Exploring the Influence of PKC-theta Phosphorylation on Notch1 Activation and T Helper Cell Differentiation

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EXPLORING THE INFLUENCE OF PKCθ PHOSPHORYLATION ON NOTCH1 ACTIVATION AND T HELPER CELL DIFFERENTIATION

A Thesis Presented

by

GRACE C. TROMBLEY

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

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September 2018

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EXPLORING THE INFLUENCE OF PKC\(\theta\) PHOSPHORYLATION ON NOTCH1 ACTIVATION AND T HELPER CELL DIFFERENTIATION

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ABSTRACT

EXPLORING THE INFLUENCE OF PKCθ PHOSPHORYLATION ON NOTCH1 ACTIVATION AND T HELPER CELL DIFFERENTIATION

September 2018

GRACE C. TROMBLEY, B.Sc., UNIVERSITY OF MASSACHUSETTS AMHERST

M.Sc., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by: Professor Lisa M. Minter

The T cell-specific kinase, Protein Kinase C theta (PKCθ) is essential to T cell activation and differentiation. PKCθ integrates T cell receptor (TCR) and CD28 signaling, and ultimately activates transcription factors necessary for full T cell activation, proliferation, survival, and differentiation into T helper (Th) subsets. Th1, Th2, Th17 and Treg cells compose the four major lineages of T helper cells, differentiated from CD4 T cells, and each have different requirements for PKCθ.

PKCθ, itself, is regulated through phosphorylation of specific residues, including tyrosine (Y)90 and threonine (T)538. Following T cell stimulation, PKCθ is phosphorylated on Y90 by the kinase, LCK, and translocates to the cell membrane. There it remains associated with LCK in a structural complex known as the immunological synapse. Loss of PKCθ in T cells produces a phenotype that is similar to loss of another important T cell protein, Notch1, suggesting these two proteins may function in the same signaling pathway. Our lab has shown that PKCθ can interact with Notch1, but how this interaction regulates Notch1 function is not known. Due to the strong overlap between cellular functions regulated by PKCθ and Notch1, understanding how these two proteins might function, cooperatively, can provide better insight into autoimmune diseases and may be useful in developing novel therapies.

We hypothesized that phosphorylation of a specific residue of PKCθ (T538) is required for Notch1 cleavage and nuclear translocation. We also hypothesize that the phosphorylation status of PKCθ (T538) will influence the ability of T cells to differentiate into specific T helper subsets. We used two means of inhibiting PKCθ function to evaluate its regulation of Notch1 in differentiated T cells: 1) we blocked the association of PKCθ and LCK, thereby preventing its movement to the immunological synapse and 2) we prevented PKCθ phosphorylation on T538.

We found that by preventing the phosphorylation of PKCθ, we also decreased the level of cleaved Notch1 in Th1, Th2, and Th17 cells. We further determined that inhibiting PKCθ decreased the amount of Notch1 that translocated to the nucleus in
Th1 and iTreg cells. Understanding how PKCθ interacts with and regulates Notch1 to influence T cell differentiation may lay the foundation for specifically modulating T cell responses.
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CHAPTER 1

INTRODUCTION

T helper cells (Th cells) are essential in directing immune responses though their role in activating and directing other immune cells. Upon recognition of a foreign antigen, Th cells will stimulate antibody production by producing cytokines that will activate other T-cell subsets, B-cells and innate immune responses. The different CD4 T cell subsets of Th cells can be defined by their distinct cytokine secretion patterns. They include T helper type 1 (Th1), T helper type 2 (Th2), T helper type 17 (Th17), and regulatory T cells (Tregs). Th1 cells are essential in cell-mediated immunity and in eliminating intracellular pathogens via the activation of infected macrophages (Zhu, J., et al. 2012). Some Th1 dominant diseases include organ-specific autoimmune disorders such as multiple sclerosis, diabetes, and rheumatoid arthritis. Th2 cells are essential in humoral immune responses and host defense against extracellular parasites via induction of B-cell antibody production (Zhu, J., et al. 2012). Some conditions associated with Th2 dominance are systemic autoimmune disorders, allergies, and asthma. Th17 cells play a role in host defense against extracellular pathogens by mediating the recruitment of neutrophils and macrophages to infected tissues (Zhu, J., et al. 2012). A few of the autoimmune inflammatory diseases associated with Th17 cells are psoriasis, multiple sclerosis, lupus, and chronic obstructive pulmonary disease. Regulatory T cells place a negative regulation on other immune cells and therefore are capable of suppressing an immune reaction and preventing autoimmune diseases and chronic
inflammatory diseases (Zhu, J., et al. 2012). Understanding the mechanisms of Treg cells could lead to development of therapeutic strategies for diseases such as cancer, diabetes, and immune mediated diseases. Data in the literature suggest that these distinct subsets have different requirements for Protein Kinase C-theta (PKCθ), these requirements being dependent upon antigen and immune response (Hayashi, K., et al. 2008).

The T cell-specific kinase, PKCθ, is essential to T cell activation and differentiation through the integration of TCR and CD28 signaling (Isakov, N., et al. 2002). This integration ultimately leads to the activation of transcription factors that are necessary for full T cell survival, activation and effector function of Th subsets. PKCθ, itself, is regulated through phosphorylation of specific residues, including tyrosine (Y)90 and threonine (T)538 (Wang, X., et al. 2012). Following T cell stimulation, PKCθ is phosphorylated on Y90 by the kinase, LCK, and translocates to the membrane (Hayashi, K., et al. 2008). There it remains associated with LCK in a structural complex known as the immunological synapse (Hayashi, K., et al. 2008).

Loss of PKCθ in T cells produces a phenotype that is similar to loss of another important T cell protein, Notch1, suggesting these two proteins may function in the same signaling pathway. Notch1, a transmembrane receptor, is known to play an essential role of the differentiation of CD4 T cells into T helper cells (Osborne, B., et al. 2007). Our lab has shown that PKCθ can interact with Notch1, but how this interaction regulates Notch1 function is not known. Because of the strong overlap between cellular functions regulated by PKCθ and Notch, understanding how these
two proteins might function, cooperatively, can provide better insight into autoimmune diseases and may be useful in developing novel therapies.

We used two means of inhibiting PKCθ function to evaluate its regulation of Notch1 in differentiated T cells: 1) we blocked the association of PKCθ and LCK, thereby preventing its movement to the immunological synapse and 2) we prevented PKCθ phosphorylation on T538. Understanding how PKCθ interacts with and regulates Notch1 to influence T cell differentiation may lay the foundation for specifically modulating T cell responses.
CHAPTER 2
LITERATURE REVIEW

The immune system is composed of two parts, the non-specific innate immunity and the specific adaptive immunity. Upon infection, the innate and adaptive immune systems become active and work together to target invading pathogens (Figure 1). The innate immune system provides immediate defense against infection by acting as a physical and chemical barrier. This part of the system is also responsible for activating the adaptive immune system through antigen presenting cells (APCs). While the innate immune system is not useful for long-term immunity, the adaptive immune system does provide long-lasting protection. The adaptive immune system creates an immunological memory; therefore, it is able to ward off repeated attacks of the same specific pathogen. The cells responsible for the adaptive immune response are the lymphocytes, a type of white blood cell. Two different lymphocytes carry out this immune response, the B cells and T cells. The B cells are produced in the bone marrow and either form into memory cells or plasma cells which are responsible for secreting antibodies.
Figure 1: (VMC 2012) Overview of the adaptive and innate immune systems.

T cells, a type of white blood cell, play a central role in the adaptive immune system. Complete activation of T cells requires signaling events through the antigen-specific receptor as well as one through the receptor for a co-stimulatory molecule. Without the co-stimulatory molecule, the T cell is incapable of producing its own growth hormone, interleukin 2 (IL-2), upon stimulation (Schwartz, R. 1990). T cells differentiate in response to activation, resulting in the T cells being capable of producing other cytokines that will serve as effector molecules through the activation of other cells. This cycle causes the T cells to proliferate, which is essential for their role in host defense. T cells can become effector cells that will develop either as T helper (Th) cells or as cytotoxic T (Tc) cells. T cells that mature in the thymus will migrate to peripheral lymphoid organs such as the spleen and lymph node (Schwartz, R. 1990). It is here that naïve T cells will encounter antigens through antigen presenting cells (APCs).
Antigens are taken up by APCs, are fragmented into peptides, and complexed with major histocompatibility complex (MHC) on the APC surface to present to T cell antigen receptors (Kwon, M., et al. 2010). Upon engagement of the MHC-peptide with the TCR, the TCR initiates signaling pathways that lead to activation of T cells. The T cell antigen receptor (TCR) is a protein complex responsible for signal transduction following recognition. These antigen receptors are composed of two disulfide-linked subunits (α and β) which come into contact with the peptide and the MHC (Schwartz, R. 1990). After attachment to these chains, the CD4 or CD8 molecule is brought into the receptor complex. MHC class I molecules are recognized by lymphocytes expressing the CD8 surface molecule while the MHC class II molecule is recognized by lymphocytes expressing the CD4 surface molecule (Figure 2). When T cells become activated upon recognition of a foreign antigen, the naïve cells will proliferate in response to their own production of the proliferative cytokine, IL-2.
CD4 and CD8 T cells bind to their respective class I or class II major histocompatibility complex (MHC) ligands on an antigen presenting cell (APC). CD4 T cells are essential to the adaptive immune response through their role in helping B cells to produce antibody and undergo maturation, as well as activating other immune cells such as CD8 T cells. Once CD4 T cells are activated, they are primed to differentiate into T helper cells that are responsible for mediating pathogen-specific immune responses. T helper cells, or Th cells, differentiated from naïve CD4 T cells, make up four major lineages: Th1, Th2, Th17 and T regulatory (Treg) cells. Their differentiation depends on function, cytokine secretion, and expression of transcription factors (Figure 3). Th1 cells produce IFNγ, making these cells essential in macrophage activation as well as in elimination of intracellular pathogens. The cytokines that direct the Th1 differentiation process are IL-12 and
IFNγ (Zhu, J., et al. 2012). Th2 cells produce IL-4, IL-5, IL-10 and IL-13, making these cells essential in Immunoglobulin E production and in the elimination of extracellular parasites (Zhu, J., et al. 2012). The cytokines that promote a Th2 phenotype are IL-4, TSLP, IL-17 and IL-2 (Zhu, J., et al. 2012). Th17 cells produce many cytokines and are essential in immune responses to extracellular bacteria and fungi (Zhu, J., et al. 2012). Cytokines involved in the Th17 differentiation process are TGFβ, IL-6, IL-21, and IL-23 (Zhu, J., et al. 2012). Regulatory T cells consist of naturally occurring Tregs (nTregs) and inducible regulatory Tregs (iTregs). The nTregs develop in the thymus while the iTregs are caused by exposure of naïve peripheral CD4 T cells with a TCR stimulant along with TGFβ and IL-2 (Zhu, J., et al. 2012). Both types of Tregs are involved in self-tolerance as well as immune modulation. The cytokines that promote the Treg differentiation process are TGFβ and IL-2 (Zhu, J., et al. 2012).
Protein Kinase C theta (PKCθ) is a serine/threonine kinase and a member of the novel protein kinase C (PKC) subfamily. PKC kinases are regulated by calcium, diacylglycerol, and phorbol esters and are separated into three major groups by their activating factors (Xu, Z., et al. 2004). These kinases have a C-terminal catalytic kinase domain and an N-terminal regulatory co-factor-binding domain (Xu, Z., et al. 2004). Expression of this protein is limited to certain tissues and cell types, which includes T cells. We know that PKCθ is required for the survival and activation of mature T cells due to its major role in coupling the activated T cell receptor and the CD28 costimulatory receptor to their signaling pathways (Isakov, N. 2012). Upon antigen stimulation, PKCθ translocates to membrane lipid rafts, and from there
localizes to the immunological synapse (IS) (Cartwright, N., et al. 2011). PKCθ−/− mice were found to have impaired T cell activation and proliferation due to defects of activator protein-1 (AP-1), nuclear factor-κB (NF-κB), and IL-2 induction (Cartwright, N., et al. 2011). This impairment was found to affect the CD4 T cells more severely than the CD8 T cells (Cartwright, N., et al. 2011). PKCθ−/− T cells have also shown defects in cytokine production and differentiation.

During TCR stimulation, phosphorylation of PKCθ occurs at multiple serine, threonine, and tyrosine residues. PKCθ consists of a N-terminal regulatory region and a C-terminal catalytic region. On the outside of the C1 domains are two variable regions, V1 and V3 (Wang, X., et al. 2012). The site of phosphorylation responsible for bringing PKCθ to the IS resides in the V3 domain of PKCθ, where it is involved in the indirect association of PKCθ and CD28 (Isakov, N. 2007). TCR and CD28 co-stimulation induces production of diacylglycerol; diacylglycerol binds to the C1 domains of PKCθ, which in turn exposes the activation loop of PKCθ (Wang, X., et al. 2012). Once this occurs, the germinal center kinase-like kinase (GLK) is able to phosphorylate Threonine 538 and subsequently induce catalytic activation (Wang, X., et al. 2012). The C2-like domain region of PKCθ contains a phosphorylated Y90 residue which is thought to interact with a SH-2 domain containing region (Wang, X., et al. 2012). Additionally, the C2-like domain may interact with a receptor for activated C kinase (RACK), a possible regulator of PKCθ translocation to the membrane (Wang, X., et al. 2012).
Figure 4: (Wang, X., et al. 2012) TCR Signaling via PKCθ. Activation of the TCR starts a signaling cascade which leads to the phosphorylation of PKCθ and induces its translocation to the membrane. This translocation facilitates the activation of transcription factors essential for T cell activation.

Localization of PKCθ to the immunological synapse (IS) is thought to access downstream targets as well as contribute to CD28-mediated co-stimulation (Cartwright, N., et al. 2011). When PKCθ is active and retained at the IS, it will initiate a signaling cascade that leads to the activation of transcription factors including nuclear factor-kB (NF-kB), AP-1, and NF-AT, all of which are essential in T cell proliferation and differentiation (Figure 4). This is due to their corresponding binding sites on the IL-2 gene promoter and are, therefore, essential for an optimal IL-2 response (Isakov, N. 2012). Alternatively, it was found in Tregs that PKCθ will translocate to the opposite cell pole, away from the IS. Other data has shown that this localization may control IS stability (Cartwright, N., et al. 2011). The IS, also
known as the supramolecular activation cluster (SMAC), is the region containing the site of contact between antigen (Ag)-specific T cells and Ag-presenting cells (APCs) (Hayashi, K., et al. 2007). The IS serves to deliver activation signals that are required for productive T cell activation. The central SMAC (cSMAC) contains a high content of TCR, PKCθ, co-stimulatory receptors CD28, CD2, CD4, and CD8, and kinases LCK and FYN (Hayashi, K., et al. 2007). The cSMAC contains two separate compartments that are responsible for different functions, the first containing TCR-associated signaling complexes that are internalized and degraded while the second compartment, containing large amounts of PKCθ and CD28, is the site of co-localization between these proteins (Isakov, N. 2012).

CD28 is a homodimeric glycoprotein that is expressed by mature T cells as a surface molecule and acts as a co-stimulatory signal receptor for T cell activation (Nunes, J., et al. 1996). CD28 co-stimulation has been found to enhance translocation of PKCθ to the lipid rafts and the IS (Hayashi, K., et al. 2007). In combination with this signaling, TCR signaling leads to T cell proliferation and differentiation through activation of transcription factors NF-κB and AP-1 (Hayashi, K., et al. 2007). There are binding sites for these transcription factors on the IL2 gene promoter, therefore signaling is followed by the activation of the IL2 gene promoter (Isakov, N. 2012). Studies have shown that co-stimulation between the TCR and CD28 induces binding of PKCθ to the cytoplasmic tail of CD28 (Isakov, N. 2012). Additionally, it was discovered that functional LCK, a Src protein-tyrosine kinase, is required in order for PKCθ to translocate to the IS (Hayashi, K., et al. 2007).
Six different phosphorylation sites that contribute to the regulation of PKCθ kinase activity and membrane translocation were discovered on PKCθ: S676, S685, S695, T219, T538, and Y90 (Figure 5; Wang, X., et al. 2012). According to previous data, preventing the phosphorylation of T538 with a T538A mutant eliminated PKCθ activity, suggesting that T538 is a critical phosphorylation site for PKCθ function (Wang, X., et al. 2012). Certain peptide sequences within the PKCθ C-terminal fragments are required for IS retention while multiple phosphorylation sites in the PKCθ Kinase Domain contribute to protein stability and kinase activity. These residues include Thr-538 in the activation loop, Ser-676 in the turn motif, and Ser-695 in the hydrophobic domain (Cartwright, N., et al. 2011). In previous studies, a T538A mutation was found to cause poor IS retention, indicating that phosphorylation of Thr-538 is responsible for the IS retention of PKCθ (Cartwright, N., et al. 2011). This demonstrates a strong correlation between PKCθ kinase activity and IS retention.

Additionally, it was discovered that specific T cell subpopulations have different requirements for PKCθ, which is dependent upon the type of antigen and immune response (Isakov, N. 2007). PKCθ is required for developing Th2-dependent immunity in vivo and is critical in Th17 responses as well (Hayashi, K., et al. 2007). As previously mentioned, when Tregs are activated, PKCθ is pulled in the opposite direction of the IS. When inhibiting PKCθ, there is an increase in the suppressive activity of Tregs (Isakov, N. 2007). PKCθ is known to mediate a negative feedback on Tregs and their functions, which subsequently will downregulate certain functions of T effector cells (Isakov, N. 2007). Under normal conditions,
suppressed T cells will show a decrease in CD69 up-regulation, decrease of IL-2 secretion, and an impaired proliferative response (Isakov, N. 2007). In PKCθ-deficient cells, it has been reported that these cells are not able to up-regulate CD69 and without translocation of PKCθ to the IS, TCR-dependent proliferation will not occur (Isakov, N. 2007). Additionally, T cells that were pulsed with suboptimal concentrations of antigen were not able to recruit PKCθ (Isakov, N. 2007).

Figure 5: (Isakov, N., 2012) Structure of PKCθ.

An essential part of the adaptive immune response is a functional CD8 T cell response. When encountering an antigen, the naïve CD8 T cells differentiate into CD8 effectors, then produce cytokines such as IFNγ as well as the effector molecules perforin and granzyme B (Cho, O., et al. 2009). These effector cytotoxic T cells are essential in the immune defense against intracellular bacteria and viruses. They produce this defense by perforin-mediated release of granzyme B, which causes apoptosis (Cho, O., et al. 2009). Another system of defense includes the expression of the FAS ligand, which works by attaching to FAS on a target cell, inducing apoptosis (Cho, O., et al. 2009).
The Notch signaling pathway is responsible for regulating interactions between neighboring cells. This communication either promotes or suppresses differentiation, proliferation and cell death (Kopan, R., et al. 2009). Notch proteins are single-pass transmembrane receptors that are cleaved multiple times before coming to the cell surface as a heterodimer. The final cleavage is completed by gamma-secretase (GSI). From this cleavage, the Notch intracellular domain (NCID) is generated (Osborne, B. et al. 2007). This intracellular signaling peptide translocates to the nucleus, where it associates with a DNA-bound Suppressor of Hairless (Su(H)) and the nuclear effector Mastermind (MAM), leading to the activation of transcriptional activity (Guruharsha, K., et al. 2012).

Notch proteins play a role in controlling cell fate decisions as well as affecting peripheral T cells (Figure 6). Notch receptors have been shown to be responsible for regulating transcription factors NF-κB, IFNγ, IL-4, and enhancer CSN2, proteins that are essential to peripheral T cell activation and differentiation (Osborne, B. et al. 2007). There are four mammalian Notch receptors, Notch1, Notch2, Notch3, and Notch4 along with five canonical Notch ligands Jagged1, Jagged2, DLL1 (Delta-Like 1), DLL2, DLL3, and DLL4 (Osborne, B. et al. 2007). Reports have shown that without Notch, lymphoid progenitors entering the thymus were not able to initiate a T cell development and subsequently developed into B-cells (Osborne, B. et al. 2007). One study demonstrated that pharmacological inhibitors that were used to block the upregulation of Notch expression in peripheral CD4 T cells also inhibited T cell proliferation along with IFN-γ production following stimulation via the TCR (Osborne, B. et al. 2007). Additionally, it was discovered that a positive feedback
loop involving Notch signaling is responsible for regulating IL-2 production and the expression of CD25, an IL-2 receptor, via T cells. (Osborne, B. et al. 2007). Another study reported that Notch proteins co-localize with CD4 in human CD4 T cells. This localization would place the proteins at the IS, where it would be capable of modulating early signaling events in activated T cells (Osborne, B. et al. 2007).

Signaling through the TCR induces activation of Notch1 in both CD4 and CD8 T cells (Cho, O., et al. 2009). Activation of Notch leads to the activation of NF-κB which subsequently leads to the expression of genes needed for a Th1 differentiation, such as T-bet and IFNγ (Osborne, B. et al. 2007). This suggests that Notch proteins have a role in the differentiation of CD4 T cells into Th1 cells. When CD4 T cells are differentiated to Th2 cells, Notch proteins are also required for the production of IL-4 by NKT cells and memory CD4 T cells (Osborne, B. et al. 2007). Notch proteins may also have a role in Treg differentiation by ligation via Notch receptors on T cells with Jagged 1 or DLL1 on APCs, which can lead to the generation of CD4+ CD25+ Tregs as well as CD8 Tregs (Osborne, B. et al. 2007).

Another study found that in Notch1, in vivo, acts in the same functional pathway as PKCθ and that Notch1 associates with PKCθ and Carma1 in the cytosol (Shin, H., et al. 2014). PKCθ is known to have distinct nuclear and cytoplasmic functions. Following T cell activation, studies have reported a rise in the activity of cytoplasmic PKCθ while no change was seen in the nuclear fraction (Isakov, N. 2007). Notch-receptor upregulation is also seen to occur with stimulation of CD4 T cells with CD3- and CD28-antibodies (Osborne, B., et al. 2007).
Notch regulates cytotoxic T cells through regulation of eomesodermin (EOMES), perforin, and granzyme B (Cho, O., et al. 2009). EOMES expression, combined with the expression of the T-bet transcription factor, is partly responsible for the differentiation of naïve T cells into effector cytotoxic T cells. T-bet plays a role in the development of CD8 T cells, demonstrating another role on Notch in the regulation in cytotoxic T cells (Cho, O., et al. 2009).

In multiple human disorders including human genetic disorders and cancer, the impairment of the Notch signaling pathway has been reported as the underlying cause. In studies with impaired Notch1 signaling, a reduced CD25 expression, a decrease in IL-2 production, and a decrease in proliferation have all been observed (Shin, H., et al. 2014). Additionally, it was reported when upregulation of Notch expression is inhibited, T-cell proliferation and IFNγ production is also inhibited (Osborne, B., et al. 2007). Notch signaling has an essential role in cell fate decisions throughout various stages of T-cell development.
Figure 6: (Nakano, T., et al. 2016) Notch signaling pathway. Upon ligand/receptor interaction, cleavages at S2 and S3 allow the Notch intracellular domain (NICD) to translocate into the nucleus where it associates with a transcription factor and activates transcription.
CHAPTER 3

METHODS

CD4 T cell isolation.

Following the humane euthanasia of a mouse, the spleen was harvested and passed through a filter to collect the splenocytes. Red blood cells were lysed with ACK buffer and white blood cells were counted with a hemocytometer. CD4 T cells were isolated using anti-mouse CD4 magnetic particles (BD Biosciences) and separated using the BD IMAG Cell Separation Magnet.

Flow cytometric analyses of protein expression.

The CD4 T cells were pretreated with either DMSO, the vehicle control, 3 μM Rottlerin, or 5 μM Complegen inhibitor, CGX0486, for 30 minutes at 37°C. Following treatment, the cells were polarized into specific T helper cell subsets, each requiring their own respective polarizing agents:

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<tr>
<td>Th1</td>
<td>Anti-IL-4</td>
<td>10 μg/mL</td>
</tr>
<tr>
<td>IL-12</td>
<td></td>
<td>1 ng/mL</td>
</tr>
<tr>
<td>Th2</td>
<td>Anti-IFN-γ</td>
<td>10 μg/mL</td>
</tr>
<tr>
<td>IL-4</td>
<td></td>
<td>1 ng/mL</td>
</tr>
<tr>
<td>Th17</td>
<td>Anti-IL-4</td>
<td>10 μg/mL</td>
</tr>
<tr>
<td>Anti-IFN-γ</td>
<td></td>
<td>10 μg/mL</td>
</tr>
<tr>
<td>IL-6</td>
<td></td>
<td>20 ng/mL</td>
</tr>
<tr>
<td>TGF-β</td>
<td></td>
<td>5 ng/mL</td>
</tr>
<tr>
<td>iTreg</td>
<td>TGF-β</td>
<td>20 ng/mL</td>
</tr>
<tr>
<td></td>
<td>IL-2</td>
<td>1.35 ng/mL</td>
</tr>
</tbody>
</table>

Cells were stimulated by plating onto anti-CD3-plus anti-CD28-coated wells and incubated at 37°C. The cells were harvested at 24-, 48- and 72-hour timepoints. The supernatant was collected and stored for cytokine analysis by ELISA. The cells
were harvested and stained with Zombie Fixable Viability Dye (BioLegend) for 15 minutes at 2-8°C, then pelleted by centrifugation for 5 minutes. Cells were resuspended in 0.2% bovine serum albumin (BSA) in PBS and incubated for 30 minutes at 2-8°C. After this incubation, the cells were stained with antibodies for Notch1IC, pPKCθ, and their respective master transcription factor, T-Bet (Th1), GATA-3 (Th2), RORγt (Th17), or FOXP3 (iTreg). Flow Cytometry was used to analyze the expression of the proteins. Lastly, cytosolic vs nuclear PKCθ as well as cytosolic vs nuclear Notch1IC levels were assessed by imaging flow cytometry. One thousand cells were visualized and fluorescent intensities were quantified using an AMNIS ImageStream X Mark II Imaging Flow Cytometer at 60X magnification. To determine nuclear localization of desired proteins, the nuclear localization wizard was applied in the IDEAS software upon masking the nuclear area (Intensity mask: Ch05-DRAQ5 staining). We also determined the percent of cells showing nuclear or non-nuclear expression of PKCθ and Notch1 by applying the AMNIS nuclear masking wizard.
CHAPTER 4

RESULTS

Mouse CD4 T cells were isolated and differentiated \textit{in vitro} into Th1, Th2, Th17, or Treg subsets. The cells were treated with an inhibitor that prevented PKC\(\theta\) from interacting with LKC (Complegen) or with a PKC\(\theta\)-specific inhibitor, Rottlerin, that prevents PKC\(\theta\) phosphorylation on the Thr538 residue. The cells were analyzed 24, 48, and 72 hours after differentiating them in culture, and we measured percent positive and median fluorescent intensity of pPKC\(\theta\), NOTCH1 and each of the signature transcription factors, in each subset.

We first wanted to confirm that the CGX inhibitor reduced PKC\(\theta\) phosphorylation, then we asked what effect this had on different T cell subsets. In Th1 cells, we observed a decrease in the percent positive cells for phosphorylated PKC\(\theta\) (T538) when the cells were treated with 5\(\mu\)M Complegen (CGX) inhibitor (Figure 7), while PKC\(\theta\) phosphorylation was not affected following treatment with 3\(\mu\)M Rottlerin. In the CGX-treated cells we also observed a decrease in the median fluorescence intensity of phosphorylated PKC\(\theta\) (T538) 48 and 72 hours after stimulation (Figure 8). In Th1 cells, Rottlerin treatment decreased the percent positive NOTCH1 cells, as well as the median fluorescent intensity of NOTCH1 at all time points (Figure 9). CGX treatment had no effect on NOTCH1 positive cells or the median fluorescent intensity (Figure 10). T-Bet, the master transcription factor of Th1 cells, was not affected by CGX treatment. When T cells were treated with Rottlerin, there was a decrease in the percent of T-Bet positive
cells (Figure 11) and median fluorescence intensity at all time points. In Th1 cells, we observed a small decrease in nuclear NOTCH1 when the cells were treated with Rottlerin. However, when treated with the CGX inhibitors, we did not observe a change in NOTCH1 nuclear localization (Figure 12). We also observed a large decrease in the median fluorescent intensity of NOTCH1, both nuclear and non-nuclear, when the Th1 cells were treated with Rottlerin (Figure 13). In the Th1 cells, we observed a decrease in nuclear PKC\(\theta\) in CGX-treated cells, and a small decrease in the Rottlerin-treated cells (Figure 14). The median fluorescence intensity of non-nuclear PKC\(\theta\) in the Rottlerin-treated cells was slightly decreased, while the CGX treated cells showed an increase in nuclear PKC\(\theta\) and a decrease in non-nuclear PKC\(\theta\) (Figure 15). In Th1 cells, we observed colocalization of PKC\(\theta\) and NOTCH1 was not much affected by the inhibitor treatments (Figure 16). The signature cytokine of Th1 cells, IFN\(\gamma\), was decreased after treatment with either inhibitor at the 48- and 72-hour time points (Figure 17). Rottlerin treatment had a greater effect on the decrease in production of IFN\(\gamma\) than the CGX inhibitor. We can conclude from these data that both CGX and Rottlerin affect PKC\(\theta\) signaling in Th1 cells, but they appear to act by different mechanisms, since we did not see identical results with the two inhibitors. Interestingly, even though we didn’t see strong effects on PKC\(\theta\) phosphorylation in Rottlerin-treated Th1 cells, there was a significant decrease in T-bet expression and IFN\(\gamma\) expression, suggesting Rottlerin may have other actions beyond inhibiting PKC\(\theta\) phosphorylation.

We next compared the effects of CGX and Rottlerin on Th2 cell differentiation. In Th2 cells, treatment with Rottlerin produced an increase at 24
hours followed by a decrease at the 48- and 72-hour time points in percent of cells positive for phosphorylated PKCθ (T538). Treatment with the CGX inhibitor also produced a decrease in percent of cells positive for phosphorylated PKCθ (T538) 48 and 72 hours after stimulation; however, this decrease was much greater than in cells treated with Rottlerin (Figure 18). The same trend was seen in the median fluorescence intensity of pPKCθ, with an initial increase followed by a decrease in Rottlerin-treated Th2 cells. Again, a greater decrease was seen in the CGX-treated Th2 cells, at the 48- and 72-hour time points (Figure 19). In Th2 cells, the percent of NOTCH1 positive cells (Figure 20), as well as the median fluorescence intensity of NOTCH1 (Figure 21), showed a decrease at all time points in the Rottlerin-treated cells. When we measured the percent of positive cells expressing GATA-3, the master transcription factor of Th2 cells, we observed a decrease at all time points in Rottlerin-treated cells. CGX-treated cells showed a slight decrease in the percent of positive cells expressing GATA-3 by 24 hours after stimulation, but no effect on the percent of GATA-3 positive cells at the 48- and 72-hour time points (Figure 22). The same trend observed in the percent of positive cells was also seen in the GATA-3 median fluorescent intensity of both the Rottlerin-treated and CGX-treated cells (Figure 23). For IL-4, the signature cytokine of Th2 cells, Rottlerin treatment had little effect on IL-4 levels. The CGX inhibitor, however, decreased IL-4 production at 24 hours but showed an increase by 72 hours (Figure 24). These data suggest that Th1 and Th2 cells differ in how sensitive they are to the effects of CGX and Rottlerin. Like Th1 cells, CGX treatment showed a greater reduction on PKCθ phosphorylation,
but unlike the very strong inhibiting effects Rottlerin had on T-bet, it had much less effect on GATA-3 expression.

We also were interested in understanding the effects of CGX and Rottlerin on Th17 cells. Th17 cells showed a decrease in the percent of phosphorylated PKCθ (T538) positive cells in cells treated with Rottlerin, and a greater decrease in percent of phosphorylated PKCθ (T538) positive cells was observed with CGX treatment, 24 and 48 hours after stimulation. By 72 hours, neither inhibitor showed any effect on the percent of phosphorylated PKCθ positive cells (Figure 25). When we measured the median fluorescent intensity of phosphorylated PKCθ (T538), we observed decreased expression of phosphorylated PKCθ throughout all time points for both inhibitors (Figure 26). In CGX-treated cells, we observed decreased percent NOTCH1 positive cells and median fluorescent intensity 24 and 48 hours after stimulation. In Rottlerin-treated Th17 cells, the percent of NOTCH1 positive cells was reduced only at 24 hours, while the CGX inhibitor had no effect on the percent of NOTCH1 positive Th17 cells at any time point (Figure 27). The median fluorescence intensity for NOTCH1 in CGX-treated cells decreased by 48 hours after stimulation. Rottlerin-treated cells showed decreased median fluorescent intensity of NOTCH1 at the 48- and 72-hour time points (Figure 28). When we examined the percent positive cells expressing RORγt, the master transcription factor of Th17 cells, neither Rottlerin nor the CGX inhibitor had any effect (Figure 29). However, when we measured the median fluorescence intensity of RORγt, we observed that both inhibitors produced increased RORγt expression 48 hours after stimulation, with no change at the 24- and 72-hour time points (Figure 30). In the treated TH17
cells, we did not observe significant changes in the percent of cells expressing nuclear NOTCH1 (Figure 31); however, we did observe significantly decreased median fluorescence intensity of nuclear NOTCH1, when Th17 cells were treated either with Rottlerin or CGX (Figure 32). In Th17 cells, we did not observe significant effects on percent of cells expressing nuclear PKC\(\theta\) (Figure 33) following treatment with either inhibitor but we did observe a slight decrease in the median fluorescent intensity of the inhibitor-treated cells, with the CGX inhibitor having a greater impact (Figure 34). In Th17 cells, unlike in Th1 or in Th2 cells, Rottlerin and CGX appeared to have very similar effects on PKC\(\theta\) phosphorylation, and Notch1 and ROR\(\gamma\)\(t\) expression.

Finally, we investigated the effects of CGX and Rottlerin on regulatory T cells. In Tregs, treatment with the CGX inhibitor caused a decrease in the percent of cells positive for phosphorylated PKC\(\theta\) (T538) 72 hours after stimulation. This inhibitor had no effect at the 24- and 48-hour time points of pPKC\(\theta\) (T538). Treatment with Rottlerin showed the opposite, with a decrease in percent of phosphorylated PKC\(\theta\) (T538) positive cells at the 24- and 48-hour time points. Rottlerin showed no effect on percent of pPKC\(\theta\) (T538) positive cells at 72 hours (Figure 35). The same trend, for both inhibitors, was observed when we measured the median fluorescence intensity of phosphorylated PKC\(\theta\) (T538) (Figure 36). For Treg cells, neither inhibitor had an effect on the percent of NOTCH1 positive cells, (Figure 37), but both inhibitors resulted in decreased median fluorescence intensity for NOTCH1 at 24 hours (Figure 38). There was no change in percent of FOXP3 positive cells after treatment with either inhibitor (Figure 39). However, Rottlerin treatment
decreased the median fluorescence intensity of FOXP3 24 hours after stimulation and increased it by 72 hours. After treatment with CGX, the median fluorescent intensity of FOXP3 decreased at the 24- and 48-hour time points. By the 72-hour time point, we observed a small increase in the FOXP3 levels (Figure 40). We observed the same trend in the percent of nuclear NOTCH1 positive cells, that we did with the percent of nuclear PKC\(\theta\) positive Treg cells. In Treg cells, we observed decreased percent of nuclear-expressing NOTCH1 cells following Rottlerin treatment, but we did not observe any change in cells treated with CGX (Figure 41). In the Rottlerin-treated cells, we also observed a large decrease in the median fluorescent intensity of nuclear and non-nuclear NOTCH1. The CGX-treated cells had a smaller decrease in NOTCH1 nuclear median fluorescent intensity while there was no change in non-nuclear NOTCH1 expression (Figure 42). We observed a slight decrease in the percent of nuclear PKC\(\theta\)-expressing cells following Rottlerin treatment (Figure 43), together with a significant decrease in the median fluorescent intensity both of nuclear and non-nuclear PKC\(\theta\) (Figure 44). In CGX-treated cells, we observed no change in the percent of nuclear PKC\(\theta\) positive cells, but we did observe decreased median fluorescent intensity of nuclear PKC\(\theta\). We observed that, in Treg cells, while many of the cells stain double-positive for NOTCH1 and PKC\(\theta\), only a small percentage of these cells show nuclear colocalization of these two proteins. When Tregs were treated with Rottlerin, we observed decreased percentages of double-positive cells, but no significant changes in the nuclear colocalization of NOTCH1 and PKC\(\theta\). In CGX-treated Treg cells, we observed slightly decreased nuclear colocalization of these two proteins (Figure
The most striking difference we observed between Tregs, and Th1 cells was the effect of CGX on PKC\(\theta\) phosphorylation. In Th1 cells, CGX-treatment significantly reduced PKC\(\theta\) phosphorylation, while in Tregs, CGX-treatment did not seem to affect PKC\(\theta\) phosphorylation. However, both Rottlerin and CGX reduced nuclear co-localization of Notch1 and PKCq in Tregs. Whether or not this observation correlates with differences in Treg suppression will need to be investigated further.
CHAPTER 5

DISCUSSION

To further understand the influence of PKCθ on Notch1 cleavage and nuclear translocation, we inhibited pPKCθ function to evaluate its regulation of Notch1 in differentiated T cells. Additionally, to explore the influence of the phosphorylation of PKCθ on the ability of T cells to differentiate into specific T helper subsets, we inhibited pPKCθ function to determine its role in T cell differentiation.

Our data suggests that the Complegen inhibitor, CGX, works to prevent the phosphorylation of PKCθ (T538) in Th1, Th2 and Th17 cells, particularly by 72 hours following stimulation. Therefore, we can conclude that by preventing the LCK association with PKCθ, we are preventing PKCθ localization to the Immunological Synapse and ultimately inhibiting the phosphorylation of PKCθ (T538).

From our data, we can also conclude that treating Th cells with the PKCθ inhibitor, Rottlerin, we are causing decreased Notch1 expression in Th1, Th2, and Th17 cells. This suggests that the PKCθ signaling is indeed essential to Notch1 cleavage and translocation. Additionally, in cells treated with Rottlerin we saw decreased levels of nuclear Notch1 in Th1, Th17 and Treg cells. This suggests that treatment with Rottlerin prevents nuclear translocation of Notch1 in Th1, Th17, and Treg cells.

Finally, in Rottlerin-treated cells, we see decreased levels of T-bet in Th1 cells and GATA-3 in Th2 cells, the signature transcription factors in both cell subsets. These data suggest that Rottlerin reduces the ability of naïve T cells to polarize into Th1 or Th2 cells, while in their optimal polarizing conditions.
Altogether, we have demonstrated that the phosphorylation of PKCθ at T538 is essential to 1) Notch1 cleavage and nuclear translocation and 2) differentiation into specific T cell subsets. Further investigation into the PKCθ-Notch1 relationship will advance our understanding of how PKCθ and Notch1 function in the same signaling pathway and may provide insight into autoimmune diseases and lay the foundation for novel therapies.
Figure 7: Percent of pPKC\(\theta\) (T538) positive Th1 cells. Complegen inhibitor treatment decreased the percent of pPKC\(\theta\) (T538) positive Th1 cells at 48 and 72 hours after stimulation. Rottlerin treatment had little to no effect.

Figure 8: MFI of pPKC\(\theta\) (T538) in Th1 cells. Complegen inhibitor treatment decreased the median fluorescent intensity of pPKC\(\theta\) (T538) in Th1 cells, especially 48 and 72 hours after stimulation. Rottlerin treatment had no effect.
Figure 9: Percent of Notch1 positive Th1 cells. Rottlerin treatment decreased the percent of Notch1 positive Th1 cells at all time points. The Complegen inhibitor treatment showed no effect.

Figure 10: MFI of Notch1 in Th1 cells. Rottlerin treatment decreased the median fluorescent intensity of Notch1 at all time points in Th1 cells. Complegen inhibitor treatment showed no effects.
Figure 11: Percent of T-Bet positive cells. Percent of T-Bet positive Th1 cells decreased slightly after Rottlerin treatment at 48 and 72 hours after stimulation. Complegen inhibitor treatment had no effect on percent of T-Bet positive cells.

Figure 12: MFI of T-Bet positive cells. Median fluorescent intensity of T-Bet in Th1 cells is decreased at all time points following Rottlerin treatment, while Complegen inhibitor treatment had no effect.
Figure 13: Percent of Nuclear Notch1 positive Th1 cells. Nuclear Notch1 in T helper 1 cells is decreased by the Rottlerin while the Complegen inhibitor had no effect.

Figure 14: MFI of Nuclear and Non-nuclear Notch1 in Th1 cells. The median fluorescent intensity in T helper 1 cells is decreased in both nuclear and non-nuclear Notch1 by Rottlerin. Nuclear Notch1 is decreased by CGX treatment.
Figure 15: Percent of Nuclear pPKCθ (T538) positive Th1 cells. In T helper 1 cells, a small decrease is seen in nuclear pPKCθ in Rottlerin treated cells with a larger decrease in nuclear pPKCθ in CGX treated cells.

Figure 16: MFI of Nuclear and Non-nuclear pPKCθ in Th1 cells. The median fluorescent intensity of nuclear pPKCθ in T helper 1 cells is increased by CGX treatment.
Figure 17: Percent of Nuclear Notch1 and pPKCθ-Colocalizing Th1 cells. No change in colocalization of pPKCθ and NOTCH1 in T helper 1 cells from either inhibitor.

Figure 18: IFNγ produced by Th1 cells. Rottlerin-treated cells showed decreased IFNγ production by Th1 cells 48 and 72 hours after stimulation. Complegen inhibitor-treatment resulted in slightly decreased IFNγ production 72 hours after stimulation.
Figure 19: Percent of pPKCθ (T538) positive Th2 cells. Both Complegen inhibitor treatment and Rottlerin treatment decreased the percent of pPKCθ (T538) positive Th2 cells, 48 and 72 hours after stimulation. Complegen inhibitor treatment resulted in a larger decrease in percent of pPKCθ (T538) positive cells 72 hours after stimulation.

Figure 20: MFI of pPKCθ (T538) in Th2 cells. Complegen inhibitor treatment decreased the median fluorescent intensity of pPKCθ (T538) in Th2 cells, 48 and 72 hours after stimulation. Rottlerin treatment resulted in increased pPKCθ (T538) expression 24 and 48 hours after stimulation.
Figure 21: Percent of NOTCH1 positive Th2 cells. Rottlerin treatment decreased the percent of Notch1 positive Th2 cells at all time points. Complegen inhibitor treatment resulted in decreased percent of Notch1 positive cells 24 and 48 after stimulation, but showed no effect by 72 hours.

Figure 22: MFI of Notch1 in Th2 cells. Rottlerin treatment decreased the median fluorescent intensity of Notch1 at all time points in Th2 cells. Complegen inhibitor treatment resulted in a slight decrease in the median fluorescent intensity of Notch1 24 and 48 hours after stimulation.
Figure 23: Percent of GATA-3 positive cells. Rottlerin treatment reduced the percent of GATA-3 positive Th2 cells at all time points. Complegen inhibitor treatment slightly reduced the percent of T-Bet positive cells 24 hours after stimulation, but not at later time points.

Figure 24: MFI of GATA-3 positive cells. Rottlerin treatment reduced the median fluorescent intensity of GATA-3 in Th2 cells at all time points. Complegen inhibitor treatment resulted in a small decrease in the median fluorescent intensity of GATA-3 at 24 and 48 hours after stimulation.
Figure 25: IL-4 levels in Th2 cells. Rottlerin treatment had little effect on IL-4 production by Th2 cells. Complegen inhibitor treatment decreased IL-4 production 24 hours after stimulation, but increased IL-4 production by 72 hours.

Figure 26: Percent of pPKCθ (T538) positive Th17 cells. Rottlerin treatment and Complegen inhibitor treatment, both, decreased the percent of pPKCθ (T538) positive Th17 cells at 24 and 48 hours after stimulation, with slightly greater reduction observed after Complegen inhibitor treatment. By 72 hours after stimulation, neither inhibitor showed a significant effect on percent of pPKCθ (T538) positive Th17 cells.
Figure 27: MFI of pPKCθ (T538) in Th17 cells. Rottlerin treatment and Complegen inhibitor treatment, both, decreased in the median fluorescent intensity of pPKCθ (T538) in Th17 cells, at all time points.

Figure 28: Percent of NOTCH1 positive Th17 cells. Rottlerin treatment reduced the percent of NOTCH1 positive Th17 cells, 24 hours after stimulation, but had no effect 48 or 72 hours after stimulation. Complegen inhibitor treatment followed the same trend, decreasing the percent NOTCH1 positive Th17 cells by 24 hours, but showing no effect 48 and 72 hours after stimulation.
Figure 29: MFI of NOTCH1 in Th17 cells. Rottlerin treatment decreased the median fluorescent intensity of NOTCH1 48 and 72 hours after stimulation. Complegen inhibitor treatment decreased NOTCH1 median fluorescent intensity 48 hours after stimulation, but no differences were observed at the 24- and 72-hour timepoints.

Figure 30: Percent of RORγt positive cells. Percent of RORγt positive Th17 cells was not affected by either Rottlerin treatment or Complegen inhibitor treatment.
**Figure 31: MFI of RORγt positive cells.** Rottlerin treatment and Complegen inhibitor treatment, both, increased the median fluorescent intensity of RORγt in Th17 cells 48 hours after stimulation. No significant differences were observed at 24- and 72-hour time points, following treatment with either inhibitor.

**Figure 32: Percent of Nuclear Notch1 positive Th17 cells.** Nuclear Notch1 in T helper 17 cells was slightly decreased by Rottlerin inhibitor while the CGX inhibitor had no effect.
Figure 33: MFI of Nuclear and Non-nuclear Notch1 in Th17 cells. The median fluorescent intensity of nuclear Notch1 in T helper 17 cells was decreased by Rottlerin and further decreased by CGX treatment.

Figure 34: Percent of Nuclear pPKCθ (T538) positive Th17 cells. The nuclear pPKCθ in T helper 17 cells is slightly decreased by Rottlerin treatment while the CGX treatment had no effect.
Figure 35: MFI of Nuclear and Non-nuclear pPKCθ in Th17 cells. The median fluorescent intensity of nuclear pPKCθ in T helper 17 cells is decreased by Rottlerin treatment and further decreased by CGX treatment.

Figure 36: Percent of pPKCθ (T538) positive Treg cells. Rottlerin treatment decreased the percent of pPKCθ (T538) positive Tregs, 24 and 48 hours after stimulation but showed no effect by 72 hours. Complegen inhibitor treatment decreased the percent of pPKCθ (T538) in Tregs, 72 hours after stimulation, but had no effect 24 and 48 hours after stimulation.
Figure 37: MFI of pPKCθ in Treg cells. Rottlerin treatment decreased the median fluorescent intensity of pPKCθ (T538) in Tregs, 24 and 48 hours after stimulation but showed no effect at the 72-hour time point. Complegen inhibitor treatment decreased the median fluorescent intensity of pPKCθ (T538) in Tregs 72 hours after stimulation but showed no effect at 24- and 48-hour time points.

Figure 38: Percent of Notch1 positive Treg cells. Neither Rottlerin treatment nor Complegen inhibitor treatment affected the percent of Notch1 positive Treg cells.
Figure 39: MFI of Notch1 in Treg cells. Rottlerin treatment decreased the median fluorescent intensity of Notch1 in Treg cells after 24 hours but did not show effects 48 and 72 hours after stimulation. Complegen inhibitor treatment decreased the median fluorescent intensity of Notch1 by 24 hours, with no effect observed at 48- and 72-hour timepoints.

Figure 40: Percent of FOXP3 positive Treg cells. Neither Rottlerin treatment nor Complegen inhibitor treatment affected the percent of FOXP3 positive Tregs.
Figure 41: MFI of FOXP3 positive Treg cells. Rottlerin treatment decreased the median fluorescent intensity of FOXP3 in Tregs by 24 hours after stimulation, but significantly increased its expression by 72 hours after stimulation. Complegen inhibitor treatment decreased the median fluorescent intensity of FOXP3 24 and 48 hours after stimulation, but resulted in a slight increase at the 72 hour time point.

Figure 42: Percent of Nuclear Notch1 positive Treg cells. Nuclear Notch1 in regulatory T cells is decreased by Rottlerin treatment.
**Figure 43: MFI of Nuclear and Non-nuclear Notch1 in Treg cells.** The median fluorescent intensity of nuclear Notch1 in regualtory T cells is decreased by CGX treatent and further decreased by Rottlerin treatment.

**Figure 44: Percent of Nuclear pPKCθ in Treg cells.** Nuclear pPKCθ in regulatory T cells is slightly decreased by Rottlerin treatment.
Figure 45: MFI of Nuclear and Non-nuclear pPKCθ in Treg cells. The median fluorescent intensity of nuclear pPKCθ in regulatory T cells is decreased by CGX treatment. Nuclear and non-nuclear pPKCθ is further decreased by Rottlerin treatment.

Figure 46: Percent of Nuclear Notch1- and pPKCθ-Colocalizing Treg cells. The nuclear colocalization of pPKCθ and Notch1 in regulatory T cells is decreased by CGX treatment.


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