MALT1 (CBM) complex and subsequent TAK1 triggering, which is required for phosphorylation of IKKs (Mocsai et al., 2010). We thereby hypothesized that DC-ASGPR-Syk signaling cascade could activate PLCγ2, PKCδ and TAK1, resulting in downstream NF-κB activation.

Indeed, DC-ASGPR ligation activated PLCγ2 and PKCδ rapidly as evidenced by their phosphorylation at Tyr1217 and Tyr311, respectively. (Figure 4.3A & 4.3B). Interestingly, when compared with Dectin-1 ligation using zymosan depleted (dZymosan), DC-ASGPR engagement by 49C11 induced phosphorylation of PLCγ2 and PKCδ with different kinetics. Consistent with previous studies in Dectin-1 signaling (Strasser et al.,

2012), Syk inhibitor R406 or SFK inhibitor PP2 completely abolished phosphorylation of PLCγ2 and PKCδ induced by DC-ASGPR ligation (Figure 4.3C & 4.3D). In addition, PLC inhibitor U-73122 blocked PLCγ2 phosphorylation upon DC-ASGPR ligation (Figure 4.4A) and also suppressed DC activation and cytokine expression (Figure 4.4B & 4.4C). These observations suggest that activation of both PLCγ2 and PKCδ is conserved in DC-ASGPR signaling pathway, and their activation is dependent on both SFK and Syk.

*Syk Activation by DC-ASGPR Ligation Is Uncoupled from NF-κB Pathway*

Given that Syk, PLCγ2 and PKCδ are all activated in DCs upon DC-ASGPR ligation, we further investigated the activation status of TAK1. Indeed, we didn't observe any TAK1 phosphorylation in DCs up to 24h of DC-ASGPR engagement (Figure 4.5A–4.5C). However, in the same experiment, phosphorylated TAK1 wasn't detected in DCs treated with dZymosan or curdlan (Figure 4.5A–4.5C), which is contrary to previous reports using mouse DCs (Strasser et al., 2012) and rendering us unable to confirm whether DC-ASGPR ligation could induce TAK1 activation in human IL-4-DCs.

In addition, we didn't detect the phosphorylation of IKKα/β or IκBα, nor the degradation of IκBα in DCs up to 24h of DC-ASGPR engagement (Figure 4.5A–4.5D), which is in contrast to Dectin-1 ligation using dZymosan or curdlan (Figure 4.5A–4.5C). All these findings suggest that DC-ASPGR signals through Syk but, in contrast to CLRs involved in pathogen recognition, it does not intrinsically activate NF-κB pathway, which is similar to the signaling property of BDCA-2 (CLEC4C) (Geijtenbeek and Gringhuis, 2009).

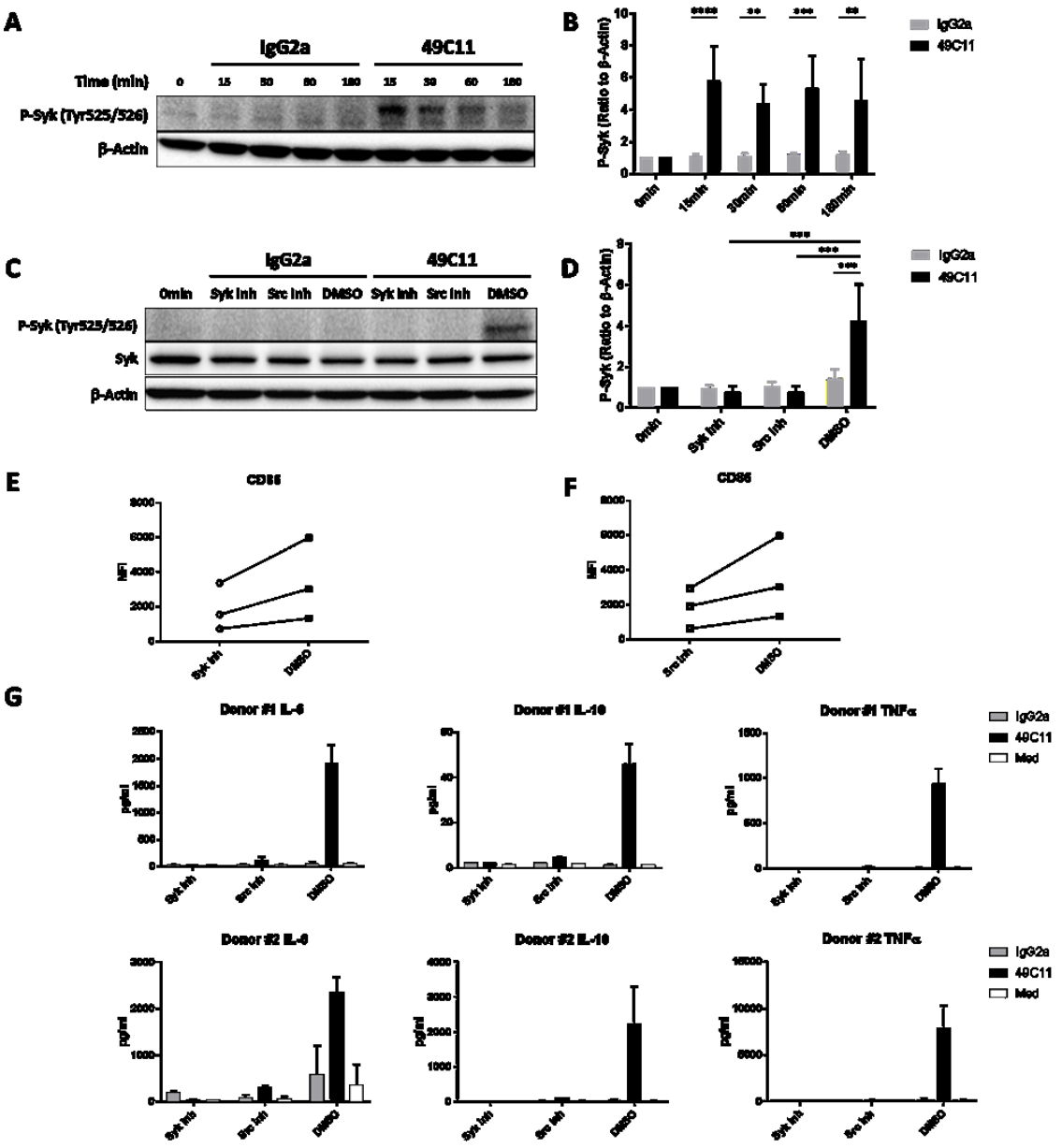


Figure 4.2. DC-ASGPR ligation activates Syk. (A and B) DC-ASGPR ligation by 49C11 phosphorylates Syk. (A) Representative data indicates the phosphorylation status of Syk upon indicated treatment at different time points. (B) Summarized data of phosphorylated Syk (P-Syk) abundance in DCs treated as in (A) generated from different donors (n=4). (C and D) Pre-incubation of DCs with Syk inhibitor R406 or Src inhibitor PP2 for 1h blocked DC-ASGPR-induced Syk phosphorylation. (C) Representative data indicates the phosphorylation status of Syk in DCs upon indicated treatment for 30min. (D) Summarized data of P-Syk abundance in DCs treated as in (C) generated from different donors (n=4). (E and F) Surface staining of CD86 on IL-4-DCs (n=3) which were pre-incubated with indicated inhibitor and then activated by plate-bound 49C11 overnight. (G) Cytokine levels in the supernatant harvested from IL-4-DCs treated as in (E and F).

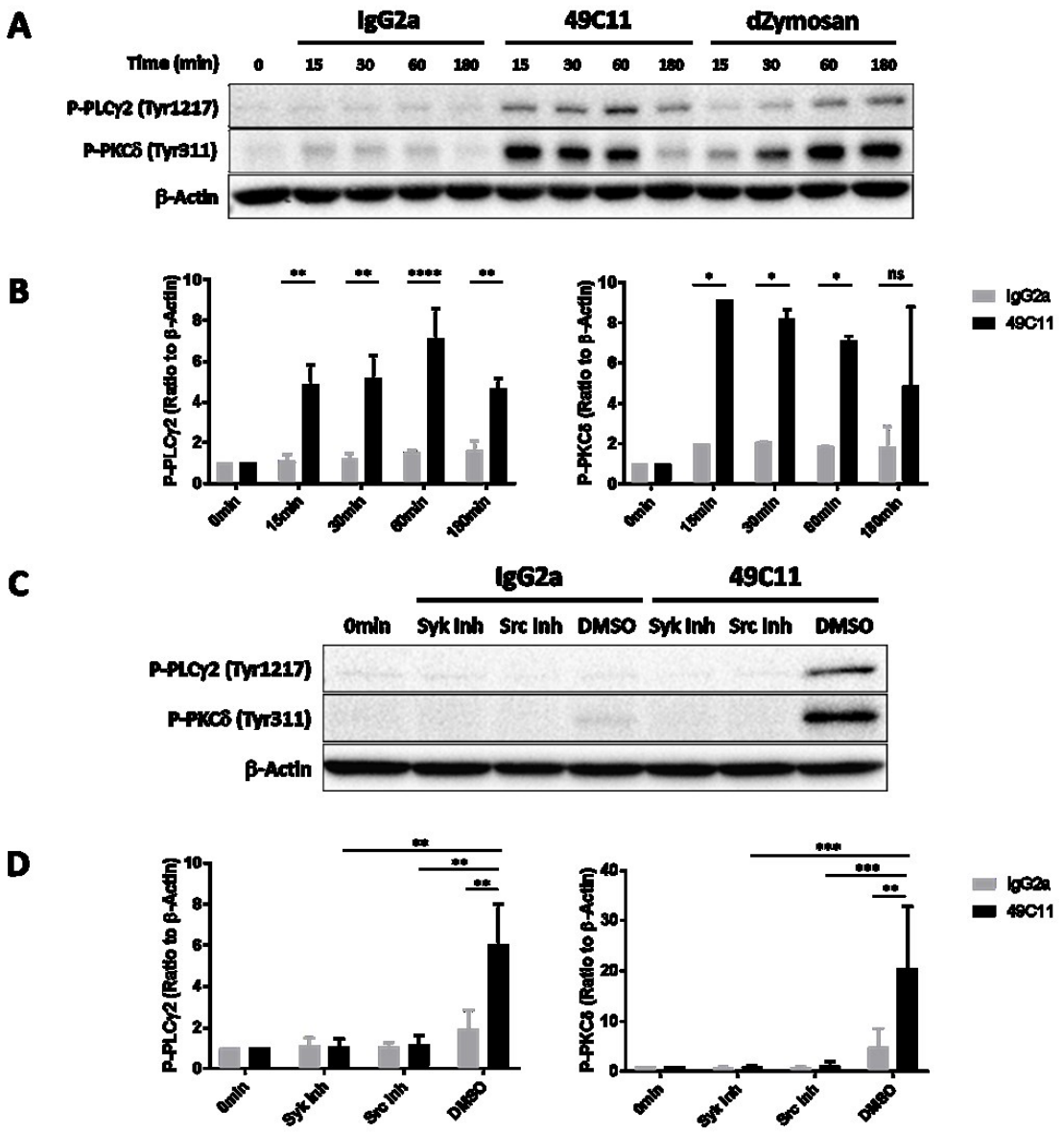


Figure 4.3. DC-ASGPR ligation activates PLCγ2 and PKCδ. (A and B) DC-ASGPR ligation by 49C11 phosphorylates PLCγ2 at Tyr1217 and PKCδ at Tyr311. (A) Representative western blot data indicates the phosphorylation of PLCγ2 and PKCδ upon indicated treatment at different time points. (B) Summarized data of PLCγ2 and P-PKCδ abundance in DCs upon indicated treatment at different time points generated from different donors (n=3). (C and D) Pre-incubation of DCs with Syk inhibitor R406 or Src inhibitor PP2 for 1h blocked DC-ASGPR-induced phosphorylation of PLCγ2 and PKCδ. (C) Representative data indicates the phosphorylation status of PLCγ2 and PKCδ after indicated treatment for 30min. (D) Summarized data of PLCγ2 and PKCδ abundance in DCs treated as in (C) generated from different donors (n=3).

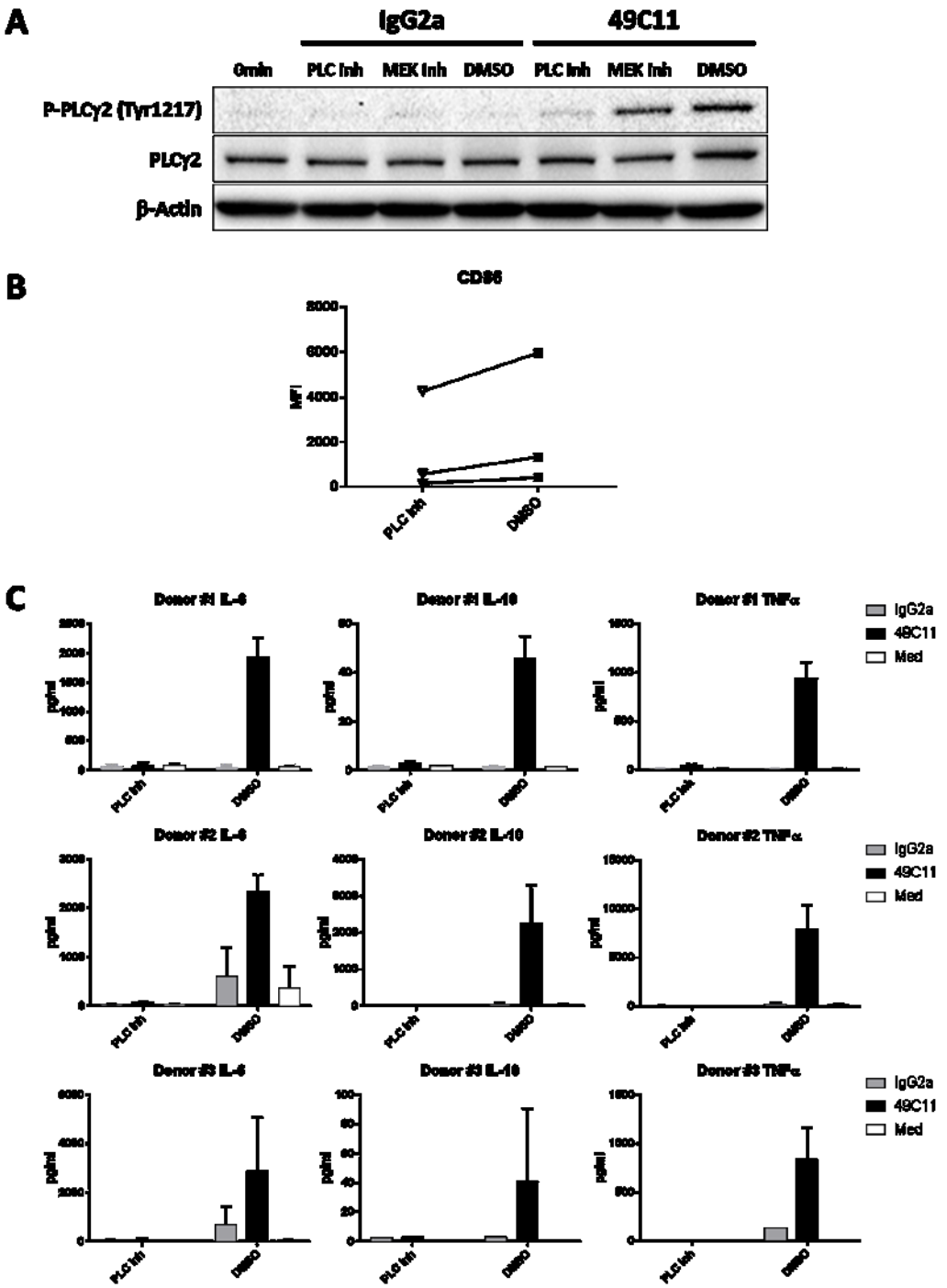


Figure 4.4. Inhibition of PLCγ2 suppresses DC activation and cytokine production upon DC-ASGPR ligation. (A) Representative data shows the phosphorylation status of PLCγ2 in DCs which were pre-incubated with PLC inhibitor for 1h and then treated by plate-bound mAb for 30min. CD86 expression (B) and cytokine production (C) of DCs (n=3) which were pre-incubated with indicated inhibitor and then activated by plate-bound mAb overnight were measured by surface staining and Luminex, respectively.

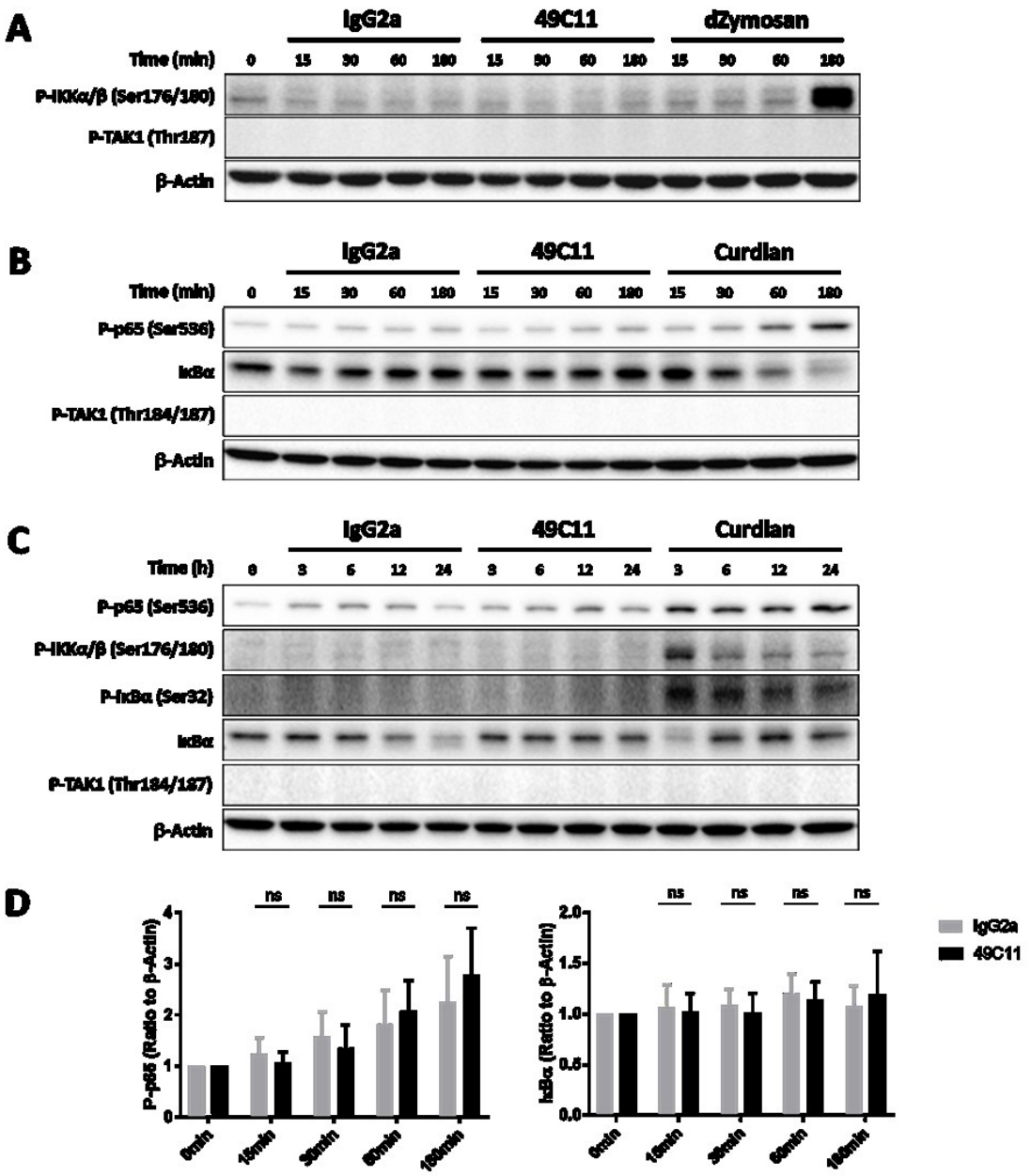


Figure 4.5. DC-ASGPR ligation by 49C11 does not induce NF-κB activation. (A–C) Representative western blot data indicate the phosphorylation status of different molecules after indicated treatment at different time points. (D) Summarized data of P-p65 and IκBα abundance in DCs upon indicated treatment at different time points generated from different donors (n=4).

*DC-ASGPR Ligation Induces Selective Activation of MAPK ERK1/2 and JNK*

We next explored MAPK activation status in DCs upon DC-ASGPR ligation by 49C11. We found that ERK1/2 and JNK were phosphorylated with different kinetics upon DC-ASGPR or Dectin-1 engagement (Figure 4.6A–4.6C). However, there was no significant enhancement of p38 phosphorylation in DCs up to 3h of DC-ASGPR ligation (Figure 4.6B & 4.6D). In addition, SFK inhibitor PP2 or Syk inhibitor R406 suppressed JNK, MEK1/2 and ERK1/2 phosphorylation in DC-ASGPR-activated DCs (Figure 4.7A). Moreover, PLC inhibitor U-73122 or MEK1/2 inhibitor U0126 abrogated DC-ASGPR-induced phosphorylation of MEK1/2 and ERK1/2 (Figure 4.7B). Given that MEK1/2 inhibitor U0126 didn't block PLCγ2 phosphorylation (Figure 4.4A), we conclude that MEK1/2 might serve as a signal intermediate downstream of PLCγ2 for ERK activation upon DC-ASGPR ligation. Furthermore, MEK1/2 inhibitor U0126 suppressed cell activation (Figure 4.8A) and cytokine production (Figure 4.8B) of DC-ASGPR-activated DCs. Taken together, these observations suggested that DC-ASGPR ligation by 49C11 induces selective MAPK activation in a Syk-dependent manner and ERK1/2 phosphorylation is further dependent on PLCγ2 and MEK1/2.

*ERK1/2 Phosphorylation by DC-ASGPR Ligation Induces Activation of p90RSK and CREB*

As DC-ASGPR ligation induced rapid and long-lasting ERK1/2 phosphorylation, we therefore examined the activation status of MSK1/2 and p90RSK that are downstream of ERK1/2 and known to be involved in the regulation of IL-10 expression upon CLR engagement in DCs (Arthur and Elcombe, 2012; van Vliet et al., 2013a). Indeed, we found p90RSK, but not MSK1, was rapidly phosphorylated in DCs upon DC-ASGPR

ligation (Figure 4.9A & 4.9B). Consistently, CREB, which is downstream of and regulated by p90RSK, was also phosphorylated upon DC-ASGPR triggering (Figure 4.9A & 4.9B). In addition, p90RSK inhibitor BI-D1870 decreased cell activation and cytokine production by DCs upon DC-ASGPR ligation (Figure 4.9C & 4.9D). Moreover, Syk inhibitor R406, Src inhibitor PP2, PLC inhibitor U-73122 or MEK1/2 inhibitor U0126 blocked phosphorylation of p90RSK and CREB (Figure 4.10A & 4.10B), suggesting that p90RSK and CREB could be activated by DC-ASGPR-initiated Syk-PLCγ2-MEK1/2-ERK1/2 signal cascade.

*DC-ASGPR Activates PI3K-Akt Pathway that Differentially Regulates the Activities of GSK-3α/β and β-Catenin*

In addition to NF-κB and MAPKs, activation of Akt is found downstream of CLRs including DC-SIGN, Mincle and Dectin-2 (Caparros et al., 2006; Lee et al., 2016; Wevers et al., 2014). We therefore investigated whether DC-ASGPR ligation could activate Akt. Indeed, rapid phosphorylation of Akt at Ser308 was selectively induced in DCs upon DC-ASGPR engagement (Figure 4.11A and 4.11B). In addition, phosphorylation of Akt substrates GSK-3α/β and β-Catenin occurred in parallel with Akt activation (Figure 4.11A and 4.11C). Indeed, upon DC-ASGPR triggering, GSK-3α/β was phosphorylated at its negative regulatory site (Ser21 of GSK-3α and Ser9 of GSK-3β), whereas β-Catenin was phosphorylated at Ser552 for activation. Moreover, PI3K inhibitor LY294002 completely abolished DC-ASGPR-mediated phosphorylation of Akt, GSK-3α/β and β-Catenin (Figure 4.11D) as well as suppressed cell activation (Figure 4.12A) and cytokine expression of DCs upon DC-ASGPR ligation (Figure 4.12B). These

results suggest that DC-ASGPR ligation by 49C11 activates Akt via a PI3K-dependent manner, which is responsible for regulation of GSK-3α/β and β-Catenin activity.

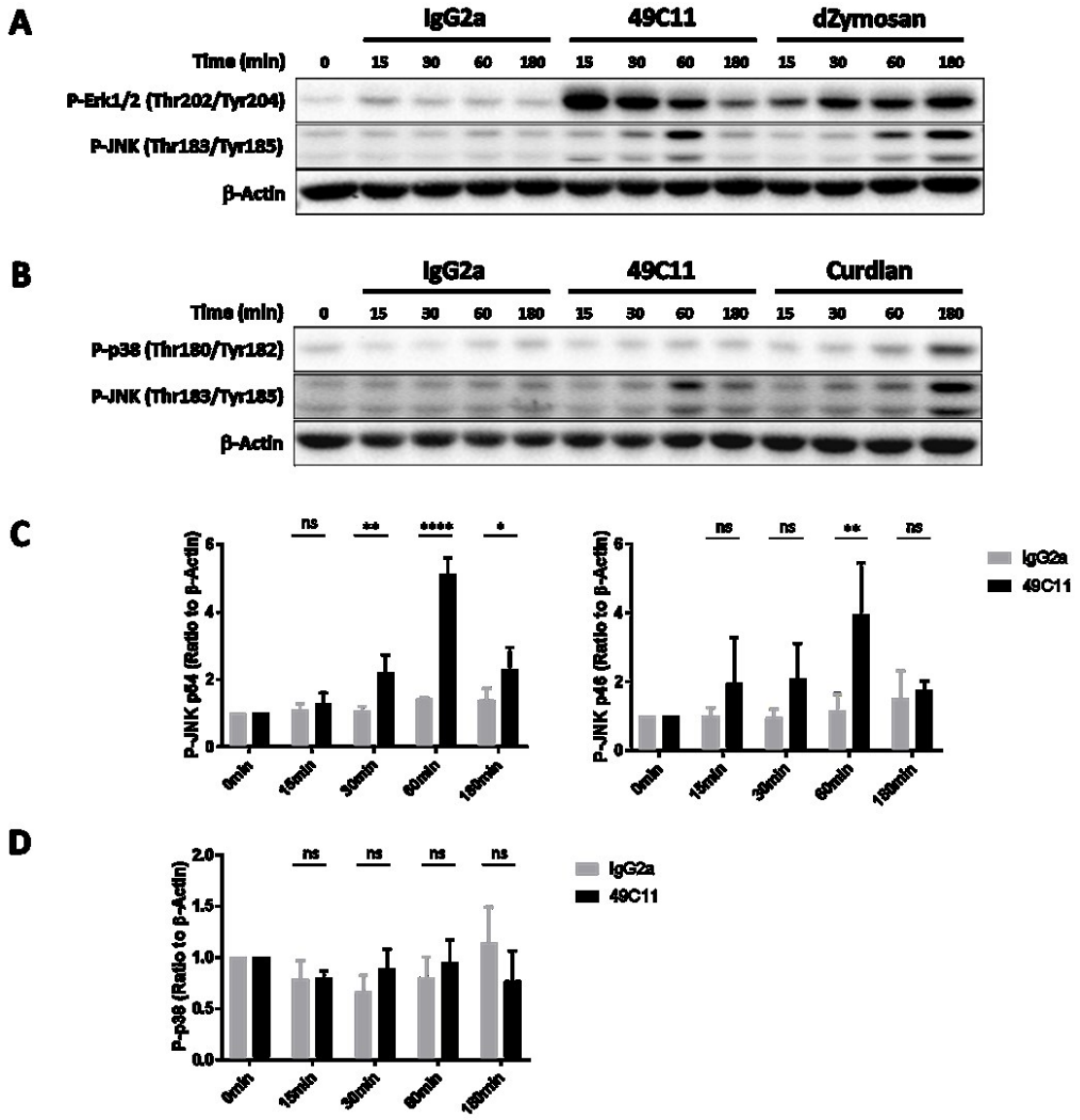


Figure 4.6. DC-ASGPR ligation activates MAPK ERK1/2 and JNK. (A & B) Representative western blot data indicate the phosphorylation status of ERK1/2, JNK and p38 upon indicated treatment at different time points. (C & D) Summarized data of P-JNK and P-p38 abundance in DCs upon indicated treatment at different time points generated from different donors (n=3).

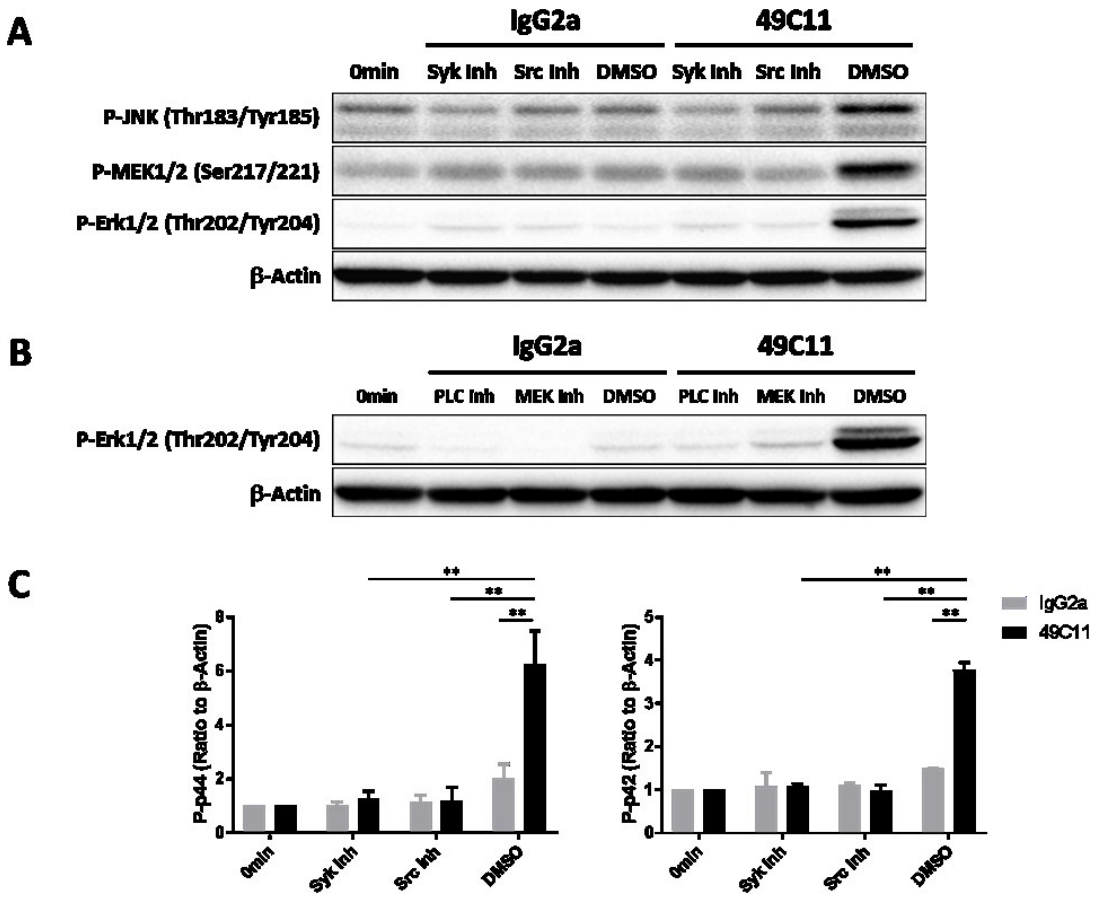


Figure 4.7. DC-ASGPR-induced MAPK activation is dependent on upstream kinases. (A) Representative data shows the phosphorylation status of JNK, MEK1/2 and ERK1/2 in DCs which were pre-incubated with indicated inhibitor for 1h and then treated by plate-bound mAb for 30min. (B) Representative data shows the phosphorylation status of ERK1/2 in DCs which were pre-incubated with indicated inhibitor for 1h and then treated by plate-bound mAb for 30min. (C) Summarized data of P-ERK1/2 abundance in DCs treated as in (A) generated from different donors (n=3).

*Discussion*

CLRs expressed by DCs interact with pathogens primarily through the recognition of mannose, fucose and glucan carbohydrate structures. Therefore, CLRs can recognize most classes of human pathogens: mannose specificity allows the recognition of viruses, fungi and mycobacteria; fucose structures are more specifically expressed by certain bacteria and helminths; glucan structures are present on mycobacteria and fungi.

Recognition by CLRs leads to internalization of the pathogenic ligand, its degradation and subsequent antigen presentation. These properties of CLRs are now giving rise to novel vaccine design (Figdor et al., 2002; Geijtenbeek and Gringhuis, 2009; Geijtenbeek and Gringhuis, 2016; Steinman and Banchereau, 2007).

Recently, more attention has been called to an even more powerful application: targeting of the signaling pathways downstream of CLRs to tailor immune responses to break tumor-induced immunosuppression, to induce Th1 cell responses against viral infections, to redirect allergic Th2 cell responses to protective Th1 cell responses or to elicit regulatory T cell responses for the control of autoimmunity and transplantation complication (Geijtenbeek and Gringhuis, 2009). Therefore, understanding the signaling properties of different CLRs will be critical to make these ideas become practical. However, CLR triggering by different ligands induces diverse signaling processes, which are complex and remain largely elusive.

So far, CLRs including Dectin-1 (CLEC7A), CLEC-2 (CLEC1B), DNGR-1 (CLEC9A), DCIR (CLEC4A) and MICL (CLEC12A) expressed on various myeloid cells including DCs, are found to signal via their cytoplasmic signaling motifs including hemITAM and ITIM, initiating different downstream signaling cascades for corresponding biological functions (Huysamen et al., 2008a; Kanazawa et al., 2002; Marshall et al., 2004; Suzuki-Inoue et al., 2006). In addition, CLRs lacking internal signaling motif are found to trigger intracellular signaling pathways via association with adaptor protein, which is normally mediated by charged residues in or near the transmembrane domains of these CLRs (Sancho and Sousa, 2012). For example, BDCA-2 (CLEC4C), Mincle (CLEC4E) and Dectin-2 (CLEC6A) associate with the ITAM-

containing adaptor protein FcRγ for signal transduction (Cao et al., 2007; Sato et al., 2006; Yamasaki et al., 2008). Ligand binding to Dectin-2 or Mincle leads to engagement of the FcRγ-Syk-PLCγ2-PKCδ-CARD9 axis and activation of the canonical NF-κB pathway similar to Dectin-1 (Gorjestani et al., 2011; Saijo et al., 2010; Schoenen et al., 2010; Strasser et al., 2012; Yamasaki et al., 2008). MDL-1 (CLEC5A) has a short cytoplasmic tail that associates noncovalently with the ITAM-containing adaptor DAP12 through a transmembrane lysine. MDL-1 has been shown to recognize mannose and fucose expressed on envelope proteins of flavivirus family members and act as a signaling receptor for proinflammatory cytokine release (Bakker et al., 1999; Chen et al., 2017; Chen et al., 2008).

Some CLRs have been described mainly as modulators of signaling initiated by other receptors. For example, DC-SIGN triggering alone is insufficient to induce expression of the gene encoding IL-10 or of other innate response genes, but DC-SIGN crosslinking leads to calcium influx and activation of Raf-1, PLCγ, Akt and ERK1/2 (Caparros et al., 2006; Gringhuis et al., 2007). The functional relevance of these intracellular signals is illustrated by that DC-SIGN ligation greatly increases IL-10 production after stimulation with the TLR4 ligand lipopolysaccharide (LPS) while at the same time blocking LPS-induced DC maturation (Caparros et al., 2006; Geijtenbeek et al., 2003b; Gringhuis et al., 2007).

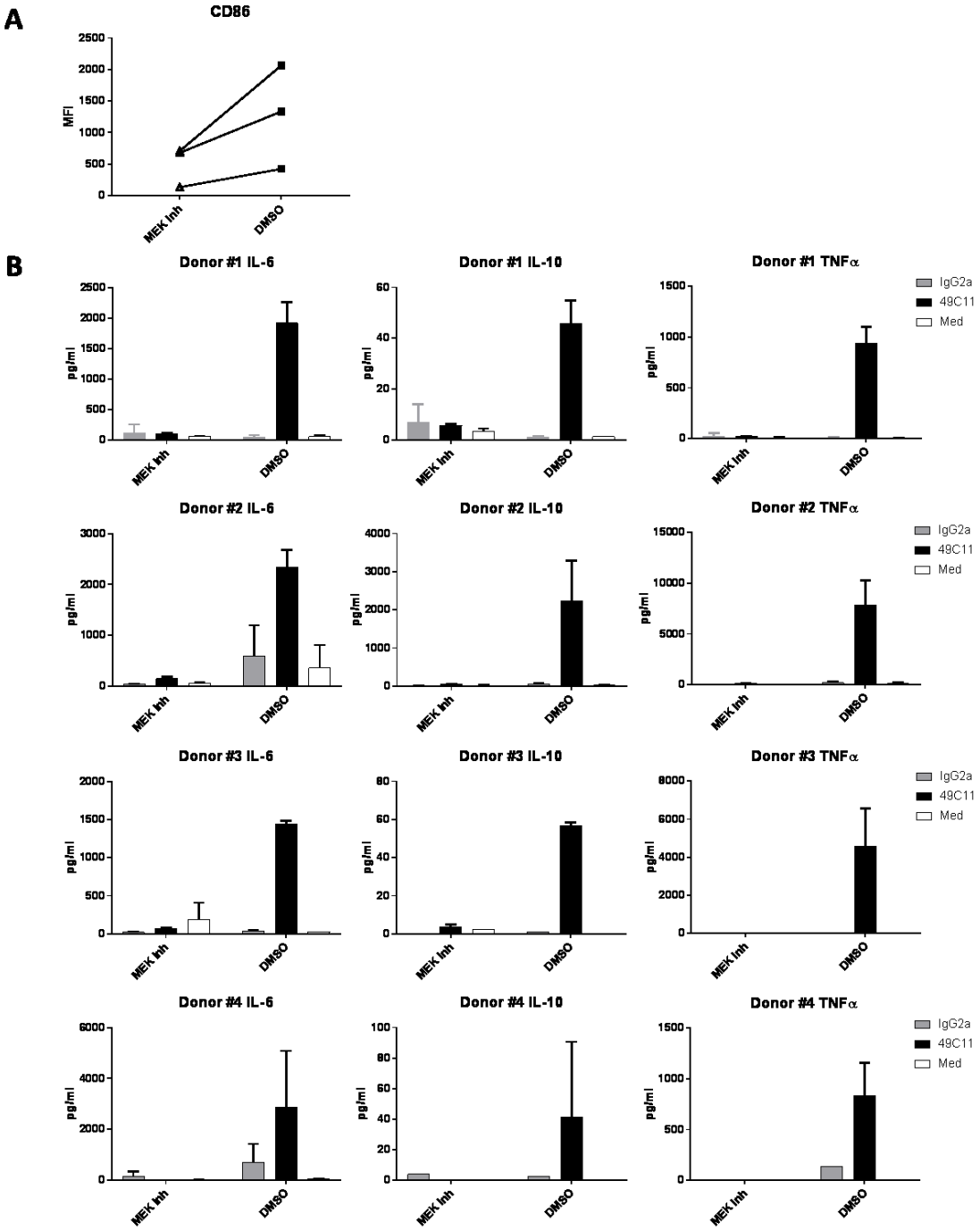


Figure 4.8. Inhibition of ERK1/2 suppresses cell activation and cytokine production of DCs upon DC-ASGPR ligation. (A) Surface staining of CD86 on IL-4-DCs (n=3) which were pre-incubated with indicated inhibitor and then activated by plate-bound 49C11 overnight. (B) Cytokine levels in the supernatant harvested from IL-4-DCs treated as in (A).

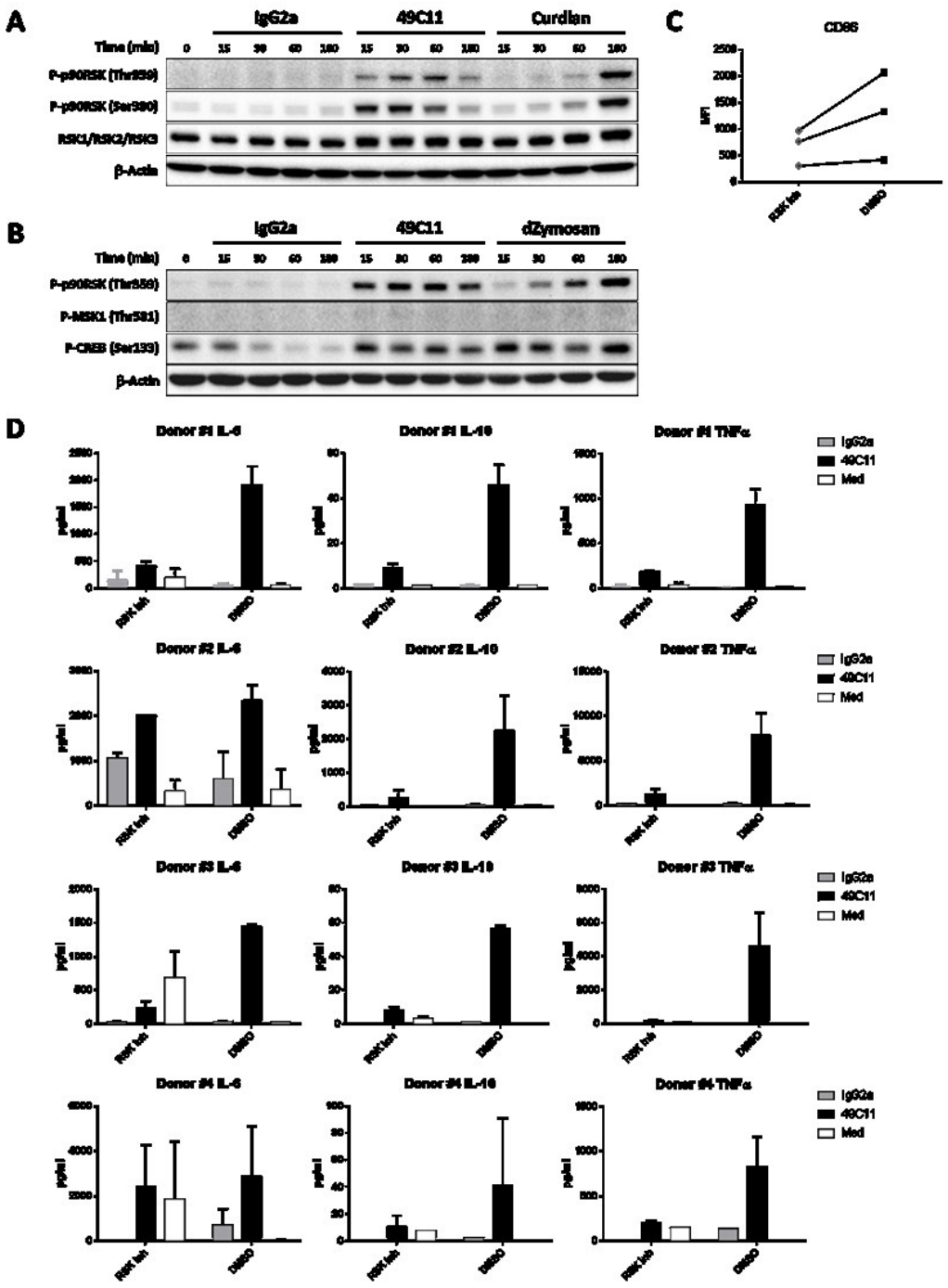


Figure 4.9. DC-ASGPR ligation by 49C11 induces activation of p90RSK and CREB. (A & B) Representative western blot data indicate the phosphorylation status of p90RSK, MSK1 and CREB upon indicated treatment at different time points. (C) Surface staining of CD86 on IL-4-DCs (n=3) which were pre-incubated with indicated inhibitor and then activated by plate-bound 49C11 overnight. (D) Cytokine levels in the supernatant harvested from IL-4-DCs treated as in (C).

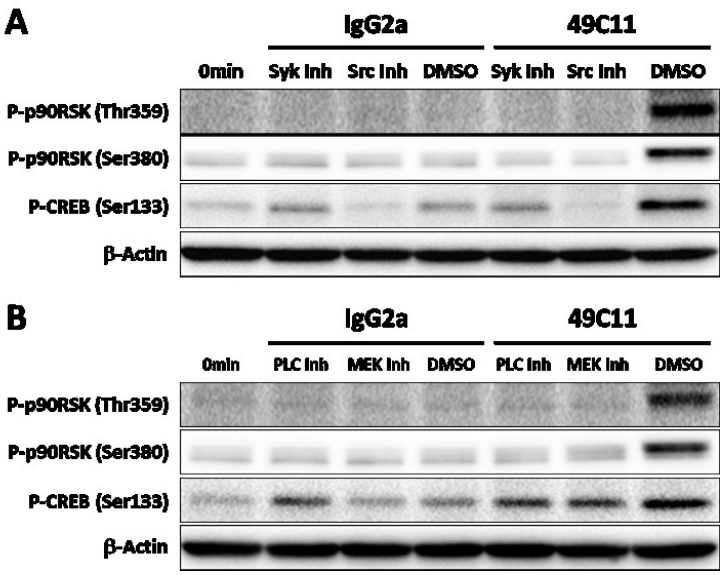


Figure 4.10. Activation of p90RSK and CREB induced by DC-ASGPR ligation is dependent on upstream kinases. (A & B) Representative data show the phosphorylation status of p90RSK and CREB in DCs which were pre-incubated with indicated inhibitor for 1h and then treated by plate-bound mAb for 30min.

In this study, we investigated human DC-ASGPR signaling pathway and report that DC-ASGPR ligation by specific mAb induces Syk activation as evidenced by its phosphorylation at Tyr525/526, which is dependent on member(s) of SFKs. The current understanding of CLR-SFK-Syk signaling cascade is that upon CLR ligation, receptor proximal SFK phosphorylates hemITAM or ITAM within the cytoplasmic tail of CLR or the adaptor protein that is associated with CLR. The phosphorylated ITAM or hemITAM then recruits and binds to Syk by providing docking sites for the tandem SH2 domains within Syk, resulting in Syk activation (Mocsai et al., 2010). The observation that SFK and Syk are engaged in DC-ASGPR signaling pathway actually provoked an important question unanswered in this study: as there is no clear ITAM or hemITAM in the cytoplasmic tail of DC-ASGPR, what is the mechanism that Syk is recruited to a proximal location to DC-ASGPR and further become activated? A possible explanation

could be that there are multiple positively charged lysine (K) residues residing in the transmembrane domain and two arginine (R) residues in the cytoplasmic tail which is proximal to the transmembrane domain, by which DC-ASGPR could associate with the ITAM-bearing transmembrane adaptor protein FcRγ or DAP12 for Syk recruitment and activation, which has been observed in many other CLRs lacking internal signaling motif (Bakker et al., 1999; Sato et al., 2006; Yamasaki et al., 2008).

Another finding in this study is that DC-ASGPR-Syk-PLCγ2-PKCδ axis does not result in NF-κB activation. It will be of significance to investigate the possible mechanism for it. Given that our antibodies for the detection of phosphorylated TAK1 does not work, we cannot rule out the possibility that DC-ASGPR ligation does not trigger TAK1 activation. If this is the case, it would be meaningful to investigate the complete formation of CBM complex, which is upstream of TAK1, in DCs upon DC-ASGPR ligation.

In addition, our results confirm that DC-ASGPR ligation by mAb induces ERK1/2 phosphorylation and provide direct evidence for p90RSK and CREB activation (Li et al., 2012; Napoletano et al., 2012; van Vliet et al., 2013a). As CREB operates downstream of p90RSK, and the IL-10 promotor contains cAMP response elements (Saraiva and O'Garra, 2010; Xing et al., 1996), DC-ASGPR likely couple to the ERK-p90RSK-CREB axis for enhanced IL-10 secretion. Moreover, we observed JNK activation upon DC-ASGPR engagement, but the specific downstream effector molecules of JNK in regulating DC-ASGPR-induced cytokine expression remain unclear.

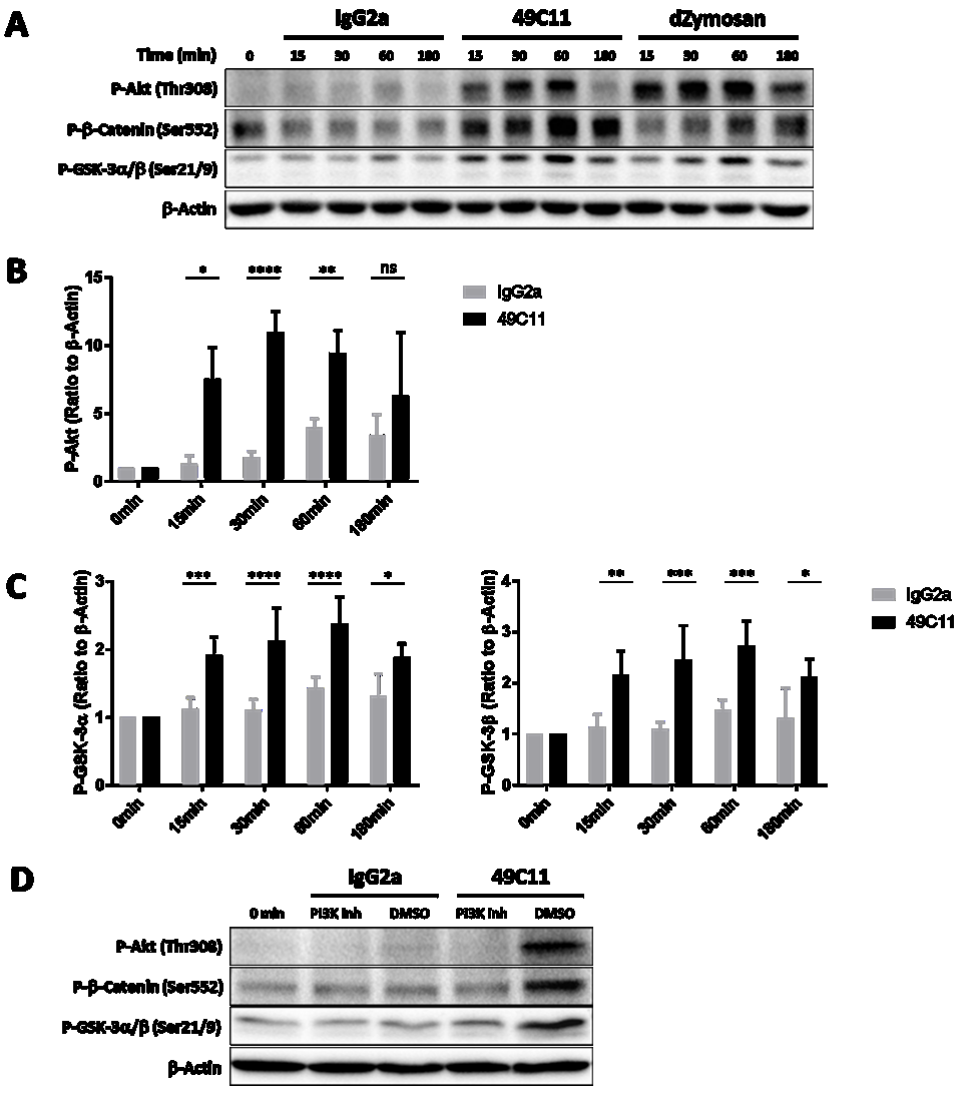


Figure 4.11. DC-ASGPR ligation activates PI3K-Akt pathway, which differentially regulates the activities of GSK-3α/β and β-Catenin. (A) Representative western blot data indicate the phosphorylation status of Akt, β-Catenin and GSK-3α/β upon indicated treatment at different time points. (B & C) Summarized data of P-Akt and P-GSK-3α/β abundance in DCs upon indicated treatment at different time points generated from different donors (n=4). (D) Representative data shows the phosphorylation status of Akt, β-Catenin and GSK-3α/β in DCs which were pre-incubated with indicated inhibitor for 1h and then treated by plate-bound mAb for 30min.

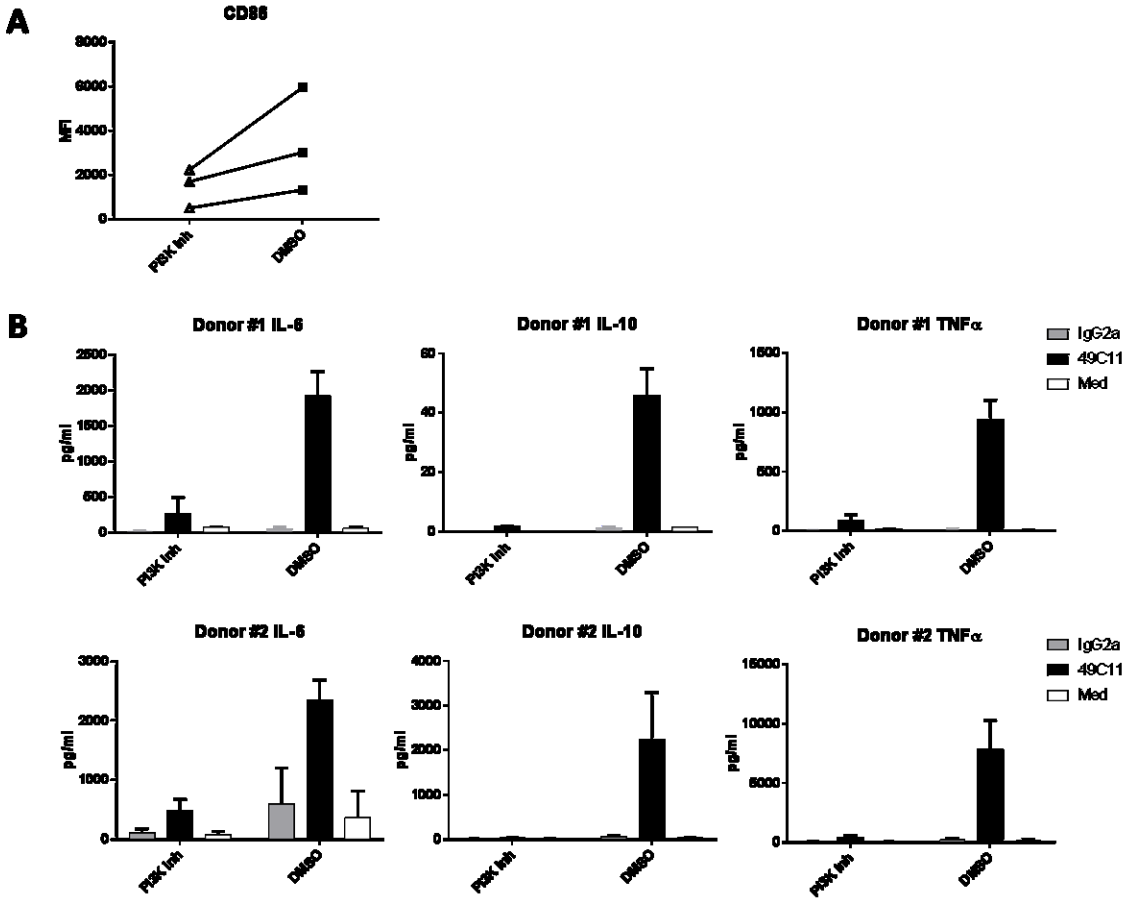


Figure 4.12. Inhibition of Akt suppresses DC activation and cytokine production upon DC-ASGPR ligation. (A) Surface staining of CD86 on IL-4-DCs (n=3) which were pre-incubated with indicated inhibitor and then activated by plate-bound 49C11 overnight. (B) Cytokine levels in the supernatant harvested from IL-4-DCs treated as in (A).

We also found that DC-ASGPR ligation by mAb induces PI3K-Akt pathway, which further regulates the activities of β-Catenin and GSK-3α/β. Recent biochemical and genetic studies have shown that inhibitory phosphorylation of GSK-3α/β prevents GSK-3α/β-mediated β-Catenin degradation, while phosphorylation of β-Catenin at Ser552 promotes its transcriptional activity (Fang et al., 2007; Rodionova et al., 2007). Moreover, it has been reported that β-Catenin is critical for the induction of vitamin A metabolizing enzymes and IL-10 in mucosal DCs (Manicassamy et al., 2010). We

postulate β-Catenin activated upon DC-ASGPR ligation could contribute to the expression of retinoic acid (RA) and IL-10 production, however, experiment to prove a direct relationship between them is highly desirable.

Studies of DC-ASGPR signaling pathway from other groups reported mixed information. For example, Chiara Napoletano *et al.* reported that DC-ASGPR ligation by synthesized peptide or mAb triggered ERK1/2 and NF-κB activation and induced DC maturation (Napoletano et al., 2012) whereas Sandra J. van Vliet *et al.* reported that DC-ASGPR ligation by mAb alone failed to induce DC maturation or cytokine expression, however, it resulted in ERK1/2 but not JNK activation. These differences could be the results of different clones of mAb, activation condition (amount of mAb coated for activation, $BS^3$ as cross-linker) used in the studies, which could mimic the binding of different ligands to DC-ASGPR and thus, induce different signaling outcomes. Last but not least, the specificity of kinase inhibitor used in the CLR signaling always remains questioned.

Taken together, DC-ASGPR ligation induces a complex signaling cascade which is responsible for phenotypic change and cytokine expression of human DCs. Our study provides a molecular explanation for the ability of DC-ASGPR-interacting ligands to preferentially evoke immune modulation.

# CHAPTER FIVE

## Conclusion

Based on the studies presented, we conclude:

- Dectin-1 ligation downregulates TSLP-induced OX40L expression on mDCs via repressing p50-RelB transcriptional activity
- Activation of mDCs via Dectin-1 suppresses $CRTH2^+$ memory $CD4^+$ T cell responses
- Dectin-1 ligation decreases the capacity of TSLP-mDCs in maintaining Th2 cell commitment
- Activation of mDCs via Dectin-1 inhibits allergen-specific Th2 cell response
- Dectin-1 signaling in mDCs decreases TSLP-mediated CCL17 production via inhibition of STAT6 activity
- DC-ASGPR ligation by mAb (49C11) induces DC activation and cytokine production
- DC-ASGPR ligation by 49C11 induces activation of Syk-PLCγ2-PKCδ cascade but not leading to NF-κB activation
- DC-ASGPR ligation by 49C11 induces ERK1/2-p90RSK-CREB cascade for IL-10 production
- DC-ASGPR ligation by 49C11 induces PI3K-Akt pathway, which differentially regulates GSK-3α/β and β-Catenin activity to fine-tune cytokine expression

# APPENDICES

# APPENDIX A

## List of Current and Future Publications

### *Publications Related to the Topics of Dissertation*

**Gu, C.**, L. Wang, L. Ni, W. Zhang, Z. Wang, S. Zurawski, L. Bover, H. Joo, M. Millard, and S. Oh. Cellular and Molecular Mechanisms for Dectin-1-Mediated Suppression of Th2-Type Immune Response (manuscript in preparation).

**Gu, C.**, L. Wang, S. Zurawski, G. Zurawski, and S. Oh. DC-ASGPR-Mediated Signaling Pathway in Human Dendritic Cells (manuscript in preparation).

### *Additional Publications*

Therapeutic HPV cancer vaccine targeted to CD40 elicits effective $CD8^+$ T-cell immunity
Wenjie Yin, Dorothée Duluc, HyeMee Joo, Yaming Xue, **Chao Gu**, Zhiqing Wang, Lei Wang, Richard Ouedraogo, Lance Oxford, Amelia Clark, Falguni Parikh, Seunghee Kim-Schulze, LuAnn Thompson-Snipes, Sang-Yull Lee, Clay Beauregard, Jung-Hee Woo, Sandra Zurawski, Andrew G. Sikora, Gerard Zurawski and SangKon Oh
Cancer Immunol Res. 2016;4(10):823-34.

Functional Specialty of CD40 and Dendritic Cell Surface Lectins for Exogenous Antigen Presentation to $CD8^+$ and $CD4^+$ T Cells
Wenjie Yin, Laurent Gorvel, Sandra Zurawski, Dapeng Li, Ling Ni, Dorothée Duluc, Katherine Upchurch, JongRok Kim, **Chao Gu**, Richard Ouedraogo, Zhiqing Wang, Yaming Xue, HyeMee Joo, Jean-Pierre Gorvel, Gerard Zurawski, SangKon Oh
EBioMedicine. 2016;5:46-58.

# APPENDIX B

## Author Contributions

### *Chapter Three*

C.G. performed experiments in Figures 3.1, 3.2, 3.3, 3.5, 3.6, 3.7, 3.8, analyzed the data, and wrote the manuscript. L.W., L.N., and W.Z. performed the experiments in Figures 3.1, 3.2, 3.3, 3.4. 3.5, 3.7 and 3.8. B.C. and L.B. provided Amb a1 and TSLP, respectively. H.J. provided general technical assistance. M.M. provided patient samples. S.O. supervised the project, analyzed the data, and wrote the manuscript.

### *Chapter Four*

C.G. performed experiments in Figures 4.1–4.12, analyzed the data, and wrote the manuscript. L.W. helped in the preparation of IL-4-DCs and provided general technical assistance. S.Z., G.Z. and Z.W. provided us with anti-DC-ASGPR mAb. S.O. supervised the project, analyzed the data, and wrote the manuscript.

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