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University of Massachusetts Amherst

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DEFINING AND MANIPULATING THE FUNCTION OF PROTEIN KINASE C-THETA IN GRAFT VERSUS HOST RESPONSES

A Dissertation Presented

by

EMRAH ILKER OZAY

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

DOCTOR OF PHILOSOPHY

May 2018

Program in Molecular and Cellular Biology
DEFINING AND MANIPULATING THE FUNCTION OF PROTEIN KINASE C-THETA IN GRAFT VERSUS HOST RESPONSES

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Approved as to style and content by:

__________________________________________________________
Lisa M. Minter, Co-Chair

__________________________________________________________
Gregory N. Tew, Co-Chair

__________________________________________________________
Barbara A. Osborne, Member

__________________________________________________________
Wilmore C. Webley, Member

__________________________________________________________
Scott C. Garman, Director

Program in Molecular and Cellular Biology
Firstly, I would like to emphasize that my thesis has completely come together through the great support of colleagues, friends, and family. Words cannot express how grateful I am to them. I would like to express my special appreciation and thanks to my advisors, Dr. Lisa M. Minter and Dr. Gregory N. Tew, for encouraging my research and for allowing me to grow as a research scientist. They have been tremendous mentors for me. I feel that I am very lucky to be your student and you made me realize and value all the great achievements I have made during my study. I cannot be more thankful and grateful for their mentorship, strong guidance, and great encouragement. I would also like to thank my committee members, Dr. Barbara A. Osborne, for everything she has provided for my dissertation. She is truly an amazing and unforgettable mentor who I really feel I am so lucky to have her in my committee. Her endless support, guidance, and scientific inputs took me where I am right now. Dr. Wilmore Webley has provided me with very valuable advice and I would like to thank for his comments and advice. I would also like to thank a lot to Dr. Richard Goldsby who made me a very strong scientist by providing me precious support, encouragement, and brilliant scientific discussions. I am sending my great thanks to my wonderful lab colleagues who have been supporting and making this time enjoyable. I felt like home in the lab since they supported me in a friendly environment. I would like to send my special thanks to Gabriela Gonzalez Perez, Federica Sgolastra, Christina Arieta Kuksin, Brittany deRonde for training me so well, Rebecca Lawlor, Joe Torres, Karthik Chandiran, Jyothi Vijayaraghavan, Sudarvili Shantalingam for their guidance and Heather Sherman, Daniel Garrigan Jr., Mine Canakci, Ankita Mitra for their precious support on my experiments. I was very lucky to
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ABSTRACT

DEFINING AND MANIPULATING THE FUNCTION OF PROTEIN KINASE C-THETA IN GRAFT VERSUS HOST RESPONSES

MAY 2018

EMRAH ILKER OZAY, B.S., ISTANBUL TECHNICAL UNIVERSITY
Ph.D., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by: Lisa M. Minter and Gregory N. Tew

Immune-mediated tissue destruction of graft-vs-host disease (GvHD) remains a major barrier to greater use of bone marrow transplantation (BMT). It is found that alloreactive donor-derived T cells activated through their T cell receptor (TCR) are primarily the major contributors to the immunopathobiology of GvHD. Protein kinase C-theta (PKC\(\theta\)), a crucial, early downstream kinase of TCR signaling, enhances T cell activation, thereby promoting alloreactive responses such as differentiation, proliferation, migration, and cytotoxicity. Thus, delineating specific ways of interfering PKC\(\theta\) signaling is beneficial for the GvHD treatment or prevention.

Here, we investigated the molecular mechanisms driven by PKC\(\theta\) in T cells by establishing preclinical all-murine and humanized GvHD mouse models as well as in vitro primary cell cultures. We found that both CD4\(^+\) and CD8\(^+\) T cells expressing high levels of activated PKC\(\theta\) contributed to the development of GvHD. Genetic deletion or chemical inhibition of PKC\(\theta\) prevented the GvHD induction via preventing expression of proinflammatory cytokines. In addition, we established an effective strategy for intracellular antibody delivery against activated PKC\(\theta\) by cell-penetrating peptide mimics (CPPMs). CPPM: Antibody (cell-penetrating antibody) complexes were readily
introduced with high efficacy into hard-to-transfect T cells, dampening PKCθ-specific downstream response to delay GvHD progression. In the context of cell-based therapy, we demonstrated that targeting PKCθ via a cell-penetrating antibody in induced regulatory T cells (iTregs) generated a novel, highly stable, super-suppressive iTregs by reprogramming their post-transcriptional organization and epigenetic signatures. These reprogrammed iTregs augmented their suppressive activity both in vitro and in vivo, hence, providing a promising cell-based therapy in preventing GvHD. In addition to super-suppressive iTregs, we utilized induced pluripotent stem cell (iPSC)-derived mesenchymal stem cells (MSCs) to prevent GvHD via manipulating PKCθ signaling in alloreactive T cells. iPSC-MSCs also attenuated GvHD severity and prolonged survival in the humanized model. Altogether, our findings demonstrate the importance of manipulating PKCθ function in preventing or treating GvHD in the context of BMT therapy for immunological diseases.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>iv</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xiv</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xv</td>
</tr>
<tr>
<td>CHAPTER</td>
<td></td>
</tr>
<tr>
<td>1. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>1.1 Immunobiology of graft-versus-host disease (GvHD)</td>
<td>1</td>
</tr>
<tr>
<td>1.1.1 Pathophysiology of GvHD</td>
<td>2</td>
</tr>
<tr>
<td>1.1.2 Alloreactive T cells</td>
<td>3</td>
</tr>
<tr>
<td>1.1.3 Treatment of GvHD</td>
<td>4</td>
</tr>
<tr>
<td>1.2 Protein kinase C-theta (PKCθ) in T cell signaling</td>
<td>6</td>
</tr>
<tr>
<td>1.2.1 Structural analysis of PKCθ</td>
<td>7</td>
</tr>
<tr>
<td>1.2.2 Role of PKCθ in CD4+ and CD8+ T cell responses</td>
<td>8</td>
</tr>
<tr>
<td>1.2.3 PKCθ in various immune pathologies</td>
<td>9</td>
</tr>
<tr>
<td>1.2.4 PKCθ as a drug target in clinic</td>
<td>10</td>
</tr>
<tr>
<td>1.3 Immune system-targeted therapeutics</td>
<td>11</td>
</tr>
<tr>
<td>1.3.1 Small molecule immunotherapeutics</td>
<td>12</td>
</tr>
<tr>
<td>1.3.2 Therapeutic antibodies</td>
<td>13</td>
</tr>
<tr>
<td>1.3.3 Cell-based therapeutics</td>
<td>14</td>
</tr>
<tr>
<td>1.3.3.1 Regulatory T cell (Treg) therapy</td>
<td>15</td>
</tr>
<tr>
<td>1.3.3.2 Mesenchymal stem cell (MSC) therapy</td>
<td>16</td>
</tr>
<tr>
<td>1.4 Biologics delivery via next generation nanocarriers for T cell engineering</td>
<td>18</td>
</tr>
<tr>
<td>1.4.1 Challenges in intracellular delivery of biologics</td>
<td>19</td>
</tr>
<tr>
<td>1.4.2 Cell-penetrating peptides (CPPs)</td>
<td>20</td>
</tr>
<tr>
<td>1.4.3 Novel cell-penetrating peptide mimics (CPPMs)</td>
<td>21</td>
</tr>
<tr>
<td>1.5 Significance and hypothesis</td>
<td>23</td>
</tr>
<tr>
<td>2. PKCθ IMPACTS ALTERNATIVE <em>IFNG</em> mRNA REGULATION BY NOTCH1-ASSOCIATED HNRNPU AND PCMT1 IN CD8+ T CELLS TO INDUCE APLASTIC ANEMIA</td>
<td>36</td>
</tr>
<tr>
<td>2.1 Introduction</td>
<td>36</td>
</tr>
</tbody>
</table>
2.2 Materials and Methods .......................................................................................... 39
  2.2.1 Animals ........................................................................................................ 39
  2.2.2 Antibodies .................................................................................................. 39
  2.2.3 BMF induction and analyses ..................................................................... 40
  2.2.4 Rottlerin administration ........................................................................... 41
  2.2.5 Histology .................................................................................................... 41
  2.2.6 T cell isolation and in vitro assays ......................................................... 41
  2.2.7 Patient samples and healthy controls ..................................................... 42
  2.2.8 Surface and intracellular staining for flow cytometry ....................... 42
  2.2.9 Validating phosphorylated PKCθ using flow cytometry .................. 43
  2.2.10 Immunoblotting of whole cell, nuclear, and cytoplasmic extracts upon rottlerin and LiCl treatments .................................................. 43
  2.2.11 AMNIS imaging flow cytometry ............................................................. 44
  2.2.12 Immunoprecipitation and western blot ............................................... 45
  2.2.13 RNA immunoprecipitation .................................................................. 45
  2.2.14 Quantitative real-time PCR (qPCR) ....................................................... 46
  2.2.15 cDNA synthesis and reverse transcription PCR (RT-PCR) ............. 47
  2.2.16 Enzyme-linked immunosorbent assay (ELISA) .................................. 48
  2.2.17 Cytometric bead array ........................................................................... 48
  2.2.18 Statistics .................................................................................................. 48

2.3 Results ................................................................................................................. 49
  2.3.1 T cells from AA mice and human express elevated pPKCθ ................. 49
  2.3.2 PKCθ regulates NOTCH1(C and IFN-γ expression .......................... 50
  2.3.3 PKCθ in CD8+ T cells is necessary to induce BMF in AA mice .......... 51
  2.3.4 Rottlerin treatment inhibits PKCθ signaling in vivo and attenuates AA in mice .......................................................... 53
  2.3.5 PKCθ and NOTCH1 interact in CD8+ T cells ...................................... 55
  2.3.6 PKCθ and GSK3β activity counteract on NOTCH1 regulation ....... 57
  2.3.7 PKCθ regulates NOTCH1-associated RNA binding proteins in CD8+ T cells ........................................................................ 58
  2.3.8 PKCθ regulates ifng mRNA processing through NOTCH1-associated PCMT1 and hnRNP interactions ........................................... 61

2.4 Discussion ............................................................................................................. 65

3. INTRACELLULAR DELIVERY OF ANTI-pPKCθ (Thr538) VIA PROTEIN TRANSDUCTION DOMAIN MIMICS FOR IMMUNOMODULATION ............................................. 86

3.1 Introduction .......................................................................................................... 86

3.2 Materials and Methods ....................................................................................... 88
  3.2.1 Materials ..................................................................................................... 88
  3.2.2 Synthesis of MePh13-b-dG5 (P13D3) ...................................................... 89
  3.2.3 Characterization of MePh13-b-dG5 (P13D3) ....................................... 89
4. TARGETING PKCθ VIA CELL-PENETRATING ANTIBODY GENERATES SUPER-SUPPRESSIVE FOXP3hi PD-1hi IFNγhi iTREGS PREVENTING GRAFT-VERSUS-HOST DISEASE .................................................. 122

4.1 Introduction ........................................................................ 122

4.2 Materials and Methods .......................................................... 125
4.2.1 Animals ......................................................................... 125
4.2.2 Antibodies and Reagents .................................................. 126
4.2.3 Human iTreg differentiation coupled with intracellular P13Ds: αpPKCθ delivery ......................................................... 127
4.2.4 Immunoblotting ............................................................... 127
4.2.5 Protein subcellular localization via AMNIS imaging flow cytometry .................................................................. 128

3.2.4 Dynamic Light Scattering (DLS) ............................................. 89
3.2.5 Native Polyacrylamide Gel Electrophoresis and Silver Staining .... 90
3.2.6 P13Ds: Anti-pPKCθ Complex Delivery into Human Peripheral Mononuclear Blood Cells (hPBMCs) ............................... 90
3.2.7 Cellular Viability Assay ...................................................... 91
3.2.8 Flow Cytometric Analyses of Marker Expression ...................... 91
3.2.9 Cell Proliferation Assay ..................................................... 92
3.2.10 Enzyme Linked Immunosorbert Assay (ELISA) for Cytokine Determination ................................................... 92
3.2.11 Nuclear vs. Cytosolic Protein Extraction ................................ 93
3.2.12 Immunoblotting .......................................................... 93
3.2.13 In vitro Human Th1 Cell Differentiation Assay ......................... 94
3.2.14 Ex vivo Delivery of P13Ds: Anti-pPKCθ Complex into hPBMCs Subsequently Transferred into NSG Mice ......................... 94
3.2.15 Assessment of GvHD ................................................................ 95
3.2.16 Cytometric Bead Array ................................................... 96

3.3 Results ................................................................................. 96
3.3.1 PTDM design and characterization ...................................... 96
3.3.2 Anti-pPKCθ (Thr538) delivery into hPBMCs ......................... 97
3.3.3 P13Ds: Anti-pPKCθ delivery into ‘unstimulated’ hPBMCs greatly reduces their activation potential ........................................ 98
3.3.4 P13Ds: Anti-pPKCθ delivery into ‘activated’ hPBMCs diminishes expression of downstream activation markers .......... 99
3.3.5 P13Ds: Anti-pPKCθ delivery alters the activity and localization of PKCθ in hPBMCs ..................................................... 101
3.3.6 Ex vivo delivery of P13Ds: Anti-pPKCθ into hPBMCs provides a survival benefit in a lymphocyte transfer, humanized mouse model of graft-versus-host disease (GvHD) .......................... 102

3.4 Discussion ............................................................................. 104
5. PKC0 MODULATES PCMT1 TO SWITCH RNA PROCESSING AND FOXP3 STABILITY IN REGULATORY T CELLS .......................................................... 170

5.1 Introduction .................................................................................. 170

5.2 Materials and Methods ................................................................. 174
  5.2.1 Animals ............................................................................... 174
  5.2.2 Antibodies and Reagents ....................................................... 175
  5.2.3 Human iTreg differentiation upon intracellular
        $P_{13}D_{5}:\alpha$PKC0 or $P_{13}D_{5}:\alpha$PCMT1 delivery .................. 176
  5.2.4 Immunoblotting ................................................................... 176
  5.2.5 In vivo RNA analysis of iTregs in humanized GvHD model .... 177
  5.2.6 Quantitative real time PCR (qPCR) ...................................... 178
  5.2.7 Reverse Transcriptase PCR (RT-PCR) for splicing and 3’UTR
        analyses ............................................................................... 179
  5.2.8 Lambda phosphatase treatment ........................................... 179
  5.2.9 hnRNPL immunoprecipitation ............................................. 180
  5.2.10 RNA immunoprecipitation ................................................ 180
  5.2.11 ChIP-qPCR ...................................................................... 181
  5.2.12 Bioinformatics for RNA-binding protein motifs ............... 182
5.2.13 Bisulfite Sequencing ................................................................. 182

5.3 Results ......................................................................................... 183
5.3.1 Modulation of splicing regulatory proteins and RNA processing upon αpPKC0 delivery in iTregs in vitro ........................................ 183
5.3.2 Tissue-, cell-, and gene-specific modulation of RNA processing in ex vivo-treated iTregs by αpPKC0 delivery .................................. 185
5.3.3 Post-translational and post-transcriptional regulation of PCMT1 in iTregs .................................................................................. 187
5.3.4 Effects of αPCMT1 delivery in iTregs ........................................... 189
5.3.5 PCMT1 as an iTreg instability marker ......................................... 193

5.4 Discussion ..................................................................................... 194

6. CYMERUS™ iPSC-MSCs SIGNIFICANTLY PROLONG SURVIVAL IN A PRECLINICAL, HUMANIZED MOUSE MODEL OF GRAFT-VERSUS-HOST DISEASE ......................................................................................... 213

6.1 Introduction ................................................................................... 213

6.2 Materials and Methods ................................................................. 216
6.2.1 Animals ..................................................................................... 216
6.2.2 Antibodies ................................................................................ 216
6.2.3 iPSC-MSC licensing and immunophenotyping ......................... 217
6.2.4 In vitro differentiation of iPSC-MSCs ......................................... 218
6.2.5 Determining post-thaw senescence ........................................... 219
6.2.6 In vitro immunopotency assay .................................................. 219
6.2.7 Assessing IDO and PD-L1 expression by IFNγ-licensed iPSC MSCs ........................................................ .................................................. 220
6.2.8 Graft-vs-Host disease induction ............................................... 221
6.2.9 iPSC-MSC administration ....................................................... 221
6.2.10 GVHD clinical scoring ............................................................ 221
6.2.11 Biomarker analysis ................................................................. 222
6.2.12 LEGENDplex™ Bead-based immunoassay ............................... 222
6.2.13 Protein subcellular localization ............................................... 223
6.2.14 Statistical analyses ................................................................. 223

6.3 Results .......................................................................................... 224
6.3.1 iPSC-derived MSCs phenotypically resemble native MSCs, respond to IFNγ licensing, and dampen PBMC activation potential ........................................................ ........................................... 224
6.3.2 iPSC-MSC administration, in vivo, reduces cytokine production and weight loss in mice with GvHD ........................................ 227
6.3.3 iPSC-MSC administration reduces BM-infiltration and expression of proinflammatory molecules in mice with GvHD ...... 228
6.3.4 iPSC-MSCs attenuate GvHD severity and prolong survival in mice..............................................................................................................230

6.3.5 iPSC-MSC treatment alters subcellular localization of pPKCθ in BM-infiltrating T cells........................................................................................................231

6.3.6 Pro-inflammatory molecules expressed by circulating PBMCs correlate with therapeutic response to iPSC-MSC administration.................................................................................................232

6.4 Discussion.........................................................................................................................233

7. CONCLUSIONS AND FUTURE DIRECTIONS................................................................253

BIBLIOGRAPHY ..................................................................................................................260
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Tables</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1 List of qPCR primers used in this study</td>
<td>169</td>
</tr>
<tr>
<td>5.1 List of splicing primers</td>
<td>212</td>
</tr>
<tr>
<td>5.2 List of 3’UTR primers</td>
<td>212</td>
</tr>
<tr>
<td>6.1 Immunopotency values of iPSC-MSCs</td>
<td>252</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figures</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1 A timeline illustrating key discoveries in animal models of acute GvHD</td>
<td>26</td>
</tr>
<tr>
<td>1.2 Immunopathogenesis of acute GvHD upon BMT</td>
<td>27</td>
</tr>
<tr>
<td>1.3 Structural details of PKCθ</td>
<td>28</td>
</tr>
<tr>
<td>1.4 Several FDA-approved therapeutic antibodies</td>
<td>29</td>
</tr>
<tr>
<td>1.5 CAR-T cell therapy</td>
<td>30</td>
</tr>
<tr>
<td>1.6 Three classes of regulatory T cells</td>
<td>31</td>
</tr>
<tr>
<td>1.7 Applications of Treg-based therapies</td>
<td>32</td>
</tr>
<tr>
<td>1.8 Treg markers and their relevance to immunotherapy</td>
<td>33</td>
</tr>
<tr>
<td>1.9 Clinical applications of nanotherapeutic agents in autoimmune diseases</td>
<td>34</td>
</tr>
<tr>
<td>1.10 Development of oxanorbornene-based block-type CPPMs</td>
<td>35</td>
</tr>
<tr>
<td>2.1 Validating a flow cytometric assay for measuring pPKCθ in murine and human T cells</td>
<td>72</td>
</tr>
<tr>
<td>2.2 T cells from AA mice and human express elevated pPKCθ</td>
<td>73</td>
</tr>
<tr>
<td>2.3 PKCθ regulates NOTCH1IC and IFN-γ expression</td>
<td>74</td>
</tr>
<tr>
<td>2.4 PKCθ in CD8+ T cells is necessary to induce BMF in AA mice.</td>
<td>75</td>
</tr>
<tr>
<td>2.5 White and red blood cell counts in PKCθ−/− and Rottlerin-treated AA mice</td>
<td>76</td>
</tr>
<tr>
<td>2.6 PKCθ splenocytes do not expand in recipient mice and CD8+ T cells require PKCθ to induce AA in mice.</td>
<td>77</td>
</tr>
<tr>
<td>2.7 Rottlerin treatment attenuates AA in mice when administered at time of BMF induction</td>
<td>78</td>
</tr>
<tr>
<td>2.8 Rottlerin treatment inhibits PKCθ signaling \textit{in vivo} and attenuates AA in mice</td>
<td>79</td>
</tr>
</tbody>
</table>
2.9 Nuclear vs. cytoplasmic distribution of NOTCH1 and PKCθ in early T cell activation.................................................................80

2.10 PKCθ and NOTCH1 interact in CD8+ T cells.................................................................81

2.11 Early regulation of PKCθ and GSK3β signaling in CD8+ T cells.................................82

2.12 PKCθ and GSK3β activity counteract on NOTCH1 regulation.................................83

2.13 PKCθ regulates ifng mRNA processing through NOTCH1-associated PCMT1 and hnRNPU interactions.........................................................84

2.14 PKCθ regulates hnRNPU that selectively binds to ifng mRNA.........................85

3.1 PTDM design and characterization................................................................109

3.2 Synthesis and characterization of P13D5......................................................................110

3.3 Anti-pPKCθ (Thr538) delivery into hPBMCs. .........................................................111

3.4 The presence of anti-pPKCθ and its effect on cellular viability.........................112

3.5 P13D5: Anti-pPKCθ delivery into ‘unstimulated’ hPBMCs greatly reduces their activation potential.................................................................113

3.6 CD69 expression in P13D5: Anti-pPKCθ-treated hPBMCs........................................114

3.7 Reversibility of P13D5: Anti-pPKCθ treatment.........................................................115

3.8 P13D5: Anti-pPKCθ delivery into ‘activated’ hPBMCs diminishes expression of downstream activation markers.................................116

3.9 Effects of P13D5-only or Anti-pPKCθ-only treatment on hPBMCs ..................117

3.10 P13D5: Anti-pPKCθ delivery alters the activity and localization of PKCθ in hPBMCs .................................................................................118

3.11 Effects of P13D5: Anti-pPKCθ on total PKCθ expression........................................119

3.12 Ex vivo delivery of P13D5: Anti-pPKCθ into hPBMCs provides a survival benefit in a lymphocyte transfer, humanized mouse model of graft-versus-host disease (GvHD)......................................................120

3.13 Characterization of CD4+ T cells, treated ex vivo, and used to induce GvHD ..................................................................................121
4.1 αpPKC0 delivery generates CD4+CD25+FOXP3hi iTregs in vitro ...............156

4.2 Flow cytometric and qPCR analysis of certain iTreg differentiation markers in vitro ................................................................................................................157

4.3 pPKC0 is diminished in both cytosol and nucleus in iTreg+αpPKC0 cells.....158

4.4 Analysis of pPKC0, pSTAT3, and pSTAT5 expression for in vitro iTreg differentiation ........................................................................................................159

4.5 iTreg+αpPKC0 cells localize functional Treg markers to nucleus while reducing nuclear pPKC0 .................................................................160

4.6 Nuclear localization of critical iTreg proteins in conventional T cells (Tconv). ........................................................................................................161

4.7 iTreg+αpPKC0 cells behave as super-suppressive iTregs and substantially express suppressive receptors, LAG-3 and PD-1, on their surface...........162

4.8 In vitro proliferation profile of responder and suppressor cells and their intracellular and surface co-inhibitory receptor expressions..........163

4.9 Adoptive transfer of super-suppressive iTreg+αpPKC0 cells that are highly efficacious in vivo in humanized GvHD model .........................164

4.10 Cellular and cytokine profiles of adoptive iTreg transfer experiment in humanized GvHD model on day 17........................................165

4.11 Super-suppressive iTreg+αpPKC0 cells are long lasting in vivo and represent a unique population of FOXP3hiPD-1hiIFNγhi iTregs .....................166

4.12 Analysis of iTregs in peripheral blood and spleen in humanized GvHD model on day 17..........................................................167

4.13 Immunophenotyping of iTregs in bone marrow, peripheral blood, and spleen in humanized GvHD model on day 17.........................................168

5.1 Effect of αpPKC0 delivery on splicing regulators and non-differentiated T cells (Tconvs) in vitro ..........................................................202

5.2 αpPKC0 delivery modulates splicing regulatory proteins and affects RNA processing in iTregs in gene-specific manner in vitro ..................203

5.3 RNA processing was altered in ex vivo-treated iTregs in tissue- and gene-specific manner in humanized mouse model of GvHD ............204

xvii
5.4 Effect of αpPKCθ delivery on RNA processing of naïve T cells *in vivo*........205

5.5 PKCθ controls PCMT1 both post-transcriptionally and post-translationally in iTregs.................................................................206

5.6 PCMT1 splicing and 3’UTR analyses in non-differentiated T cells (Tconvs) *in vitro* and *in vivo*......................................................207

5.7 Inhibiting PKCθ diminishes hnRNPL association with PCMT1 at both protein and RNA levels in iTregs .............................................208

5.8 hnRNPL binding sites on 3’UTR sequences of iTreg genes.....................209

5.9 Cytoplasmic vs. nuclear mRNA association with hnRNPL in αpPKCθ- or αPCMT1-treated iTregs......................................................210

5.10 PCMT1 can be a good target to increase stability of iTregs....................211

6.1 iPSC-derived MSCs phenotypically resemble native MSCs and respond to IFNγ licensing..............................................................239

6.2 iPSC-derived MSCs exhibit tri-lineage differentiation capacity and reduced senescence *in vitro*. ...................................................240

6.3 iPSC-derived MSCs phenotypically resemble native MSCs, respond to IFNγ licensing, and dampen PBMC activation potential ..................241

6.4 PBMCs co-cultured with iPSC-MSCs show reduced proliferation and differentiation potential ..........................................................242

6.5 iPSC-MSCs attenuate disease severity and provide a survival benefit in a pre-clinical model of GvHD...................................................243

6.6 iPSC-MSC administration, *in vivo*, reduces cytokine production and weight loss in mice with GvHD......................................................244

6.7 iPSC-MSC administration reduces BM-infiltration and expression of proinflammatory molecules in mice with GvHD .........................245

6.8 iPSC-MSC administration reduces immune cell infiltration to target organs and expression of proinflammatory molecules..........................246

6.9 Clinical scoring and treatment cohorts.....................................................247

6.10 iPSC-MSCs attenuate GvHD severity and prolong survival in mice ...........248
6.11 iPSC-MSC-treatment alters cellular localization of pPKCθ in BM-infiltrating T cells ................................................................. 249

6.12 iPSC-MSC-treatment alters subcellular localization of pPKCθ in BM-infiltrating T cells ................................................................. 250

6.13 Pro-inflammatory molecules expressed by circulating PBMCs correlate with therapeutic response to iPSC-MSC administration .................. 251

7.1 Models for the role of PKCθ in the context of regulating ifng mRNA stability in activated CD8 T cells ............................................................ 254

7.2 Models for the role of regulating PKCθ responses in Anti-pPKCθ iTregs expressing high PD-1 and IFNγ (super-suppressive iTregs) in the context of regulating Treg stability through PCMT1-hnRNPL interactions ................................................................. 258
CHAPTER 1

INTRODUCTION

1.1 Immunobiology of graft-versus-host disease (GvHD)

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is a well-established therapy that involves the intravenous infusion of stem cells collected from a donor’s bone marrow, peripheral blood, or umbilical cord blood to reestablish the hematopoietic system in patients (recipient) who have genetic diseases, hematologic malignancies, and bone marrow failure due to defective bone marrow or immune system (Boeri et al., 2016). Upon transplantation, donor-derived T cells enable a successful engraftment, thus rejecting underlying disease, and provide anti-pathogenic protective immunity (Negrin, 2015). The success of the transplantation depends on the similarity of histocompatibility match between the donor (graft) and the recipient (host). Histocompatibility is linked to a genomic region that has genes encoding major histocompatibility complex (MHC). Differences in the genes of MHC region between the donor and the recipient are the main cause of the induction of acute graft-versus-host disease (GvHD) that exhibits life-threatening (or sometimes life-ending) complications (Negrin, 2015; Petersdorf, 2013). Patients, who survive acute GvHD, can develop the chronic form of the disease that leads to significant morbidity and mortality even after months and years of transplantation (Couriel et al., 2004). The immunobiology of GvHD has been extensively studied in mouse models of this disease that led to major findings in current understanding of GvHD and therefore, it is important to understand the findings from the mouse studies to unravel novel mechanisms and discover potential therapeutics.
for the purpose of translating these into humans (Figure 1.1) (Van Elssen et al., 2017; Eswaraka and Giddabasappa, 2016; Schroeder et al., 2017). GvHD appears to occur as a dysregulated, uncontrolled cascade of immunological events. These events are characterized in three phases; T cell priming-phase, effector-phase, and tissue damage-phase (Hülsdünker and Zeiser, 2015). Rapidly after allo-HSCT, donor-derived T cells home lymph nodes and spleen, in which they become activated and upregulate molecules allowing the entry to main target GvHD organs such as bone marrow, gut, liver, and skin. Later, activated T cells trafficking to these GvHD target organs destruct the tissues resulting in manifestation of several symptoms including diarrhea, weight loss, fur loss, low activity, poor posture, and mortality (Bäuerlein et al., 2013; Beilhack et al., 2005).

Current treatments are applied only once GvHD is established and effective in just 50% of the patients. Therefore, additional research efforts should be made for preventive therapies for larger cohorts (Couriel et al., 2004).

#### 1.1.1 Pathophysiology of GvHD

The scenario of the pathogenesis of GvHD is composed of three major phases: T cell priming-phase, effector-phase, and tissue damage-phase (Figure 1.2) (Boieri et al., 2016; Hülsdünker and Zeiser, 2015). In the initial T cell priming-phase, pre-conditioning regimens of chemotherapy, radiotherapy, or immunotherapy triggers cytokine storm (production of interleukin-1 (IL-1), tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ)) resulting from the damage in host tissues (Couriel et al., 2004; Kato et al., 2014; Ferrara and Antin, 2016; Gyurkocza and Sandmaier, 2014). In effector-phase, recipient and donor antigen-presenting cells (APCs) together with the cytokine storm
induce donor-derived CD4+ T cell activation and expansion through the interactions of T cell receptor (TCR) and costimulatory molecules with their cognate ligands expressed on the surface of the APC (Socié and Blazar, 2009). Even though the risk of alloreactive T cell activation is lower in MHC-matched donor transplantations, minor histocompatibility antigens (MiHA, non-self-peptides) play a huge role in the activation in both MHC-matched and MHC-mismatched donor transplantations. Subsequent to the activation, the activated CD4+ T cells differentiate into Th1 effector cells owing to the nature of T cell-APC interaction. Th1 cells produce inflammatory cytokines, such as IL-2 and IFN-γ, as well as chemokine receptors that amplify the allogeneic immune response and attract them to target GvHD organs (Zhang et al., 2016; Spierings, 2014). In the third phase, activated donor Th1 effector cells activate cytotoxic CD8+ T cells that mediate apoptosis in target host cells through the production of TNF-α, perforin, and granzyme B as well as Fas-FasL-mediated pathway (Schmaltz et al., 2001; Korngold et al., 2003). In addition to CD8+ T cells, macrophages and natural killer (NK) cells also produce TNF-α to activate themselves and other immune cells including neutrophils and B cells, thereby stimulating the production of additional inflammatory cytokines, IL-1, IL-6, IL-10, IL-12, and TNF-α itself (Ferrara et al., 2003; Schmaltz et al., 2003; Fowler et al., 2004; Korngold et al., 2003). Eventually, all this cytokine dysregulation via allogeneic interaction perpetuates the tissue destruction characteristic to GvHD.

1.1.2 Alloreactive T cells

There are studies defining the subsets of alloreactive T cells that play crucial role in mediating and regulating GvHD. So far, studies provided evidences for the importance
of naïve T cells, Th1 cells, Th2 cells, Th17 cells, regulatory T cells (Tregs), natural killer T (NKT) cells, and CD8+ T cells (Socié and Blazar, 2009). In murine models, expansion of CD8+ killer T cells has been sustained by CD4+ T cells as CD4-knockout mice showed either deletion or persistence of non-functional CD8+ T cells (Coghill et al., 2011). Additional evidence emerged from the studies of donor CD4+ T lymphocyte infusions enabled expansion of CD8+ T cells that mediate GvHD (Zorn et al., 2002). Although CD8+ T cells are the killers of target cells, both CD4+ and CD8+ T cells contribute to the pathogenesis of GvHD.

During GvHD reaction, donor-derived T cells initially migrate to secondary lymphoid organs such as spleen and lymph nodes. T cells receiving activation signals from APCs subsequently migrate to the target organs to induce the pathogenesis of GvHD. Chemokine receptor (CCR5, CCR6, CXCR3, CXCR4, CX3CR1), CD62L (L-selectin), and β7 integrin expressions on the alloreactive T cells are required for infiltrations into the target tissues (Socié and Blazar, 2009; Boieri et al., 2016).

1.1.3 Treatment of GvHD

To date, there is only one treatment approved in 2016 by Food and Drug Administration (FDA) for acute GvHD. Ruxotinib (a Janus kinase 1/2 inhibitor), developed by Jakafi; Incyte/Novartis, is currently being used to treat acute GvHD in children and adults. Clinical studies show that the overall response is fair, yet, the best-tolerated dosage and treatment regimen are still being investigated (Khandelwal et al., 2017). Besides, therapies with cyclosporine (calcineurin inhibitor), methylprednisolone (corticosteroid), daclizumab (IL-2R antibody), horse and rabbit anti-thymocyte globulin
(hATG and rATG), etanercept (TNF inhibitor), infliximab (TNF-α antibody), mycophenolate mofetil (inosine monophosphate dehydrogenase inhibitor), pentostatin (purine analog), and sirolimus (mTOR inhibitor) have shown average response rate of 50%, median survival around 6 months, high infection risk, and relapse-related GvHD mortality. Among these, mycophenolate mofetil has been found the most effective, however, futility analysis concluded that it would not improve the endpoint of GvHD-free survival. In general, overall response rate was approximately 40-60% although their effects on overall survival remains unclear (Jaglowski and Devine, 2014).

Over the last fifteen years, the focus has been put on *ex vivo* cell engineering of donor grafts due to successful accomplishments in reducing the risk of GvHD while retaining tumor- and pathogen-specific T cell responses (Couriel et al., 2004). Several strategies were developed to deplete or anergize alloreactive T cells. For instance, toxin-conjugated monoclonal antibody was used to eliminate activated cells expressing CD25 (IL-2 receptor α chain) (Martin et al., 2004). In addition, *ex vivo* expansion of Tregs is another graft engineering strategy to decrease the incidence of GvHD upon bone marrow transplantation (BMT). CD4<sup>+</sup>CD25<sup>+</sup> Tregs were initially described as a T cell subset that prevented autoimmunity after neonatal thymectomy in mice (Nakamura et al., 2001). They have immunosuppressive properties enabling peripheral tolerance and protecting from aberrant immune responses. It was shown that depletion of Tregs from the donor bone marrow graft accelerated GvHD response, whereas addition of donor Treg to the graft at 1:1 ratio with conventional T cells repressed the GvHD reaction in mouse models. Moreover, mesenchymal stem cells (MSCs) were found to support hematopoiesis in the bone marrow as well as immunosuppression in acute GvHD (Boeri et al., 2016;
Mattar and Bieback, 2015). However, potential mechanism of action of MSCs in GvHD has not been well-characterized in preclinical animal models, thereby hampering consistent clinical efficacy (Tolar et al., 2011). All in all, a better understanding of the biology of immunosuppressive cell subsets as well as improved and standardized techniques for their characterization, isolation, and expansion should be considered to develop powerful ex vivo cell engineering therapies for acute GvHD prevention or treatment.

1.2 Protein kinase C-theta (PKCθ) in T cell signaling

Protein kinase C is a kinase family that has different isoforms activated either by proteolysis or translocation to plasma membrane, where it binds to specific cofactors. Diacylglycerol (DAG) and inositol 1, 4, 5-trisphosphate (IP₃) are the cofactors that activate PKC enzymes (Isakov and Altman, 2012; Inoue et al., 1977). These enzymes transduce signals from cell surface receptors that are associated with phospholipase C and phospholipid hydrolysis. This family includes 3 subfamilies and 10 isoforms that are structurally and functionally related (Pfeifhofer-Obermair et al., 2012). The first subfamily contains conventional PKCs (cPKC; α, βI, βII, γ) that are regulated via DAG-binding C1 domains and Ca²⁺ binding (Ho et al., 2001). The second group includes novel PKCs (nPKC; δ, ε, η, θ) that are DAG-dependent but Ca²⁺-independent for their activity. The third group has atypical PKCs (aPKC; ζ, λ, ι) that are both DAG- and Ca²⁺-independent (Rosse et al., 2010). Immunological studies show that distinct PKC isoforms have different ways of activating function of T cells and thus, they can serve as drug targets for T cell mediated adaptive immune responses (Baier and Wagner, 2009).
Among the PKC enzymes, protein kinase C-theta (PKC\(\theta\)) exhibits a selective pattern of tissue distribution with a predominant expression in T lymphocytes, platelets, and skeletal muscle. It is translocated to the center of immunological synapse (IS) in activated T cells where the integration of T cell receptor (TCR) and CD28 costimulatory signal occurs for its full activation (Yokosuka et al., 2008). Upon its full activation, three transcription factors including NF-\(\kappa\)B, AP-1, and NF-AT initiate signals that are critical for T cell activation, proliferation, and differentiation (Pfeifhofer et al., 2003). Recently, it has been suggested that PKC\(\theta\) can also translocate to the nucleus and binds to chromatin to regulate microRNA and T cell-specific gene expression programs (Sutcliffe et al., 2011).

1.2.1 Structural analysis of PKC\(\theta\)

PKC\(\theta\) consists of an N-terminal regulatory region and a C-terminal catalytic region that are linked by hinge region (Figure 1.3A). There are important modifications occurring on certain domains that regulate PKC\(\theta\) function (Brezar et al., 2015). For instance, C2-like domain has phosphorylated Tyr90 residue and is involved in membrane translocation of PKC\(\theta\). Two C1 domains bind to DAG; C1b has a higher affinity for DAG than C1a. C1a domain contains a pseudosubstrate sequence (RRGAIKQA) that binds to substrate binding region, inhibiting PKC\(\theta\) kinase activity in the absence of allosteric effectors (Wang et al., 2012). Hinge region also called as V3 domain is involved in the association of PKC\(\theta\) with CD28 via lymphocyte-specific protein tyrosine kinase (LCK), thereby promoting PKC\(\theta\) membrane translocation (Kong et al., 2011). Figure 1.3B shows 3D conformational structure of PKC\(\theta\) enzyme and important sites for
PKCθ activation. The full activation of the enzyme involves two steps: DAG binding to C1 domain and subsequent Thr538 phosphorylation at activation loop of PKCθ (Seco et al., 2012). TCR/CD28 engagement enables the generation of DAG and DAG binding to the C1 domain leads to exposure of the activation loop. Consequently, Thr538 is accessible to get phosphorylated by germinal center kinase-like kinase (GLK) resulting in catalytic activation (Chuang et al., 2011). Besides Thr538, there are other phosphorylation sites on PKCθ that have been identified to play different roles in regulation of the enzyme (Brezar et al., 2015).

1.2.2 Role of PKCθ in CD4+ and CD8+ T cell responses

*In vitro* and *in vivo* studies of PKCθ-deficient (Prkcq−/−) mice in different disease models revealed that distinct T cell subpopulations have differential requirements for PKCθ during immune response (Anderson et al., 2006). Hence, PKCθ was reported to be essential for Th17-mediated experimental autoimmune encephalomyelitis (EAE) as a model of multiple sclerosis (MS) and Th2-type immune responses to allergens or helminth infections (Marsland et al., 2004; Kwon et al., 2012). On the other hand, *Prkcq−/−* mice had intact resistance to *Leishmania major* infection, which is a Th1-mediated immune response and PKCθ was nonessential for CD8+ T cell-mediated antiviral responses suggesting the compensation by innate immunity signals (Valenzuela et al., 2009). Furthermore, PKCθ was necessary for Th2 and Th17 cell development and had moderate effect on Th1 cell development (Marsland et al., 2007). Overall, PKCθ has a positive effect in effector T cell (Teff) activation and an advantage of adaptive immune responses. However, the nature of PKCθ in T regulatory cells (Treg) seems different.
Tregs are responsible for suppression of Teff functions and PKCθ mediates a negative role in Treg functions. Activation of Tregs caused PKCθ sequestration in cytosol which prevents its translocation into the center of IS and inhibition of PKCθ activity augmented Treg suppression activity (Zanin-Zhorov et al., 2010, 2011). Moreover, Prkcq−/− mice had impairment of Treg development in the thymus. However, mature PKCθ-deficient Treg cells had intact activity (Gupta et al., 2008).

1.2.3 PKCθ in various immune pathologies

Perturbations of PKCθ activity can result in a variety of diseases such as autoimmune and inflammatory diseases, cancer, and diabetes (Hage-Sleiman et al., 2015). Firstly, increased PKCθ activity has become a hallmark of autoimmune disorders that leads to activation of self-reactive T cells differentiating into Teffs and attacking self-tissues (Kwon et al., 2010). In addition, overexpression of GLK increases PKCθ phosphorylation leading to autoimmunity in systemic lupus erythematosus (SLE) as well as in rheumatoid arthritis (RA) (Chuang et al., 2011; Chen et al., 2013). Moreover, additional studies were done in Prkcq−/− mice revealing the requirement of PKCθ in GvHD and autoreactive T cell-mediated immune responses (Valenzuela et al., 2009). Interestingly, PKCθ-deficient T cells retained the ability to induce graft-versus-leukemia (GvL) response in allogeneic BM-transplanted mice. In addition, PKCθ contributes to allograft rejection using an adoptive transfer model where the rejection of allograft by Prkcq−/− mice was delayed (Manicassamy et al., 2008). Besides its role in regulating autoimmune and immunosuppressive responses, PKCθ is involved in inflammatory brain conditions and muscle dystrophy (Rigor et al., 2012; Madaro et al., 2012). Moreover,
PKCθ controls phosphorylation of BAD, a pro-apoptotic protein, which prevents Fas-mediated apoptosis in leukemic T cells. In breast cancer cell proliferation, Akt is activated by PKCθ and causes estrogen receptor-alpha (ERα) expression. PKCθ also mediates lipid metabolism and insulin resistance, which is a cause of type II diabetes. High level of fatty acids in plasma increases DAG which in turn activates PKCθ and insulin-stimulated insulin receptor substrate I (IRS1) is phosphorylated on Ser307 residue. This phosphorylation leads to insulin resistance (Gao et al., 2004).

1.2.4 PKCθ as a drug target in clinic

The fact that role of PKCθ is dispensable for anti-viral responses and GvL following allogeneic BM transplantation strengthened the possibility that PKCθ may be a potential drug target and inhibiting PKCθ may selectively suppress allograft rejection and autoimmunity without disrupting anti-viral and anti-tumor immunity (Isakov, 2012). For this reason, pharmaceutical companies developed small molecules capable of modulating PKCθ function. For instance, AEB071 (Sotrastaurin) is currently in the early phase of clinical trials although it inhibits other PKC isoforms. Unfortunately, most of the existing small molecule inhibitors for PKCθ are toxic because of their lack of specificity reflecting the conserved domains through all PKC isoforms (Evenou et al., 2009). Since these small molecule inhibitors are ATP competitors, they need to be used at high concentrations and as a result, current investigations are being done development of allosteric inhibitors (Lamba and Ghosh, 2012). Numbers of studies have shown that successful selective targeting of PKCθ may serve as a novel therapy for several T-cell
mediated disease conditions such as multiple sclerosis (MS), RA, inflammatory bowel disease, and GvHD (Chand et al., 2012).

There have been extensive efforts to develop isozyme-selective PKCθ inhibitors. These contain: ATP-competitive small molecule inhibitors, phorbol esters and derivative activators, inhibitors binding to C1 domain that mimic the binding of DAG, and peptides disrupting protein-protein interactions. Although the molecules were used for selective inhibition of PKCθ, results in clinical trials have not been promising. The biggest problem is inadequate preclinical study since animal models do not exhibit the pathological role of PKCθ very well and therefore the data is not reproducible (Mochly-Rosen et al., 2012; Altman and Kong, 2014).

1.3 Immune system-targeted therapeutics

Clinical advances with immune system-targeted therapeutics have revolutionized the treatment of cancers and immunological disorders including autoimmune diseases, infectious diseases, lymphoproliferative disorders, and GvHD (Dhanak et al., 2017; Bluestone and Tang, 2015; Ostrov, 2015; Khanna et al., 2017; Postow et al., 2015). Although there are key achievements made in clinic, most patients treated with these therapeutics exhibit various histopathologies and only have a short period of benefit. Hitherto, several immune-based therapies, such as Yervoy, Opdivo, and Keytruda accelerated the research in academia and pharmaceutical companies so as to develop next generation immune system-targeted therapeutics for the purpose of treating multiple diseases together with consideration of personalized medicine (Dhanak et al., 2017).
Current approaches in treating several diseases benefit from three major immune-related therapeutics: small molecule immunotherapeutics, therapeutic antibodies, and cell-based therapeutics (Fischbach et al., 2013; Leavy, 2010; Bluestone and Tang, 2015). These therapeutics offer the flexibility of combination therapies in clinical setting to enhance or extend effective treatment. Importantly, clinical experience with these therapies asserts the need for higher selectivity, higher potency, and lower toxicity. Besides, a better understanding the mechanism of action for each therapeutics is obligatory to maximize the therapeutic effect for a particular disease (Bluestone and Tang, 2015; Woodsworth and Holt, 2017).

1.3.1 Small molecule immunotherapeutics

There are several developed small molecule therapeutics that are attractive and synergistic to biologics. They act either as immunostimulatory or immunosuppressive molecules. Immunostimulatory therapeutics include toll-like receptor (TLR) agonists, STING agonists, retinoic acid receptor-related orphan receptor gamma-t (ROR-\(\gamma\)t) agonists, adenosine receptor 2a (A2a) antagonists, indoleamine 2, 3-dioxygenase (IDO) inhibitors. (Dhanak et al., 2017). On the other hand, immunosuppressive agents have been developed for three major signaling pathways. NF-kB inhibitors (bortezomib, oxycodone, BAY-11, DHMEQ), p38 MAPK inhibitors (TAK-7125, clomethiazole, succinobucol), and JAK/STAT inhibitors (lestaurtinib, rixolitinib, CP-690550) have been extensively used to treat many immunological disorders (Ivanenkov et al., 2008; Gupta et al., 2011)
1.3.2 Therapeutic Antibodies

Monoclonal antibodies have emerged to be potential therapeutics in many diseases such as cancer, infection, and autoimmune disorders due to target specificity in the treatment and prophylaxis of the disease. Genetic engineering allows manipulating mouse monoclonal antibodies into humanized versions that are useful for clinical use. To date, there are more than 200 antibodies in clinical trials and FDA approved several of them that are used in treatments of cancer, transplant rejection, RA, and anti-viral prophylaxis (Figure 1.4). It is important to note that therapeutic antibodies take 20% of all biopharmaceuticals in clinical trials, which makes them the second largest biopharmaceutical product category after vaccines (Brekke and Sandlie, 2003; Dübel and Reichert, 2014). The top five selling therapeutic antibodies are split in two areas: oncology and immune disorders (Elvin et al., 2013). Especially, antibody-mediated immune checkpoint blockade (ICB) via anti-CTLA-4 and anti-PD-1 antibodies has exhibited substantial clinical benefit and highly durable anti-tumor effect in various cancer immunotherapies although responsiveness to this therapy is limited to 20-30% of total patients (Callahan et al., 2016). They look promising in treating these diseases due to their greater safety and selectivity. However, the biggest limitation of therapeutic antibodies is that clinical applications are currently limited to cell surface or extracellular targets because of their inability to pass through the cellular membrane (Imai and Takaoka, 2006). In this case, their mechanisms of action are usually through ligand or receptor blockade, receptor downregulation, cell depletion, and non-specific signaling induction. Inevitably, there are side effects of blocking whole signaling pathway and there is a need for more specific intervention via targeting intracellular molecules of
interest in order to improve clinical relevance of the antibody therapy (Chan and Carter, 2010; Beck et al., 2010)

1.3.3 Cell-based therapeutics

Using biological cells as therapeutic devices has a lot of advantages over small molecule immunotherapeutics and therapeutic antibodies. Firstly, cells are highly selective that have complex sensing and response systems. Their distribution is controlled by directed cell migration, whereas small molecules and therapeutic antibodies are transported via diffusion and controlled by pharmacokinetics/pharmacodynamics (PK/PD). They can make decisions and exhibit varied and regulable behaviors (Fischbach et al., 2013). Cells can execute their function in a context-dependent fashion and maintain homeostatic environment. Also, cells have tremendous phenotypic and functional diversity. It is a great advantage that the diversity is encoded in the genome of each cell so that it can be logically reprogrammed. Novel effector components of the reprogrammed cells can be generated by altering, adding, or removing biological molecules that fulfill the requirements of functional cells (Woodsworth and Holt, 2017). Genetic engineering of cells allows us to personalize therapies for certain diseases. As such, chimeric antigen receptor T (CAR-T) cell therapy is the new emerging concept proven for the treatment of B cell malignancies (Zhang et al., 2015; Dai et al., 2016; Maude et al., 2015). In CAR-T cell therapy, patient-derived cytotoxic T cells are genetically modified to express a synthetic receptor incorporating a single-chain variable fragment (scFv) to an intracellular domain comprising various T cell signaling components (Figure 1.5). Hence, CAR-T cells can target all the malignant cells that
express cognate antigens for the scFv (Rosenberg and Restifo, 2015). Much of the existing work in cell-based therapy focused on CD8+ T cells in cancer immunotherapy. However, induced pluripotent stem cells (iPSCs), Tregs, and MSCs can also be efficiently used in immune-based therapies (Wu and Hochedlinger, 2011; Takahashi and Yamanaka, 2006; Parmar and Shpall, 2016; Hamada et al., 2005).

1.3.3.1 Regulatory T cell (Treg) therapy

Tregs are immunosuppressive cell subsets of the immune system and can be classified into three types: thymic-derived Tregs, peripherally-derived Tregs, and in vitro induced Tregs (iTregs) (Figure 1.6). iTregs carry the characteristics, to some extent, of peripherally-derived Tregs in vivo (Safinia et al., 2015). Adoptive Treg immunotherapy, with high therapeutic efficacy and safety, is promising due to lower adverse effects, thereby representing a powerful treatment toward personalized medicine. However, it remains essential to assess the long-term behaviors of these cells upon transfer in order to establish effective treatment protocols (Tang and Bluestone, 2013; Beres and Drobyski, 2013). Tregs provide transplantation tolerance and long-lasting benefit in preventing immunological disorders (Figure 1.7). Clinical trial design of Treg immunotherapy is well-established and follows several steps. In the first step, Tregs are isolated from the donor. Later, they are expanded polyclonally or in an alloantigen-specific fashion in ex vivo cultures. They are induced via certain cytokines and small molecules, including transforming growth factor-beta (TGF-β), rapamycin, and low-dose IL-2, to enhance their function. Finally, these iTregs are carefully characterized and purified prior to the
adoptive transfer. (Figure 1.8) (Trzonkowski et al., 2015; Singer et al., 2014; Hahn et al., 2015).

It is found that the most suitable Treg populations for human adoptive transfer studies are either CD4+CD25hi, CD4+CD25hiCD127−, or CD4+CD25+ICOS+ Tregs. Both thymus-derived Tregs and iTregs expanded in large scale ex vivo are found to ameliorate the disease in xenogeneic model of GvHD (Chakraborty et al., 2013). Another example of Tregs appeared to be the most effective for acute GvHD treatment is CD62L-expressing CD4+CD25+ Tregs (Ermann et al., 2005). Elucidation of novel Treg signaling pathways and manufacturing of ex vivo-expanded suppressive Tregs will be essential to harness the tolerogenic potential of Treg immunotherapy.

1.3.3.2 Mesenchymal stem cell (MSC) therapy

MSCs are able to differentiate a variety of cell types and easily expandable in vitro. It has been shown that adult MSCs exert immunomodulatory effects that alter T cell responses. They suppress T cell proliferation and proinflammatory cytokine production, thereby regulating Th1/Th2 balance (Puissant et al., 2005; Yañez et al., 2006; Glennie et al., 2005). Furthermore, they support the function and stability of Tregs (Selmani et al., 2008). In addition to T cells, they also inhibit maturation of dendritic cells and IL-2 induced NK cell activation (Burchell et al., 2010; Ramasamy et al., 2007; Spaggiari et al., 2006). On the other hand, iPSCs and embryonic stem cells (ESCs) are capable of inhibiting lymphocyte proliferation and NK cell-mediated killing (Trivedi and Hematti, 2008; Yen et al., 2009; Tan et al., 2011). Interestingly, MSCs can be plastic and behave differently depending on the local microenvironment or disease status. For
example. Adult MSCs reduce Th1-mediated response in GvHD and SLE, however, BM-derived MSCs cause a shift from Th2 to Th1 response in airway allergic inflammatory diseases (Le Blanc et al., 2004; Rafei et al., 2009; Cho et al., 2009; Cho and Roh, 2010).

The mechanisms how MSCs inhibit aberrant T cell responses involve soluble factors and cell-to-cell contact (Crop et al., 2010). Such soluble factors include TGF-β, prostaglandin E2 (PGE2), hepatocyte growth factor (HGF), IDO, nitric oxide (NO), and IL-10. Also, MSCs licensed by IFN-γ express programmed death ligand 1 (PD-L1), vascular cell adhesion molecule-1 (VCAM1), and galectin-1 in order to have a contact with T cells, thereby inducing Treg function (English et al., 2009; Sheng et al., 2008; Gieseke et al., 2010).

MSCs are excellent candidates for immunomodulation of aberrant immune responses. BM- or fat tissue-derived MSCs have been found to significantly reduce T cell proliferation and proinflammatory cytokine expression in animal models of inflammatory disorders including colitis, immune thrombocytopenia, experimental autoimmune encephalomyelitis (EAE), SLE, and GvHD (Zhang et al., 2009; Xiao et al., 2012a; Zappia et al., 2005; Sun et al., 2009; Guo et al., 2011). It is very promising that autologous, allogeneic, and even xenogeneic MSCs showed great survival benefits from the disease (Firinci et al., 2011). A progressive understanding of MSCs in animal models has led to launch clinical trials as an immunomodulatory prophylaxis in GvHD, diabetes, organ transplantation, and Crohn’s disease. Several MSC products have been approved for clinical applications: Cartistem for degenerative arthritis, Prochymal for acute GvHD, and Cupistem for anal fistula. Although they show great potential in treating immune disorders, enormous variability in cell quality of different donors and different
transfusion patterns limit their ultimate therapeutic benefit. Thus, sizeable production of homogeneous MSC population, critical evaluation of appropriate cell sources, and more preclinical studies should be considered to avoid undesired effects in clinical setting (Gao et al., 2016).

1.4 Biologics delivery via next generation nanocarriers for T cell engineering

The range of applications of nanocarriers constitutes drug delivery, cancer and gene therapy, imaging, and cell tracking through biosensors (Škalko-Basnet, 2014). In drug delivery, these delivery agents are currently used for large therapeutics called biologics. Biologics can be encapsulated, embedded, conjugated, or adsorbed onto the nanocarriers which then provide optimized delivery strategy (Mahapatro and Singh, 2011). Besides the strong demand to develop alternative therapeutic strategies to address unmet clinical needs in immunotherapy, next generation nanocarriers help to achieve unprecedented clinical efficacy as a means of modulation of pathophysiological dynamics at the molecular level (Pagels and Prud’Homme, 2015).

Therapeutic approaches using nanocarriers in T cell immunomodulation have gained substantial success in manipulating various disease pathologies including cancer, GvHD, rheumatoid arthritis, multiple sclerosis, type I diabetes, SLE, scleroderma, psoriasis, Sjögren’s syndrome, and Crohn’s disease (Kingwell, 2016; Gharagozloo et al., 2015; Getts et al., 2015). Mainly, peptide- and polymeric-based nanocarriers had higher success rates in biologics delivery and notable biological consequences (Figure 1.9). For example, polyethylene glycol (PEG)-conjugated anti-TNF-α fragment, Certolizumab pegol, neutralized TNF-α function by preventing the interaction of TNF-α with specific
receptors. Also, IL-2/IL-15 receptor β chain was targeted by polyethylenimine (PEI)-complexed siRNA nanoparticles to suppress inflammatory cytokines. Iron oxide nanoparticles coated with mono-specific, type I diabetes-relevant peptide-MHC class I monomers have used to expand CD8$^+$ Tregs. Another interesting approach is rapamycin delivery in elastin-like polypeptide micelle nanoparticles in order to alter cytokine- and mTOR-related gene expression and reduce rapamycin toxicity for the treatment of Sjögren’s syndrome (Gharagozloo et al., 2015).

1.4.1 Challenges in intracellular delivery of biologics

There are many proteins and peptides that are biologically active and becoming as potent therapeutics. For instance, enzymes have a long history of clinical investigations and certain diseases associated with enzyme deficiency can only be treated by administration of exogenous enzymes. The second category is peptide hormones that are commonly used for anticancer treatments. The third category of protein therapeutics includes antibodies against certain specific molecules. Currently, antibodies for T cell engineering can only exert their function extracellularly implying that low permeability of cell membranes puts an obstacle for protein-based therapeutics (Torchilin, 2009; Beck et al., 2010; Chan and Carter, 2010). Therefore, intracellular delivery of biologically active molecules is a main challenge to achieve more specific targeting via antibodies (Stewart et al., 2016).

The major limitation about the use of biologics is because they cannot easily cross cellular membranes (not even applicable for hard-to-transfect cells), thus can only target surface molecules which can cause off-target effects. Additionally, the chemical nature
(size, charge, hydrophobicity, conformation, molecular weight etc.) of a particular cargo determines whether the nanocarrier allows cellular penetration with a desired delivery efficiency. Using next generation nanocarriers, these molecules can be delivered intracellularly via receptor-mediated endocytosis. However, each molecule entering via endocytic pathway is trapped in endosome and eventually degraded in lysosome. Therefore, delivery mechanism of these macromolecules is quite important. Besides, specific targeting, uptake efficiency, proper subcellular localization, and payload activity are other key challenges in a successful and efficient intracellular delivery of biologics (Guillard et al., 2015). Nevertheless, current available methodologies for intracellular delivery are restricted by the cargo instability due to chemical modifications with the nanocarriers, thus impairing the occurrence of desired biological effect (Massignani et al., 2010).

1.4.2 Cell-penetrating peptides (CPPs)

A novel approach to deliver such biologics involves usage of cell-penetrating peptides (CPPs), also known as protein transduction domains (PTDs), which are short sequences of peptides capable of translocating through the membrane. Intracellular delivery with CPPs has a lot of advantages over conventional delivery methods since they have a potential therapeutic application (Torchilin, 2009; Guillard et al., 2015). CPPs are categorized as three classes: protein-derived CPPs, chimera-derived CPPs, and synthetic CPPs (deRonde and Tew, 2015). The first protein-derived CPP discovered was HIV-1 TAT protein. Residues between 48-60 were arginine-rich and found to be solely responsible for cellular penetration. After discovery of TAT, additional protein-derived CPPs (penetratin, pVEC), chimera-derived CPPs (Pep-1, transportan), and synthetic
CPPs (polyarginine, MAP) were developed (deRonde and Tew, 2015). Some of these CPPs additionally have hydrophobic component as well as cationic region that increases membrane transduction activity (Sgolastra et al., 2014). Although they are capable of crossing cellular membranes, their structures are synthetically challenging because of their complexities (Sgolastra et al., 2017). They also require covalent attachment to their cargo, therefore can lead to alterations in the biological activity (Fawell et al., 1994; Juliano et al., 2009). Additionally, CPPs can be time-consuming and restrictive since each cargo needs to have a specific chemistry for conjugation (Becker-Hapak et al., 2001).

1.4.3 Novel cell-penetrating peptide mimics (CPPMs)

Uptake performance of CPPs is somewhat limited. Recent studies demonstrate the incorporation of key features of CPPs into simpler, tunable scaffolds gives rise to extensive opportunities for novel delivery agents and improved uptake for a broad range of cell types (Sgolastra et al., 2013). Therefore, a non-covalent protein delivery strategy is primarily selected, in which the cargo is delivered as a part of supramolecular complex. The first non-covalent strategy introduced to the field was the development of Pep-1. Pep-1 has both hydrophilic translocation moiety, nuclear localization signal of SV40 large T-antigen, and tryptophan-rich hydrophobic motif through a short linker (deRonde and Tew, 2015; Sgolastra et al., 2017, 2014). Although Pep-1 has been used in a variety of cell types, it does not deliver into hard-to-transfect cell types, such as primary cells and T cell lines (Backlund et al., 2016; Sarapas et al., 2017; Tezgel et al., 2017; Sgolastra et al., 2017).
Following the investigations on peptide chirality and hydrogen bonding, several research groups have developed synthetic CPPs to mimic CPP function, yet, enhancing cellular uptake efficiency. However, the structures were not flexible, and those synthetic CPP-cargo complexes were not stable under different physiological conditions (Sgolastra et al., 2014, 2013; deRonde and Tew, 2015).

In addition to peptide-based scaffold system, polymeric scaffolds were developed that enabled the use of different chemistries, new chemical compositions and polymer architectures, and non-covalent attachment to the cargo. These molecules are called cell-penetrating peptide mimics (CPPMs), also known as protein transduction domain mimics (PTDMs), that exhibit highly efficient delivery for biologics, even to hard-to-transfect cells, with higher potency. CPPMs have the combinatorial characteristics of TAT (guanidinium-rich), and Pep-1 (sequence of hydrophobic region and cationic domain). The hydrophobic region consists of phenyl-functionalized repeat units, which were shown to be more efficient in cellular penetration with respect to aromatic or aliphatic groups (Figure 1.10) (deRonde and Tew, 2015; Sgolastra et al., 2017; Tezgel et al., 2011). Several studies reported that oxanorbornene-based, block-type CPPMs have superior efficiencies for intracellular siRNA, protein, and antibody delivery as compared to their homopolymer counterparts and currently available CPPs (Tezgel et al., 2017; Sgolastra et al., 2017; DeRonde et al., 2015; Tezgel et al., 2012; Ozay et al., 2016). More importantly, these CPPMs have demonstrated greater ability to manipulate immunological responses in the context of T cell engineering (Ozay et al., 2016; Tezgel et al., 2012).
1.5 Significance and hypothesis

Acute graft-versus-host disease (GvHD) is a life-threatening complication of allogeneic bone marrow transplantation (allo-BMT) (Negrin, 2015; Petersdorf, 2013). Currently available treatments are only effective 50% of the patients, yet, the patients can become refractory and have relapses. Therefore, additional research efforts should be made for preventive therapies for larger cohorts (Couriel et al., 2004). To date, the immunopathogenesis of GvHD in humans has not yet to be well-characterized (Van Elssen et al., 2017; Schroeder et al., 2017). Here, we use humanized mouse models to recapitulate the human immune system so as to contribute to unravel novel mechanisms and explore potential translational therapeutics for human clinical treatments.

GvHD appears to occur as a dysregulated, uncontrolled cascade of immunological events. These events are characterized in three phases; T cell priming-phase, effector-phase, and tissue damage-phase (Hülsdünker and Zeiser, 2015). Alloreactive donor-derived T cells are activated through their T cell receptor (TCR) and play major role in the progression of GvHD. Therefore, we seek to investigate the molecular mechanisms of CD4+ and CD8+ T cell subsets leading to aberrant immune cell activation, thus causing the generation of alloreactivity. In this context, protein kinase C-theta (PKCθ), a crucial, early downstream kinase of TCR signaling, is a mediator of T cell activation and promote alloreactive responses such as proinflammatory cytokine production, differentiation, proliferation, migration, and cytotoxicity. Thus, delineating specific ways of interfering PKCθ signaling appears to become highly beneficial for the GvHD treatment or prevention.
This study will have significant impact on immunomodulation of T cells for the purpose of manipulating and intervening immune-mediated disorders as well as in the research field to further elucidate the novel molecular interactions of PKCθ in T cell signaling. We aim to utilize all three modes of immune-targeted therapeutics to manipulate PKCθ signaling and assess the degree of preclinical efficacy. As a small molecule therapeutics, we will use a pharmacological inhibitor, Rottlerin, to inhibit PKCθ phosphorylation at Thr538. As therapeutic antibody approach, we will develop intracellular antibody delivery strategy via complexation of a next generation nanocarrier, cell-penetrating peptide mimics (CPPMs), and a phospho-specific antibody (αpPKCθ-Thr538) to specifically interfere with PKCθ function. Finally, we will use engineered iTregs and iPSC-derived MSCs as cell-based therapies to manipulate PKCθ signaling, thereby treating or preventing GvHD.

Overall, the main hypothesis of this study is that specific targeting of PKCθ function by using three modes of immune-targeted therapeutics provides therapeutic benefit for the immunopathogenesis of GvHD.

To test this hypothesis, the following specific aims are proposed:

Specific Aim 1: Investigate the molecular mechanisms driven by PKCθ in T cells by establishing preclinical all-murine and humanized GvHD mouse models as well as in vitro primary cultures

Specific Aim 2: Determine the therapeutic effect of rottlerin treatment in all-murine model of immune mediated-bone marrow failure

Specific Aim 3: Establish an intracellular antibody delivery strategy against functional PKCθ by using cell-penetrating peptide mimics
**Specific Aim 4:** Assess the therapeutic efficacy of *ex vivo*-engineered iTregs and iPSC-derived MSCs in preclinical, humanized mouse model of GvHD

The knowledge gathered from this study will unravel novel molecular mechanisms driven by PKCθ in the context of T cell activation. In addition, this study will shed light on both the powerful strategy of intracellular antibody delivery by CPPMs and potential therapeutic targeting of PKCθ in the context of T cell immunomodulation. Using all-murine and humanized GvHD mouse models will empower us to investigate the durability of response by three types of immune-targeted therapeutics used in this study. The results of this study will be a sound strategy that may ultimately expand therapeutic options for various pathologies that involve PKCθ.
Figure 1.1 A timeline illustrating key discoveries in animal models of acute GvHD. Acute GvHD is a consequence of allogeneic hematopoietic stem cell transplantation that leads to the destruction of target organs, such as bone marrow, gut, liver, and skin, by alloreactive donor-derived T cells. The timeline shows all the important findings that have contributed to the current knowledge of the pathogenesis of acute GvHD (Adapted from Boieri et al, 2016).
Figure 1.2 Immunopathogenesis of acute GvHD upon BMT. Firstly, patient (recipient) undergoes to pre-conditioning regimen including radiation, chemotherapy, or immunotherapy prior to bone marrow transplantation (BMT). During the pre-conditioning, cytokine storm is generated in the recipient (host) tissues, which activates host- and donor-derived antigen presenting cells (APCs). These cells present antigens to donor CD4+ T cells which differentiate into Th1 effector cells. Th1 cells further activate CD8+ T cells, thereby differentiating them into killer cytotoxic T lymphocytes (CTL) that secrete destructive signaling molecules. As a result, they cause tissue damage characteristic to GvHD.
Figure 1.3 Structural details of PKCα. (a) PKCα consists of three main regions: regulatory domain, hinge region, and kinase domain. There are specific phosphorylation sites regulating different modes of PKCα action. In addition, several motifs are identified to contribute to its function. (b) 3D conformational structure of PKCα. (Adapted from Brezar et al., 2015 and Wang et al., 2012)
<table>
<thead>
<tr>
<th>Common Name</th>
<th>Cognate Antigen</th>
<th>Indication</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ipilimumab</td>
<td>CTLA-4</td>
<td>Metastatic melanoma</td>
<td>Binding and inactivation of CTLA-4, which allows cytotoxic T lymphocytes to kill tumor cells</td>
</tr>
<tr>
<td>Pembrolizumab</td>
<td>PD-1</td>
<td>Melanoma, head, neck, and bladder cancer, non-small-cell lung cancer</td>
<td>Binding and inactivation of PD-1, which allows cytotoxic T lymphocytes to kill tumor cells</td>
</tr>
<tr>
<td>Nivolumab</td>
<td>PD-1</td>
<td>Hodgkin lymphoma, head and neck cancer, lung cancer, kidney cancer, melanoma</td>
<td>Binding and inactivation of PD-1, which allows cytotoxic T lymphocytes to kill tumor cells</td>
</tr>
<tr>
<td>Rituximab</td>
<td>CD20</td>
<td>Non-Hodgkin’s lymphoma, Chronic lymphoblastic leukemia, Rheumatoid arthritis</td>
<td>Sensitizes cells to chemotherapy; apoptosis</td>
</tr>
<tr>
<td>Trastuzumab</td>
<td>HER-2</td>
<td>Breast cancer</td>
<td>Prevent cell growth and proliferation</td>
</tr>
<tr>
<td>Alemtuzumab</td>
<td>CD52</td>
<td>Chronic lymphoblastic leukemia, T-cell lymphoma</td>
<td>Specific targeting: antibody-dependent cellular cytotoxicity</td>
</tr>
<tr>
<td>Adalimumab</td>
<td>TNF-α</td>
<td>Rheumatoid arthritis, Crohn’s disease, psoriasis</td>
<td>Binds soluble and membrane bound-TNF; neutralizes TNF; inhibiting binding to TNF receptor</td>
</tr>
</tbody>
</table>

**Figure 1.4 Several FDA-approved therapeutic antibodies.** These antibodies are currently used in clinic to treat various cancers as well as immunological diseases. (Adapted from Chan and Carter, 2010)
Figure 1.5 CAR-T cell therapy. The top panel shows the insertion of a conventional TCR into T lymphocytes of patients. Later, they are expanded and infused back to the patient. The bottom panel shows engineered CAR insertion into a patient’s T cell followed by their expansion and re-infusion. TCRs and CARs are structurally different. TCRs have one alpha and one beta chain, whereas CARs are constructed by linking the variable regions of the antibody heavy and light chains to intracellular signaling chains (such as CD3-zeta, CD28, and 41BB) alone or in combination with other signaling moieties. (Adapted from Rosenberg and Restifo, 2015)
Three classes of regulatory T cells. There are thymic-derived Tregs (tTregs) that develop in thymus and express multiple surface markers. Naive T cell migrating to peripheral tissues can differentiate into different CD4+ T cell subsets including peripherally-derived Tregs (pTregs). The have robust expression of FOXP3. In addition to these naturally-occurring Tregs, CD4+ T cells can differentiate into induced Tregs (iTregs) in vitro in the presence of several cytokines including IL-2 and TGF-β. (Adapted from Safinia et al., 2015)
**Figure 1.7 Applications of Treg-based therapies.** CD4+CD25<sup>hi</sup> or CD4+CD25<sup>hi</sup>CD127<sup>lo</sup> Tregs are freshly isolated from a donor or from a patient’s blood and expanded *in vitro* to generate sufficient numbers of antigen-specific cells. (A) In case of HSCT, donor or cord blood Tregs could be used for GvHD prophylaxis. (B) Patient’s Tregs are used to prevent solid organ transplantation. (C) Patient’s Tregs could be used to treat certain autoimmune diseases. (Adapted from Trzonkowski et al., 2015)
<table>
<thead>
<tr>
<th>Marker</th>
<th>Alternative name or identifier</th>
<th>Function</th>
<th>Relevance to Treg immunotherapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foxp3</td>
<td>Forkhead box protein 3</td>
<td>Transcription factor, master regulator of Treg development and function</td>
<td>Identifies Treg lineage in mice, expressed in human CD4+ Tregs</td>
</tr>
<tr>
<td>CD25</td>
<td>IL-2 receptor a-chain</td>
<td>IL-2 receptor component</td>
<td>Expressed by CD4+ Foxp3+ Tregs but also other T cells</td>
</tr>
<tr>
<td>CD127</td>
<td>IL-7 receptor a-chain</td>
<td>IL-7 receptor</td>
<td>Negative Treg marker</td>
</tr>
<tr>
<td>ICOS</td>
<td>Inducible T cell costimulator</td>
<td>Costimulator on T cells</td>
<td>Involved in Treg expansion and IL-10 production</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T lymphocyte antigen 4</td>
<td>Coinhibitory receptor</td>
<td>Important mechanism of Treg suppressive function</td>
</tr>
<tr>
<td>CD45RO</td>
<td>Leukocyte common antigen (RO isoform)</td>
<td>Protein tyrosine phosphatase, receptor type, C</td>
<td>Positive Treg marker, also identifies memory T cell</td>
</tr>
<tr>
<td>GITR</td>
<td>Tumor necrosis factor superfamily member 18 (TNFRS18)</td>
<td>Cell signaling</td>
<td>Important mechanism of Treg suppressive function</td>
</tr>
</tbody>
</table>

Figure 1.8 Treg markers and their relevance to immunotherapy. Currently, there are numerous Treg markers associated with specific function of the cells. In this list, these are the most commonly used markers to immunophenotyped Tregs. (Adapted from Singer et al., 2014)
<table>
<thead>
<tr>
<th>Therapeutic agent</th>
<th>Commercial name and company</th>
<th>Autoimmune disease</th>
<th>Clinical status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pegylated IFNβ</td>
<td>PLEGRIDY™; Biogen Idec</td>
<td>Relapsing-remitting multiple sclerosis</td>
<td>Clinical trial, Phase III</td>
</tr>
<tr>
<td>Certolizumab pegol</td>
<td>Cimzia®; UCB Pharma, Smyrna, GA</td>
<td>Crohn’s disease, rheumatoid arthritis, psoriatic arthritis</td>
<td>FDA approved</td>
</tr>
<tr>
<td>Glatiramer acetate</td>
<td>Compaxone®; Teva Pharmaceuticals</td>
<td>Relapsing-remitting multiple sclerosis</td>
<td>FDA approved</td>
</tr>
<tr>
<td>Rapamycin (Sirolimus)</td>
<td>Rapamune®; Pfizer Inc.</td>
<td>Type 1 diabetes</td>
<td>Clinical trial, Phase I</td>
</tr>
<tr>
<td>Emapticap pegol</td>
<td>Spiegelmer®; NOX-E36; NOXXON Pharma AG</td>
<td>Type 2 diabetes mellitus, systemic lupus erythematosus</td>
<td>Clinical trial phase I, (NCT00976729)</td>
</tr>
</tbody>
</table>

**Figure 1.9 Clinical applications of nanotherapeutic agents in autoimmune diseases.** Currently, there are five different immune-targeted therapeutics modified by nanocarriers to treat several autoimmune diseases including multiple sclerosis and rheumatoid arthritis. (Adapted from Gharagozloo et al., 2015)
Figure 1.10 Development of oxanorbornene-based block-type CPPMs. (a) The first inspiration for the development of cell-penetrating peptides (CPPs) is coming from HIV-1 TAT protein. Pep-1, the first chimera-derived CPP, was developed and had both hydrophilic and hydrophobic characteristics that enable increased cellular penetration ability as compared to TAT. Similar to Pep-1, polyarginine (R9), a synthetically made CPP, exhibited better delivery efficiency as well as lower cellular toxicity. However, in recent discoveries, multiple polymeric-based structures that mimic the properties of CPPs were developed, designated as CPPM. Among these scaffolds, oxanorbornene-based CPPMs were found to be the most efficient delivery agents for various cargos including siRNA, proteins, and antibodies. (b) Structure of phenyl-functionalized, guanidinium-rich polyoxanorbornene-based block-type CPPM. (Adapted from deRonde and Tew, 2015)
CHAPTER 2

PKC\(\theta\) IMPACTS ALTERNATIVE \textit{IFNG} MRNA REGULATION BY NOTCH1-ASSOCIATED HNRNPU AND PCMT1 IN CD8\(^+\) T CELLS TO INDUCE APLASTIC ANEMIA

2.1 Introduction

Severe aplastic anemia (AA) is a rare acquired, immune-mediated bone marrow failure (BMF) syndrome characterized by pancytopenia and hypocellular bone marrow. It results primarily from autoimmune destruction of hematopoietic stem and progenitor cells and compromised stromal cell integrity (Young et al., 2008). In most cases of AA, the etiology is unknown and AA can be fatal if not treated (Dezern and Brodsky, 2011). Evidence suggests autoreactive T helper type-1 (Th1) lymphocytes are instrumental in mediating disease. Circulating T cells from AA patients express high levels of the Th1 transcriptional regulator, T-BET, as well as intracellular NOTCH1, which we previously showed directly regulates T-BET (Solomou et al., 2006; Roderick et al., 2013). In diseased BM, elevated IFN-\(\gamma\) and TNF-\(\alpha\) suppress hematopoiesis and damage stromal cells lining the hematopoietic niches through bystander effects (Giannakoulas et al., 2004; Chen et al., 2004). Bone marrow transplantation (BMT) is curative for AA although most of the patients develop graft-versus-host disease (GvHD) due to genetic mismatch and non-responsive immunosuppressive therapy upon BMT; therefore, elucidating cellular mechanisms that drive AA is critically important for identifying novel therapeutic targets (Dezern and Brodsky, 2011; Scheinberg, 2012).
Signaling through the T cell receptor (TCR) and costimulatory molecule, CD28, induces phosphorylation of protein kinase C-theta (pPKCθ), a novel, Ca\(^{2+}\)-independent member of the PKC family, through a process that can be selectively inhibited \textit{in vitro} by the serine/threonine kinase inhibitor, rottlerin, at low concentrations (Ono et al., 1988; Springael et al., 2007). Physical redistribution of pPKCθ within fluid microdomains of T cell membranes allows its accumulation at the T cell-APC interface (Kong et al., 2011). There it facilitates assembly of the CARMA1-BCL10-MALT1 (CBM) complex, a macromolecular signaling aggregate critical for T cell survival, proliferation, and differentiation (Wang et al., 2004; Matsumoto et al., 2005). The transmembrane receptor, NOTCH1, interacts physically with PKCθ and is indispensable for CBM complex assembly, suggesting PKCθ and NOTCH1 function within intersecting signaling pathways (Shin et al., 2014). In mature T cells, NOTCH1 mediates survival, proliferation, differentiation, and cytokine production in response to antigenic stimulus (Osborne and Minter, 2007; Adler et al., 2003; Palaga et al., 2003; Zhang et al., 2011). A role for aberrant NOTCH1 signaling in autoimmune disease, including AA, is also emerging (Roderick et al., 2013; Palaga and Minter, 2013). Moreover, PKCθ potentially seems to be a unique therapeutic target for prevention of graft-versus-host disease (GvHD), caused by bone marrow transplantation to cure AA, since interfering with its downstream function increases the threshold for T cell activation (Ozay et al., 2016).

It is reported that aplastic anemia is mainly driven by the augmented production of IFN-γ (encoded by \textit{ifng} gene in mouse) that accelerates the destruction of hematopoietic stem cells and their progenitors by the increased population of activated CD8\(^+\) T cells in the bone marrow (Chen et al., 2015). Especially, the critical component
of BMF causing the anemia is through IFN-γ-producing CD8⁺ T cells (Gravano et al., 2016). So far, NOTCH1 has been shown to directly upregulate T-BET expression and subsequently leads to elevated IFN-γ expression (Roderick et al., 2013; Dongre et al., 2014). However, inhibiting NOTCH1 partially reduces IFN-γ production suggesting that it regulates IFN-γ via an intermediary molecule (Dongre et al., 2014). Furthermore, PKCθ has been correlated with upregulated IFN-γ expression through T-BET regulation. Patient with aplastic anemia exhibited increased binding of T-BET to ifng promoter due to hyperactivation of T-BET. However, T-BET and IFN-γ levels returned to normal once PKCθ function was blocked using rottlerin (Solomou et al., 2006; Ozay et al., 2016). An interesting observation about the constant expression of IFN-γ in AA mouse model showed the increased half-life of ifng mRNA upon deletion of AU-rich element (ARE) on 3’ untranslated region (3’ UTR) of ifng mRNA. ifng AREs caused destabilization of the mRNA due to aberrant modifications of RNA-binding proteins (RBPs) bound to those elements (Lin et al., 2014; Hodge et al., 2014; Schoenborn and Wilson, 2007; Hodge et al., 2002; Matoulkova et al., 2012). RBPs bound to ifng ARE were not properly phosphorylated, thus, mediated mRNA decay (Ogilvie et al., 2009).

Recently, PKCθ kinase activity was shown to regulate SC35 phosphorylation from its RNA recognition motif and, thus, altered its association with target mRNA and splicing function (McCuaig et al., 2015). SC35 and several other RBPs cooperatively regulate mRNA processing upon T cell activation (Lin et al., 2008; Lemaire et al., 1999; Chandler et al., 1997; Cazalla et al., 2002; Zhu et al., 2001). In light of these explanations, here we explored a role for pPKCθ and NOTCH1 signaling on ifng mRNA regulation in activated CD8⁺ T cells in the context of AA. We investigated how both
pPKCθ and NOTCH1 are required to drive IFN-γ-driven AA *in vivo*. Later, we demonstrated *in vitro* that pPKCθ and NOTCH1 directly controlled binding of a RNA stability and export factor, hnRNPU, and a protein-RNA methyltransferase, PCMT1, to *ifng* mRNA upon T cell activation. Our data, derived from AA patient samples and an established mouse model of AA, demonstrate pPKCθ is required for progression in AA, and likely mediates *ifng* mRNA processing and stability in CD8+ T cells through NOTCH1-associated RNA binding proteins in the context of AA progression.

### 2.2 Materials and Methods

#### 2.2.1 Animals

Animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Massachusetts Amherst. F1 progeny (9-12 weeks) were obtained from BALB/c×C57BL/6 crosses (Jackson Laboratory; Bar Harbor, Maine). PKCθ+/− (C57BL/6) ×GFP+/− (C57BL/6) crosses generated PKCθ+/−GFP+/− mice.

#### 2.2.2 Antibodies

Flow cytometric antibodies used in this study were purchased as: (1) BD Biosciences: mouse CD4 (PerCP, Clone: RM4-5), human CD4 (APC, Clone:RPA-T4), mouse CD8 (PE/Cy7, Clone: 53-6.7), human CD8 (PE/Cy7, Clone: RPA-T8), IFN-γ (PE, Clone: DB-1), (2) eBioscience: NOTCH1 (PE, Clone: mN1A), T-BET (eFluor660, Clone: 4B10), (3) Cell Signaling Technology: PKCθ (PE, Clone: E1I7Y), pPKCθ
(Unconjugated, Thr538), Anti-rabbit IgG (H+L), F(ab’)2 fragment secondary antibody (AF488), (4) GeneTex: NOTCH1 (FITC, Clone: mN1A), (5) ThermoFisher: Anti-rabbit IgG (H+L), F(ab’)2 fragment secondary antibody (Qdot625).

Western blot antibodies used in this study were acquired as: (1) Cell Signaling Technology pPKCθ (anti-rabbit, Thr538), PKCθ (anti-rabbit, Clone: E1I7Y), NOTCH1 (anti-rabbit, Clone: D1E11), NOTCH1IC (anti-rabbit, Val1744), GSK3β (anti-rabbit, Clone: D5C5Z), pGSK3α/β (anti-rabbit, Ser21/9), Histone Deacetylase 1 (anti-rabbit, polyclonal), hnRNPLL (anti-rabbit, polyclonal), (2) Sigma Aldrich: Actin (anti-mouse, Clone: AC40), Tubulin (anti-mouse, Clone: B-5-1-2), (3) Santa Cruz Biotechnology: pSC35 (anti-mouse, Clone: SC-35), (4) ProteinTech: hnRNPU (anti-rabbit, polyclonal), (5) LifeSpan Biosciences (LSBio): PCMT1 (anti-rabbit, polyclonal).

2.2.3 BMF induction and analyses

F1 progeny were irradiated (3Gy,137Cs source); 4 to 6 h later, BMF was induced with 5×10^7 splenocytes (intraperitoneal injection) from age- and sex-matched C57BL/6 (WT or PKCθ−/−) donors. Mice were harvested day +17 or +31. For survival studies, mice were humanely euthanized when no longer able to eat or drink. BM cells were flushed from tibias and femurs with 5% FBS/PBS. Splenocytes were passed through 40 µm filters, RBCs lysed with ACK buffer, and enumerated using trypan blue exclusion. Circulating WBCs and RBCs were counted using a HemaTrue Hematology Analyzer (Heska).
2.2.4 Rottlerin administration

For proof-of-concept studies, rottlerin (10mg/kg/day) was administered 1 h post-BMF induction, continuing daily until day +17, when animals were humanely euthanized. For therapeutic administration, rottlerin treatment began day+10 (20mg/kg/day) and ceased day +17. Mice were harvested day +17 or followed for survival.

2.2.5 Histology

Sterna harvested day +17 were fixed overnight in 10% NBF (VWR), decalcified 48 h (Cal-Rite; Richard Allen Scientific), preserved in 70% ethanol at 4°C until processed, paraffin-embedded, sectioned, and stained with hematoxylin and eosin.

2.2.6 T cell isolation and in vitro assays

Spleens were isolated, processed through 40 µm filters (BD Biosciences) and ACK lysis buffer. CD4+ and CD8+ T cells were isolated using anti-mouse CD4 and CD8 magnetic particles (IMag; BD Biosciences) and plated at 2.25–3×10^6 cells/well in 12-well plates pre-coated with anti-CD3ε and anti-CD28 (from145-2c11 and 37N hybridoma cell lines, respectively) adsorbed with anti-Hamster IgG (Sigma-Aldrich). WT T cells were treated with DMSO or rottlerin (3µM; Sigma-Aldrich) at time of or 24h after, plating as specified.
2.2.7 Patient samples and healthy controls

PBMCs from six, treatment-naïve patients with severe AA were obtained from the National Marrow Donor Program Research Sample Repository. PBMCs from six healthy donors (STEMCELL Technologies) were included as controls. Control or patient PBMCs (10^6 cells/ml) were pre-incubated with DMSO or rottlerin (3µM; Sigma-Aldrich) 30min at 37°C before stimulating 24 to 72h with 5µg/ml plate-bound anti-CD3ε (UCHT1) and 2.5µg/ml anti-CD28 (clone 37407; both R&D Systems). IFN-γ was determined using standard ELISA assays (BD Biosciences).

2.2.8 Surface and intracellular staining for flow cytometry

Murine samples were surface-stained with PerCP-anti-CD4 (RM4-5) and PE/Cy7-anti-CD8a (53-6.7). For intracellular staining, cells were fixed/permeabilized as above, stained with PE-anti-NOTCH1 (mN1A; eBioscience) and PE-anti-IFN-γ (DB-1; BD Biosciences) following manufacturers’ protocols. For IFN-γ staining, cells were harvested and restimulated on anti-CD3ε-coated plates 5h with Brefeldin A (GolgiPlug; BD Biosciences). Human samples were surface-stained with APC-anti-CD4 (RPA-T4) and PE/Cy7-anti-CD8 (RPA-T8). For intracellular staining, cells were fixed/permeabilized as above and stained with PE-anti-NOTCH1 (mN1A; all eBioscience). Samples were acquired on an BD LSR flow cytometer and analyzed using FACS Diva (BD Biosciences) or FlowJo (Tree Star) software.
2.2.9 Validating phosphorylated PKCθ using flow cytometry

Whole cell lysates were made in RIPA buffer (150 mM NaCl, 1% IgeCal-CA 360, 0.1% SDS, 50 mM Tris, pH 8.0, 0.5% sodium deoxycholate). 40µg total protein from DMSO- or rottlerin–treated, stimulated murine WT CD4⁺ and CD8⁻ T cells was resolved by SDS-PAGE, immunoblotted with anti-pPKCθ (Thr538; Cell Signaling Technology) and anti-actin (AC-40; Sigma-Aldrich), and amplified by ECL (Amersham). Aliquots from the same replicate were stained for CD4 or CD8 expression, fixed/permeabilized following manufacturer’s instructions (Cytofix/Cytoperm Kit, BD Biosciences), incubated 30 min with anti-pPKCθ (Cell Signaling Technology) washed, and incubated 30min with AlexaFluor488-anti-rabbit IgG (Cell Signaling Technology). Samples were acquired using an BD LSR flow cytometer as described.

2.2.10 Immunoblotting of whole cell, nuclear, and cytoplasmic extracts upon rottlerin and LiCl treatments

Murine splenic CD8⁺ T cells were harvested from WT or PKCθ⁻/⁻ spleens via magnetic separation. WT CD8⁺ T cells were pre-treated with either 3 µM rottlerin or 5 mM LiCl (Sigma Aldrich) for 30 minutes at 37°C. Subsequently, they were either left unstimulated (t=0 h) or stimulated for 2 and 24 hours on anti-CD3- and anti-CD28-coated wells. At corresponding time points, whole cell lysates were made in RIPA buffer (150
mM NaCl, 1% IgeCal-CA 360, 0.1% SDS, 50 mM Tris, pH 8.0, 0.5% sodium deoxycholate). Cytosolic and nuclear proteins were extracted using NE-PER™ Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific) per the manufacturer’s instructions. Lysates were denatured and 1X SDS Laemmli buffer was added into the samples prior to transfer onto 8% SDS-PAGE for molecular weight separation. Proteins were transferred on a nitrocellulose membrane and blocked in Blotto (5% dry milk, 0.2% Tween-20 in PBS). Membranes were probed overnight with primary antibodies. Later, membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies (Amersham). Membranes were incubated in Clarity™ Western ECL substrates (BioRad) prior to developing them on the X-ray film.

2.2.11 AMNIS imaging flow cytometry

Murine splenic CD8+ T cells with or without rottlerin treatment were harvested at 24 hours of anti-CD3 and anti-CD28 stimulation. Cells were fixed and permeabilized according to the manufacturer’s directions using the foxp3 staining buffer kit (BD Biosciences, Billerica, MA) and stained for NOTCH1 FITC and pPKCθ (Thr538) followed by Qdot625-labeled secondary antibody. Nuclei were stained using the cell-permeable DRAQ5™ fluorescent probe (ThermoFisher scientific, Waltham, MA). The cells were visualized and quantified using an ImageStream®X Mark II imaging flow cytometer (EMD Millipore, Billerica, MA). Subcellular colocalization and corresponding similarity scores of notch1 and pPKCθ (Thr538) proteins were determined using
colocalization wizard and on the IDEAS ® software to quantify the percentage of CD8⁺ T cells colocalized notch1 and pPKCθ.

2.2.12 Immunoprecipitation and western blot

Murine splenic CD4⁺ T cells were harvested at 24 hours of anti-CD3 and anti-CD28 stimulation. Cells were lysed in immunoprecipitation lysis buffer (50mM HEPES, pH 7.8, 250mM NaCl, 1% NP-40, Pro tease + Phosphatase inhibitors). DynaBeads (Protein G) were coupled with 3 µg of NOTCH1 (D1E11, Cell Signaling Technology) or rabbit IgG XP control antibody (DA1E, Cell Signaling Technology) in presence of 1% BSA in PBS and incubated for 2 hours at 4°C with rotation. After the incubation, the antibody-coupled DynaBeads were washed six times with 1 mL of immunoprecipitation wash buffer (Tris-HCl, pH 8.0, 200 mM NaCl, 0.1% NP-40). Finally, cell lysates were incubated with antibody-coupled DynaBeads for 1 hour at 4°C using rotator. Subsequently, they were washed beads six times with 0.5 mL of immunoprecipitation wash buffer. 1X SDS Laemmli Buffer was added into the samples for running on 8% SDS-PAGE for western blot. The blots were probed with several antibodies specific to each protein for further analysis.

2.2.13 RNA immunoprecipitation

Murine splenic CD8⁺ T cells were harvested at 24 hours of anti-CD3 and anti-CD28 stimulation. Cells were lysed in RNA immunoprecipitation lysis buffer (50mM
HEPES, pH 7.8, 250mM NaCl, 1% NP-40, 1X Protease + Phosphatase inhibitors, 100 U/ml RNase inhibitor). DynaBeads (Protein G) were coupled with 3 µg of hnRNPU (ProteinTech), hnRNPLL (Cell Signaling Technology), PCMT1 (LSBio) or IgG controls (mouse IgG: Santa Cruz Biotechnology, rabbit IgG: Cell Signaling Technology) antibody in presence of 1% BSA in PBS and incubated for 2 hours at 4°C with rotation. After the incubation, the antibody-coupled DynaBeads were washed six times with 1 mL of immunoprecipitation wash buffer (Tris-HCl, pH 8.0, 200 mM NaCl, 0.1% NP-40). Finally, cell lysates were incubated with antibody-coupled DynaBeads for 1 hour at 4°C using rotator. Subsequently, they were washed beads six times with 0.5 mL of immunoprecipitation wash buffer + 100U/mL RNase inhibitor. The samples were divided into two equal fractions. 1X SDS Laemmlli Buffer was added into one of the fractions to run on 8% SDS-PAGE for western blot. The blots were probed with antibodies for multiple proteins. The other half of the fraction was used to extract total RNA via Quick-RNA Isolation Kit (Zymo Research) according to the manufacturer’s protocol to further use on RT-PCR experiments.

2.2.14 Quantitative real-time PCR (qPCR)

Total RNA was isolated from murine samples with the and concentrated with the via Quick-RNA Isolation Kit (Zymo Research) according to the manufacturer’s protocol. 1 µg of total RNA was reverse transcribed to cDNA using dNTPs (New England Biolabs, Inc.), M-MuLV reverse transcription buffer (New England Biolabs), oligo-DT (Promega), RNase inhibitor (Promega), and M-MuLV reverse transcription (New
England Biolabs, Inc.) on a Mastercycler gradient Thermal Cycler (Eppendorf). qPCR primers (Pair #2) for *ifng* are listed in 2.2.15. qPCR was performed in duplicate with 2x SYBR Green qPCR Master Mix (BioTool) using the RealPlex® system (Eppendorf). qPCR conditions were as follows: 95°C for 1 min, 95°C for 25 s, 62°C for 25 s (40 cycles), 95°C for 1 min, 62°C for 1 min, and 95°C for 30 s. Relative gene expression was determined using the ΔΔCt method. The results are presented as the fold change in gene expression normalized to the housekeeping gene β-actin for mouse cells and relative to untreated controls.

### 2.2.15 cDNA synthesis and reverse transcription PCR (RT-PCR)

300 µg of total RNA was used to convert cDNA (as previously described) with random hexamers (IDT) via reverse transcription (Promega). Specific primers were designed for *Actb* FW-((GGCTGTATTCCCTCCATCG), REV-(CCAGTTGGTAAACATGCCATGT) 154 bp amp, *Ifng* Pair#1 FW-(ATGAACGCTACACACTGCATC), REV-(CCATCCTTTTGGCCAGTTCCCTC) 182 bp amp, *Ifng* Pair#2 FW-(GGCCATCAGCAACAACATAA), REV-(AGATACAACCCCGCAATCACA) 499 bp amp, *Fasl* FW-(CGGTGGTATTTTCTGGTTCTGG), REV-(CTTGTGGTTTAGGGGCTG) 380 bp amp, *Notch1* FW-(CCCTTGCTCTGCCTAACGC), REV-(GGAGTCCTGGCATCGTGG) 162 bp amp. and used to set up PCR by using Phusion® High Fidelity DNA polymerase (New England Biolabs). PCR conditions were the same as manufacturer’s protocol for Phusion®. Later, PCR amplicon was used to load on a 2% agarose gel for detection via electrophoresis.
2.2.16 Enzyme-linked immunosorbent assay (ELISA)

CD8⁺ T cell culture supernatants were collected at designated timepoints and analyzed for cytokine secretion. 96-well Maxisorp plates were coated overnight at 4°C with the appropriate capture antibody (anti-mouse IFN-γ or anti-mouse IL-2; BD Biosciences, San Diego, CA). Nonspecific protein binding was prevented by blocking wells with 10% FBS in PBS for 2 hours at room temperature on a rocker. Culture supernatants and standards were diluted appropriately and added to wells. The plate was incubated overnight at 4°C, with continuous rocking. Biotinylated detection antibodies were added to wells followed by TMB substrate reagents (BD Biosciences) at a 1:1 ratio. Color development was monitored, and the reaction was terminated by the addition of stop solution (2 N H₂SO₄). Absorbance was read at 450 nm using a microplate reader. Cytokine concentrations were determined relative to the standard curves generated.

2.2.17 Cytometric bead array

Plasma cytokine levels were determined using Th1/Th2/Th17 cytometric bead array kits (BD Biosciences) following manufacturer’s protocols. Data were acquired on an LSRII flow cytometer and analyzed using FCAP array software (BD Biosciences).

2.2.18 Statistics

Data are the mean ± SEM; in vitro and in vivo experiments were repeated at least three times. Unpaired, two-tailed student’s t test, one-way with Tukey’s post-test (Prism 5; GraphPad Software) were used for statistical comparison. Survival benefit was
determined using Kaplan–Meier estimates with applied log-rank test. P values of $\leq 0.05$ were considered significant.

2.3 Results

2.3.1 T cells from AA mice and human express elevated pPKCθ

pPKCθ signaling is required for full T cell activation (Palaga and Minter, 2013). We used conventional immunoblotting to validate a flow cytometric approach for assessing intracellular pPKCθ (Thr538) (Figure 2.1a, b), then examined expression of pPKCθ in disease-inducing T cells using a well-characterized, lymphocyte transfer model of AA (Roderick et al., 2013). To induce disease, we transferred splenocytes from C57BL/6 (WT) mice into F1 hybrid offspring of a C57BL/6 by BALB/c cross then evaluated disease severity on day +17. Compared to irradiated-only control mice, we detected significantly more pPKCθ in BM- and spleen-infiltrating CD4$^+$ and CD8$^+$ T cells of AA mice (Figure 2.2a-d), suggesting pPKCθ may contribute to BMF in a mouse model of AA. In addition to this, studies reported that PBMCs from AA patients express elevated Th1-associated proteins and rottlerin treatment (inhibition of PKCθ phosphorylation) decreases Th1 markers (Solomou et al., 2006). Moreover, IFN-γ expression by aberrant T cells has been shown to drive AA (Lin et al., 2014). We previously noted elevated NOTCH1$^{IC}$ in peripheral T cells from AA patients, and showed it was enriched at the promoter regulating T-BET, which is the master transcription factor for $ifng$ gene expression (Roderick et al., 2013). Intrigued by these overlapping findings, we asked whether rottlerin treatment also affected NOTCH1$^{IC}$ expression in AA patient
samples. We first measured pPKCθ in PBMCs from a cohort of treatment-naïve AA patients. Compared to healthy controls, PBMCs from AA patients expressed significantly more pPKCθ in CD4+ and CD8+ T cells (Figure 2.2e, f). Next, we asked how inhibiting PKCθ in patient PBMCs affects NOTCH1IC expression. We stimulated PBMCs from healthy donors or from AA patients for 72 hours in the presence of rottlerin or DMSO. NOTCH1IC levels were greatly reduced in rottlerin-treated CD4+ and CD8+ T cells from healthy donors and AA patients, compared to DMSO-treated cells (Figure 2.1c, d). Thus, although AA patient samples express significantly more pPKCθ, they respond equivalently-well to rottlerin-treatment. We subsequently quantified IFN-γ produced by stimulated PBMCs, treated with rottlerin or DMSO. PBMCs from both cohorts responded to rottlerin treatment by secreting significantly less IFN-γ (Figure 2.1e), compared to DMSO-treated controls, indicating rottlerin effectively reduces expression of key inflammatory proteins associated with AA pathology. Together, these findings demonstrate pPKCθ is elevated in T cells from mice and humans with AA, and PBMCs from AA patients respond to interrupted PKCθ signaling by down-regulating NOTCH1IC expression and IFN-γ secretion.

2.3.2 PKCθ regulates NOTCH1IC and IFN-γ expression

Individually, NOTCH1 and PKCθ were shown to be essential for T cell activation but the signaling hierarchy between PKCθ and NOTCH1 in T cells has not been previously investigated (Osborne and Minter, 2007; Adler et al., 2003; Chen et al., 2015; Dongre et al., 2014; Lin et al., 2015). We stimulated WT T cells with or without rottlerin, at concentrations shown to selectively inhibit pPKCθ over the structurally-similar
pPKCδ, then assessed NOTCH1IC expression (Gschwendt et al., 1994; Roderick et al., 2013). Rottlerin-treated, stimulated CD4+ and CD8+ T cells showed significantly reduced NOTCH1IC, as measured by median fluorescence intensity (MFI; Figure 2.3a, c). We noted equivalently reduced NOTCH1IC when PKC0−/− T cells were stimulated under similar conditions (Figure 2.3b, d).

NOTCH1 can regulate IFN-γ expression in murine T cells; therefore, we asked whether inhibiting PKC0 affects NOTCH1-mediated IFN-γ secretion (Dongre et al., 2014; Roderick et al., 2013). Compared to DMSO-treated cells, abrogating PKC0 phosphorylation with rottlerin significantly reduced IFN-γ in CD4+ and CD8+ T cells (Figure 2.3e, g). We also observed low IFN-γ in stimulated PKC0−/− CD4+ and CD8+ T cells (Figure 2.3f, h). Altogether, these results suggest evidence for PKC0 regulates NOTCH1 and IFN-γ expression in activated T cells.

2.3.3 PKC0 in CD8+ T cells is necessary to induce BMF in AA mice

Aberrant PKC0 signaling facilitates development of various diseases, including T-ALL, GvHD, and numerous autoimmune conditions; therefore, we asked whether the elevated pPKC0 we observed was a cause or a consequence of AA (Ozay et al., 2016; Isakov, 2012; Zhang et al., 2013). We transferred splenocytes from WT or PKC0−/− mice and evaluated disease at +17 or +31 days post-BMF-induction. Mice receiving PKC0−/− splenocytes showed greater BM cellularity (Figure 2.4a, b), did not experience the marked weight loss that is characteristic of this AA model (Figure 2.4c), and had significantly higher levels of circulating white and red blood cells (Figure 2.5a, b) compared to AA mice receiving WT splenocytes. Hallmark characteristics of human AA
include BM-infiltration by autoreactive T cells and elevated pro-inflammatory cytokines, such as IFN-γ and TNF, findings paralleled in AA mice (Zeng and Katsanis, 2015; Young, 2013). Compared to AA mice induced with WT cells, mice receiving PKC0−/− splenocytes had negligible BM-infiltrating CD4+ and CD8+ T cells (Figure 2.4d), and significantly lower circulating IFN-γ and TNF (Figure 2.4e, f). Thus, at +17 days, and even up to +31 days after BMF-induction we did not detect overt disease when we transferred PKC0−/− splenocytes. To confirm PKC0−/− cells were unable to induce BMF, we transferred WT or PKC0−/− splenocytes into AA mice and monitored their survival. Mice receiving WT splenocytes succumbed to disease, on average, 21 days after disease-induction. However, mice receiving PKC0−/− cells survived until they were removed from the study +200 days after lymphocyte transfer (Figure 2.4g).

PKC0−/− T cells exhibit defective IL-2 production, and this impairs their proliferation both in vitro and in vivo (Saibil et al., 2007; Deenick et al., 2010). We therefore asked whether PKC0−/− T cell expansion was compromised in AA mice. We transferred WT-GFP+ or PKC0−/−-GFP+ splenocytes and tracked donor cell expansion in vivo, by monitoring GFP+ cells in blood, spleen, BM, and lymph nodes of AA mice during disease progression. We noted robust expansion of WT-GFP+ cells beginning day+12 in all lymphoid tissues we examined (Figure 2.6a). In sharp contrast, PKC0−/−-GFP+ cells were barely detectable in these tissues (Figure 2.6b), consistent with previous reports using a similar, albeit non-identical, in vivo model. These data demonstrate PKC0 signaling is necessary for BMF induction and progression in a mouse model of AA.
We next sought to determine how PKC\(\theta\) in CD4\(^+\) and CD8\(^+\) T cells contributes to disease progression. We induced BMF in AA mice using various combinations of WT-GFP\(^/-\) and PKC\(\theta\)^{-/-}-GFP\(^+/\) cells (Figure 2.6c) and determined whether CD4\(^+\) or CD8\(^+\) T cells, or both, were essential for mediating disease. When WT CD8\(^+\) T cells were co-transferred, we found BMF was independent of PKC\(\theta\) expression in the non-T cell compartment. We observed similar trends in weight change and circulating WBCs and RBCs except when WT CD8\(^+\) T cells were co-transferred; the disease was more severe (Figure 2.6d-f). Unexpectedly, we were unable induce BMF when we transferred PKC\(\theta\)^{-/-} CD8\(^+\) T cells, regardless of whether PKC\(\theta\) was expressed in CD4\(^+\) T cells or APCs transferred at the same time (Figure 2.6g-i, compare graphs above blue circles with those above red boxes). Collectively, these results provide strong evidence that PKC\(\theta\) functions in the T cell compartment to mediate BMF in AA mice, and its expression in CD8\(^+\) T cells is absolutely required to mediate disease.

### 2.3.4 Rottlerin treatment inhibits PKC\(\theta\) signaling \textit{in vivo} and attenuates AA in mice

We administered rottlerin to WT mice for one week to verify the effects of PKC\(\theta\) inhibition on NOTCH1\(^{IC}\) expression \textit{in vivo} (Figure 2.7a). In proof-of-principle experiments, we induced AA in mice and administered rottlerin daily, beginning one hour after disease induction. Rottlerin effectively prevented BMF in treated mice (Figure 2.7b-j). Rottlerin treatment did not adversely affect host naïve T cells, which were present in
spleens at levels equivalent to those of irradiated control mice (Figure 2.7k) and suggests rottlerin preferentially targets proliferating T cells.

We next evaluated the therapeutic potential of inhibiting PKC0 under clinically relevant conditions. We administered rottlerin (20mg/kg/day) to AA mice for 1 week beginning day +10 and continuing until day +17, at which time treatment was discontinued. Control animals received DMSO according to the same dosing schedule. Some mice were humanely sacrificed on day +17 to evaluate disease. As a result of rottlerin treatment, BM cellularity was nearly equivalent to that of irradiated control mice (Figure 2.8a, b). Mice displayed minimal weight changes (Figure 2.8c) and had robust circulating white and red blood cell counts (Figure 2.5c, d). Furthermore, percentages of BM-infiltrating CD4\(^+\) and CD8\(^+\) T cells were significantly lower than in DMSO controls (Figure 2.8d). Circulating IFN-\(\gamma\) and TNF in rottlerin-treated AA mice were significantly diminished compared to DMSO-treated animals (Figure 2.8e, f). When we analyzed bone marrow-infiltrating CD8\(^+\) T cells specifically, we found that they had significantly lower pPKC0, NOTCH1, T-BET, and IFN-\(\gamma\) expression in rottlerin-treated animals although we did not observe any change in total PKC0 (Figure 2.8g-k). These data strongly suggest rottlerin, especially through its effects on PKC0–expressing, bone marrow-infiltrating CD8\(^+\) T cells, dampens the immune processes responsible for mediating disease progression in BMF, even when given for a defined length of time at the peak of disease.

We further evaluated the survival benefit of targeting PKC0 in AA mice. We induced mice with BMF and treated them either with DMSO or rottlerin for only one week, as described above, then allowed disease to develop without further therapeutic intervention.
All mice treated with DMSO succumbed to lethal BMF by day +25. Remarkably, rottlerin enhanced survival in greater than 50% of treated mice (Figure 2.8l). Collectively, this report provides compelling evidence PKCθ is elevated during immune-mediated BMF in humans and mice. Moreover, therapeutic targeting of PKCθ effectively attenuates BMF in AA mice and prolongs their survival.

2.3.5 PKCθ and NOTCH1 interact in CD8⁺ T cells

Previously, we showed that PKCθ and NOTCH1 interacted physically in CD4⁺ T cells upon T cell activation (Shin et al., 2014, 2006). Upon T cell stimulation, fully active PKCθ has a phosphorylation at Thr538 and NOTCH1 gets cleaved by gamma secretase to generate its intracellular form (NOTCH1IC) (Ozay et al., 2016; Roderick et al., 2013; Dongre et al., 2014). Since we observed PKCθ expression in CD8⁺ T cells was necessary to drive AA and these data showed overlapping observations with contribution of NOTCH1 to the progression of AA, we asked whether PKCθ and NOTCH1 cooperatively act in CD8⁺ T cells (Roderick et al., 2013). We first looked at cytoplasmic and nuclear distribution of both total NOTCH1 and PKCθ as well as their active form, NOTCH1IC and pPKCθ (Thr538) upon T cell stimulation. We performed western blot experiments and calculated adjusted densities based on the cytoplasmic and nuclear loading controls, Tubulin and HDAC1 levels, respectively (Figure 2.9a, b). The spatiotemporal analysis showed that total PKCθ and pPKCθ (Thr538) are mainly localized in the cytosol at early time points (Figure 2.9c, d). However, total NOTCH1 and NOTCH1IC were already localized both in the cytosol and nucleus in unstimulated cells.
and its expression was increased upon T cell stimulation. Interestingly, PKC\(\theta^+\) and rottlerin-treated CD8\(^+\) T cells had significantly lower levels of total NOTCH1 and NOTCH1\(^{IC}\) (Figure 2.9e, f). We observed the robust protein expression by 24 hours upon stimulation. At this time point, we detected both total PKC\(\theta\) and total NOTCH1 predominantly localized in the cytosol but also in the nucleus (Figure 2.10a, b). We observed that rottlerin diminished total PKC\(\theta\) and inhibited its phosphorylation (Figure 2.10a, c). Additionally, nuclear localization and protein levels of total NOTCH1 and NOTCH1\(^{IC}\) were still significantly reduced in rottlerin-treated and PKC\(\theta^-^-\) CD8\(^+\) T cells (Figure 2.10b, d). These results demonstrate that PKC\(\theta\) activation affects NOTCH1 localization and generation of NOTCH1\(^{IC}\) in CD8\(^+\) T cells.

Next, we investigated the physical interaction of NOTCH1 and PKC\(\theta\) in CD8\(^+\) T cells upon T cell stimulation. We immunoprecipitated total NOTCH1 and probed for total PKC\(\theta\) and pPKC\(\theta\) (Thr538) at 24 hours of stimulation when we saw robust NOTCH1 expression in these cells. We observed that NOTCH1 interacts with PKC\(\theta\) and its phosphorylated form, pPKC\(\theta\) (Thr538) both in the nucleus and cytosol. However, this interaction was diminished upon rottlerin treatment suggesting that PKC\(\theta\) activation was necessary for physical interaction with NOTCH1 (Figure 2.10e). Moreover, we performed imaging flow cytometry to quantitatively assess their colocalization in higher sensitivity. In consistent with immunoprecipitation data, we saw that rottlerin treatment significantly reduced the colocalization of pPKC\(\theta\) (Thr538) and NOTCH1 in CD8\(^+\) T cells (Figure 2.10f). All these results indicate that PKC\(\theta\) and NOTCH1 cooperatively act in CD8\(^+\) T cells upon activation.
2.3.6 PKCθ and GSK3β activity counteract on NOTCH1 regulation

PKCθ and NOTCH1 appeared to be important in CD8+ T cell activation and PKCθ activity positively regulated NOTCH1 activation. The molecular mechanism how PKCθ activates NOTCH1 remained unclear. Based on the literature findings, PKCθ may compete with other kinases to regulate NOTCH1 cleavage and activity (Prager et al., 2007; Shin et al., 2014; Foltz et al., 2002; Kirschbaum et al., 2001; Walter et al., 1997). Glycogen synthase kinase 3-beta (GSK3β) was reported to regulate NOTCH1 cleavage, transcriptional activity, and its intracellular localization (Espinosa et al., 2003; Foltz et al., 2002; Han et al., 2012). Interestingly, GSK3β also regulates PKCθ-dependent signaling pathways (Gruber et al., 2009b; Moore et al., 2013; Taylor et al., 2016; Hoesel and Schmid, 2013; Steinbrecher et al., 2005). It was also found that PKCθ partially inhibits GSK3β activity by phosphorylating it from Ser 9 (Gruber et al., 2009b). Therefore, we investigated whether GSK3β and PKCθ affects NOTCH1 collaboratively or not during T cell activation. We inhibited GSK3β activity by lithium chloride (LiCl) treatment as it was reported to increase its Ser9 phosphorylation and inhibited its activity. First, we looked at spatiotemporally whether PKCθ affected GSK3β Ser9 phosphorylation in CD8+ T cells. We did not see any change at early time points in total GSK3β levels (Figure 2.11a). In consistent with the reports, total GSK3β levels were significantly diminished in rottlerin-treated and PKCθ−/− CD8+ T cells whereas, LiCl-treated CD8+ T cells did not change their total GSK3β levels (Figure 2.12a). pGSK3β (Ser9) levels were significantly dropped in PKCθ−/− CD8+ T cells and, in contrast, LiCl-
treated CD8⁺ T cells increased pGSK3β levels as compared to DMSO-treated controls
during early stimulation (Figure 2.11b). At 24 hours of stimulation, effects on PKC0 inhibition were more prominent as pGSK3β was significantly inhibited both in the
cytosol and the nucleus. LiCl treatment increased the cytosolic pGSK3β although it did
not reach significance (Figure 2.12b). These data demonstrated that GSK3β was active
due to the loss of Ser9 phosphorylation when PKC0 was inhibited or absent.

When we measured the effect of GSK3β inhibition on PKC0 and NOTCH1, we saw that
cytosolic pPKC0 was significantly decreased while total PKC0 levels did not change
upon LiCl treatment at both early and late stimulation (Fig 2.11c, d, 2.12c, d). On the
other hand, we found that cytosolic NOTCH1 was significantly increased upon GSK3β inhibition at 24 hours of stimulation while NOTCH1IC was not affected (Figure 2.12e, f).
Also, cytosolic NOTCH1 was similar in both the treatments at early time points,
however, we noticed a significant decrease in NOTCH1IC levels upon GSK3β inhibition
at 2 hours of stimulation (Figure 2.11e, f). These results suggested that GSK3β and PKC0
counteract to each other and they both regulate NOTCH1 in different ways.

2.3.7 PKC0 regulates NOTCH1-associated RNA binding proteins in CD8⁺ T cells

To better define the molecular mechanisms behind cooperative regulation of
NOTCH1 and PKC0, we focused on common regulatory pathways that could be affected
by both NOTCH1 and PKC0. Consistent with previous reports, we also found that both
PKC0 and NOTCH1IC translocated to the nucleus and strongly associated with each
other. NOTCH1 acts as a transcriptional coactivator of immune-related genes and is important for transcriptional elongation (Dongre et al., 2014; Keerthivasan et al., 2011; Minter et al., 2005). A novel role of nuclear PKCθ was recently identified as a means of phosphorylating the splicing regulator, SC35, and increasing its localization in nuclear speckles (McCuaig et al., 2015). On the other hand, GSK3β was also reported to phosphorylate SC35 and reduces SC35 localization in the nuclear speckles (Hernández et al., 2004). Interestingly, PKCθ and GSK3β phosphorylation site on SC35 overlapped indicating opposite roles in RNA processing by these kinases (Hernández et al., 2004; McCuaig et al., 2015). Since we showed that PKCθ and GSK3β activity regulated SC35 in different ways in different cell types, one could suggest that PKCθ and GSK3β may differentially control RNA binding proteins in CD8 T activation. First, we wanted to see the regulation of pSC35 in CD8+ T cells. Interestingly, we found that pSC35 was solely localized in the cytosol in CD8+ T cells and as expected, it was reduced in rottlerin-treated or PKCθ−/− CD8+ T cells whereas, was increased in LiCl-treated CD8+ T cells at 24 hours of stimulation (Figure 2.11g). This result suggested that GSK3β activity could limit PKCθ function in phosphorylating SC35.

We assessed several other RNA binding proteins that could locate in the nucleus and regulated by PKCθ and GSK3β. We, for the first time, found that heterogeneous nuclear ribonucleoprotein-U (hnRNPU), heavily localized in the nucleus, was regulated by both kinases in opposite fashion. More surprisingly, we found only one RNA binding protein, protein-L-isoaspartate-(D-aspartate) O-methyltransferase 1 (PCMT1), that was similarly regulated by PKCθ and GSK3β and heavily localized in the cytosol. hnRNPU is
known to shuttle between nucleus and cytosol and is mainly responsible to regulate mRNA stability and stable mRNA nuclear export (Yugami et al., 2007; Valente and Goff, 2006; Järvelin et al., 2016; Davis et al., 2002). On the other hand, PCMT1 has been shown to methylate proteins and is in complex with RNA nuclear export complex (Farrar et al., 2005; Dufu et al., 2010). Interestingly, based on our KEGG MOTIF search, we found that it contains a unique RrmJ/FtsJ-like methyltransferase domain that methylates 28S ribosomal RNA (rRNA) at the ribose 2'-OH group residing in 60S subunit of ribosome (Tan et al., 2002; Bügl et al., 2000), thereby suggesting a potential rRNA methylation by PCMT1. We also wanted to confirm subcellular distribution of these proteins in CD8+ T cells. Although cytosolic hnRNPU was not heavily affected, nuclear hnRNPU was significantly diminished by the absence of PKCθ (Figure 2.12g). We observed that inhibition of PKCθ activity by rottlerin treatment significantly diminished both cytosolic and nuclear hnRNPU levels at later time point (Figure 2.12g). In contrast, inhibition of GSK3β activity by LiCl treatment significantly increased both cytosolic and nuclear hnRNPU at later time point (Figure 2.12g). Moreover, both PKCθ and GSK3β inhibition significantly reduced PCMT1 levels at later time point (Figure 2.12h). These data indicated that PKCθ was found to regulate novel RNA binding proteins, hnRNPU and PCMT1. Moreover, PKCθ and GSK3β regulated hnRNPU in opposite ways, however, affected PCMT1 in a similar fashion.

Next, we investigated NOTCH1 association with hnRNPU and PCMT1 in WT and PKCθ−/− CD8+ T cells. We immunoprecipitated both PCMT1 and hnRNPU at 24 hours after stimulation, and then probed with PKCθ, NOTCH1IC, pGSK3β (Ser9), PCMT1, and hnRNPU. Firstly, we found that hnRNPU and PCMT1 interacted with each
other. We identified both PCMT1 and hnRNPU protein complexes regulated by PKCθ although we found no physical association of PKCθ with those RNA binding proteins. In the absence of PKCθ, PCMT1 association with hnRNPU was significantly abrogated. On the other hand, NOTCH1IC and pGSK3β interactions with hnRNPU were not affected while their interactions with PCMT1 were diminished. Surprisingly, there was an increase in the NOTCH1IC-PCMT1 association in the absence of PKCθ (Figure 2.13a). These observations were also consistent when PKCθ phosphorylation was inhibited upon rottlerin treatment although NOTCH1IC binding to PCMT1 was not affected (Figure 2.13b). Therefore, PKCθ itself but not its activity may be critical only for NOTCH1 association with PCMT1 and could be negligible for NOTCH1-hnRNPU interactions. All these results indicated that PKCθ selectively regulates NOTCH1-associated PCMT1 and hnRNPU protein complexes in CD8+ T cells.

2.3.8 PKCθ regulates ifng mRNA processing through NOTCH1-associated PCMT1 and hnRNPU interactions

Our results showed that hnRNPU was strongly localized in the nucleus while PCMT1 was in the cytosol. In addition, we found that hnRNPU and PCMT1 formed complexes with NOTCH1IC in CD8 T cells and their interactions were influenced by the absence of PKCθ. Therefore, we proposed whether PKCθ regulates RNA binding properties of NOTCH1-associated hnRNPU and PCMT1 complexes upon CD8 T cell activation in the context of AA progression. We know that NOTCH1 and PKCθ both
regulate IFN-γ production to drive the disease (Roderick et al., 2013; Minter et al., 2005; Ozay et al., 2016; Dongre et al., 2014; Palaga et al., 2003). Considering IFN-γ production as the main driver of the disease pathogenesis and its mRNA stability was controlled by RBP elements in AA, we investigated \( \text{ifng} \) mRNA processing in CD8\(^+\) T cells. First, we identified UTR regions and exons of \( \text{ifng} \) mRNA (Figure 2.14a). hnRNPU has been shown to associate with AU- and GU-rich elements on exons and 3’UTR sequences (Cok et al., 2003; Meininger et al., 2016; Vlasova-St. Louis and Bohjanen, 2014; Xiao et al., 2012b). In order to show if mature \( \text{ifng} \) mRNA associates with hnRNPU, we performed RNA immunoprecipitation (RNA-IP) followed by RT-PCR with primers designed for mature, stable \( \text{ifng} \) mRNA (Figure 2.14a). Upon T cell stimulation, we saw a strong binding of mature form of \( \text{ifng} \) mRNA to hnRNPU and this binding was significantly diminished in PKC\(\theta^{-/-}\) CD8\(^+\) T cells (Figure 2.13c). We also proved the strong interaction of \( \text{ifng} \) mRNA and hnRNPU by immunoprecipitating another T cell-associated RNA binding protein, hnRNPLL. hnRNPLL is known as a master regulator for RNA processing in T cells (Cho et al., 2014; Wu et al., 2008). We also showed that \( \text{ifng} \) mRNA bound to hnRNPLL, however, not as strong as hnRNPU (Figure 2.14b). This binding was gene-specific as we did not observe any significant hnRNPU association with mRNAs of other disease-related genes, \( \text{fasl} \) and \( \text{notch1} \) (Figure 2.14c, d). Because we did not detect \( \text{ifng} \) mRNA-hnRNPU association in PKC\(\theta^{-/-}\) CD8\(^+\) T cells, we asked whether these cells lacked \( \text{ifng} \) ARE on their 3’UTR. Therefore, we designed primers that could amplify \( \text{ifng} \) ARE specifically (Figure 2.14a). Subsequent to RNA-IP for hnRNPU, we performed RT-PCR with these newly designed primers. Interestingly, we found that only WT CD8\(^+\) T cells had the expected amplicon for \( \text{ifng} \) 3’UTR suggesting hnRNPU bound to \( \text{ifng} \)
mRNA only in WT CD8\(^+\) T cells. More surprisingly, we found a longer amplicon (approximately 1,300 bp long) that was found to bind hnRNPU in PKC\(^{0/−}\) CD8\(^+\) T cells. This result suggested that \(ifng\) mRNA was immature (probably unstable) in PKC\(^{0/−}\) CD8\(^+\) T cells due to intron retention caused by dysregulation of RNA processing (Figure 2.13d).

We further raised the question if PKC\(^{0/−}\) CD8\(^+\) T cells lacked \(ifng\) mRNA at all since we did not get any amplification via RT-PCR with any of the primer pairs in RNA-IP experiment. Therefore, we amplified the total \(ifng\) mRNA present in cells with both primer pairs at 24 hours of T cell stimulation. Although we saw different amplicons, both WT and PKC\(^{0/−}\) CD8\(^+\) T cells predominantly had mature \(ifng\) mRNA indicating that the loss of hnRNPU association was not because of the absence of \(ifng\) transcript (Figure 2.13e). We also wanted to investigate if inhibiting PKC\(0\) activity reduced \(ifng\) mRNA-hnRNPU association. We found that rottlerin-treated cells had \(ifng\) mRNA and 3'UTR ARE sequence as it was shown by RT-PCR via two different primer pairs (Figure 2.13e). Additionally, rottlerin-treated CD8\(^+\) T cells still had mature \(ifng\) mRNA-hnRNPU interaction indicating that PKC\(0\) itself but not its kinase activity was required to have \(ifng\) mRNA-hnRNPU association (Figure 2.13f). In our previous results, we showed that rottlerin treatment also significantly diminished IFN-\(γ\) production similar to PKC\(^{0/−}\) CD8\(^+\) T cells, yet we still found hnRNPU interacting with stable \(ifng\) mRNA transcript as seen in WT CD8\(^+\) T cells.

It is shown that hnRNPU, localized both in the nucleus and the cytosol, mediates stable mRNA export to the cytosol once it is bound to mRNA. Nevertheless, stably
exported mRNA needs to be properly loaded to the ribosomes for efficient translation (Gross et al., 2003; Kawai et al., 2006; Chaudhury et al., 2010). Therefore, we postulated that stable, mature ifng mRNA may not be properly loaded to the ribosomes for the translation. Our findings noted that PCMT1 was similarly dysregulated in rottlerin-treated and PKCθ−/− CD8+ T cells. Moreover, PCMT1 has a unique RrmJ-like methyltransferase domain that is known to methylate 28S rRNA thus, could mediate translational elongation (Edward and Maden, 1998; Sloan et al., 2017). Taking these findings into consideration, we hypothesized that PCMT1-ifng mRNA association would be reduced in both rottlerin-treated and PKCθ−/− CD8+ T cells thereby preventing its ribosomal loading for efficient translation. To test the hypothesis, we also performed RNA-IP for PCMT1 and ifng mRNA. Unlike ifng mRNA-hnRNPU association in rottlerin-treated CD8+ T cells, we found that there was a significant decrease in ifng mRNA-PCMT1 association upon rottlerin treatment and the interaction was completely lost in PKCθ−/− CD8+ T cells (Figure 2.13f). For further confirmation, we also quantified the amount of ifng mRNA associated with either hnRNPU or PCMT1 via RNA-IP combined with qPCR. qPCR results showed that while only rottlerin-treated CD8+ T cells had significantly reduced ifng mRNA-PCMT1 association, PKCθ−/− CD8+ T cells exhibited significant loss of both ifng mRNA-hnRNPU and ifng mRNA-PCMT1 interactions (Figure 2.13g). All these data demonstrated that PKCθ impacts ifng mRNA processing regulated by NOTCH1-associated hnRNPU and PCMT1 protein complexes in CD8+ T cells in the context of AA progression.
2.4 Discussion

Although the immune-mediated pathology of AA is well-described, we know surprisingly little about the mechanisms that drive its progression. Our results firmly place contribution of PKC\(\theta\) and NOTCH1 in mediating AA-associated pathogenesis. Cross-talk between NOTCH and PKC has been reported in multiple systems. Signaling cascades mediated by PKC\(\alpha\) and NOTCH4 converge in some instances of endocrine resistant breast cancer, and earlier reports demonstrated synergy between PKC\(\theta\) and NOTCH\(^{IC}\) in T-ALL models with activating NOTCH\(^{IC}\) mutations (Yun et al., 2013; Giambra et al., 2012). How PKC\(\theta\) modulates NOTCH1 activity in normal T cells remains ill-defined. Our data reveal a previously undescribed requirement for PKC\(\theta\) regulating NOTCH1\(^{IC}\) to modulate its expression.

How PKC\(\theta\) regulates Th cell-mediated processes remains controversial. Reports suggest it is required for Th2 responses, but is dispensable for generating Th1-mediated antiviral, as well as memory T cell responses (Marsland et al., 2005, 2004). Accumulating evidence supports a function for PKC\(\theta\) in autoimmunity, including experimental autoimmune encephalomyelitis (EAE), rheumatoid arthritis, and myosin-induced autoimmune myocarditis (Tan et al., 2006; Healy et al., 2006; Marsland et al., 2007). In some of these models, especially EAE, PKC\(\theta\) is also required for pathogenic Th17 responses that are responsible for an overwhelming proportion of central nervous system destruction (Kwon et al., 2012). Th17 cells have also been identified in AA patients; however, their contribution to disease pathology has not been extensively explored (De Latour et al., 2010). Whether PKC\(\theta\) drives Th1 or Th17 differentiation, its
therapeutic targeting might provide a means of interrupting the activity of these pro-inflammatory T cell subsets during AA progression. In a mouse model of graft-versus-host disease, PKCθ was required to induce pathology, but not to clear viral pathogens or residual leukemic cells (Valenzuela et al., 2009). Thus, inhibiting PKCθ in T cells may abrogate their pathogenic activity, while preserving appropriate responses to infectious stimuli, a critical consideration in the treatment of AA.

Members of the PKC family share a high degree of structural homology which makes designing isoform-specific inhibitors challenging (Altman and Kong, 2014; Hage-Sleiman et al., 2015). Currently, several PKC inhibitors are in clinical trials, many being tested for potential use in solid organ transplant or to treat various malignancies (Hage-Sleiman et al., 2015). Although some, such as sotrastaurin (AEB071) show promise in early in-human testing, none specifically and uniquely inhibit PKCθ (He et al., 2014). However, we recently developed and utilized a novel strategy to target PKCθ in human T cells via highly specific, cell-penetrating antibodies (Ozay et al., 2016). Thus, different targeting methods could be utilized in understanding of PKCθ-regulated responses in T cells and, moreover, in creating inhibitors that can selectively modulate PKCθ-mediated biological responses. Using genetic and chemical approaches, we determined PKCθ is required to induce BMF in AA mice. We further identified a CD8+ T cell-specific requirement for PKCθ to drive disease progression. Experimental evidence suggests PKCθ signaling is complex and its activity in CD4+ versus CD8+ T cells is likely context-dependent (Saibil et al., 2007; Marsland and Kopf, 2008). In vitro, PKCθ−/− CD8+ T cells exhibit a marked survival defect that only moderately affects PKCθ−/+ CD4+ T cells
Moreover, we showed that PKCθ regulates NOTCH1-mediated downstream signaling pathway critical for AA pathogenesis. We found that either genetic deletion or chemically inhibition of PKCθ in activated CD8⁺ T cells reduced NOTCH1IC, T-BET, and IFN-γ expression in the mouse model. On the other hand, we found PKCθ regulates GSK3β activity in CD8⁺ T cells. In consistent with the previous reports, it appeared that GSK3β also controls NOTCH1 localization and expression, yet different than PKCθ. Data in this report, together with evidence from the literature, suggest PKCθ and GSK3β compete to differentially regulate NOTCH1 signaling pathway. However, it is likely that there are PKCθ-dependent and NOTCH1-independent gene regulation networks and further investigation is needed to fully elucidate details of these interacting signaling cascades.

Aplastic anemia is mainly driven by the proinflammatory cytokine, IFN-γ, that accelerates the destruction of hematopoietic stem cells and their progenitors by activated CD8⁺ T cells in the bone marrow (Chen et al., 2015). Especially, IFN-γ-producing CD8⁺ T cells were found to be detrimental as adoptive transfer experiments with CD8⁺ T cells resulted in the fast progression of the disease (Gravano et al., 2016). IFN-γ production by NOTCH1-T-BET signaling axis was reported to cause AA progression (Roderick et al., 2013; Minter, 2013; Minter et al., 2005). Additionally, PKCθ has been correlated with upregulated IFN-γ expression through T-BET regulation in AA patients (Solomou et al., 2006). In our findings, we showed that genetic and chemical inhibition of PKCθ in CD8⁺ T cells abrogated NOTCH1-T-BET-IFN-γ signaling axis. Also, we showed that PKCθ directly bound and modulated NOTCH1IC expression and nuclear localization. Thus, it is
likely that PKC0 directly impacts NOTCH1 to control IFN-γ production.

Mechanistically, constant expression of IFN-γ in AA mouse model was controlled through mRNA stability by RNA-binding proteins (RBPs) bound to those elements (Hodge et al., 2014). RBPs bound to ifng ARE were post-translationally modified to control ifng mRNA stability (Ogilvie et al., 2009). We, for the first time, demonstrated that PKC0 regulated NOTCH1-associated novel RNA binding proteins, hnRNPU and PCMT1. Genetic deletion or chemical inhibition of PKC0 reduced ifng 3′UTR stability and prevented the interaction of RNA binding proteins (RBPs) and ifng mRNA suggesting the dysregulated post-transcriptional ifng mRNA processing.

The mRNA transcripts encoding some of the major effector molecules of an immune response are tightly controlled during T cell activation and differentiation program depending on the need of a cell to form different isoforms of immunomodulatory proteins (Ganguly et al., 2016). Therefore, we need to better understand how RBPs are affected by T cell signaling to alter 3′UTR shortening, intron retention, alternative exon formation, mRNA stability, nuclear export, and translation efficiency (Kafasla et al., 2014; Moulton et al., 2013; Wang et al., 2001a; Gaudreau et al., 2012; Yabas et al., 2011). Various RBPs including hnRNPU modified by T cell signaling differentially operate T cell activation, cytokine signaling, and apoptosis (Ganguly et al., 2016; Palanisamy et al., 2012; Kafasla et al., 2014; Yugami et al., 2007). hnRNPU is a predominantly nuclear protein that contains arginine-glycine-rich region as well as RNA recognition motif. Also, it is a nuclear matrix protein, therefore called scaffold attachment factor A (SAF-A), that mediates mRNA nuclear export (Xiao et al., 2012b).
We also found a novel RNA binding protein, PCMT1, has an important role in CD8+ T cell activation. PCMT1 was reported to be involved in critical cellular processes such as RNA maturation, stability, export, translational, and post-translational control (Enünlü et al., 2003; Yang et al., 2013; MacKay et al., 2012; Shi et al., 2017). Proteomic studies showed that the absence of PCMT1 diminished the amount of two crucial RNA regulators, poly(rC)-binding protein 2 (PCBP2) involved in mRNA stability and DX39B involved in mRNA export to the cytoplasm, in the whole proteome (Yang et al., 2013). Additionally, PCMT1 is a part of mRNA nuclear export complex and interacts with several key RNA binding proteins such as hnRNPU, hnRNPD, hnRNPA2, and hnRNPM (Dufu et al., 2010). Among those, hnRNPU is methylated by a protein methyltransferase implicating the change of receiving extracellular stimuli that influences RNA maturation and transport (Herrmann et al., 2004). We found that PCMT1 shares structural similarities with two other common protein and RNA methyltransferases, PRMT (S-adenosylmethionine-dependent protein arginine methyltransferase) and PIMT (PRIP-interacting protein with methyltransferase domain), respectively (Herrmann et al., 2009; Enünlü et al., 2003). These observations raised the question about the possibility of co-regulation of hnRNPU and PCMT1. Based on our findings, PCMT1 and hnRNPU interacted with each other in CD8+ T cells. More interestingly, we found that they formed individual complexes associated with NOTCH1IC. Although we observed that PKCθ did not physically interact, its genetic deletion or chemical inhibition altered NOTCH1 association with PCMT1 but not with hnRNPU. However, PKCθ impacted hnRNPU binding to ifng mRNA. In the absence of PKCθ, hnRNPU did not associate with mature, stable ifng mRNA. Instead, we saw a longer, unstable ifng mRNA transcript associated
with hnRNPU. However, rottlerin-treated CD8+ T cells still showed ifng mRNA-hnRNPU association indicating that there are other compensatory mechanisms in activated WT CD8+ T cells. Surprisingly, both rottlerin-treated and PKC0−/− CD8+ T cells failed to associate their ifng mRNA with PCMT1. Considering PCMT1 playing a role in mRNA loading to ribosomes and efficient translation, we think that IFN-γ production was significantly diminished in rottlerin-treated and PKC0−/− CD8+ T cells implying loss of CD8+ T cell function in the context of AA progression. The possible explanation why we did not observe any difference in ifng mRNA-hnRNPU association in rottlerin-treated CD8+ T cells could be because, as shown in Figure 5e, they still had nuclear PKC0-NOTCH1 complex while these cells had less cytosolic NOTCH1-PKC0 complex. Given the fact that hnRNPU in the nucleus and PCMT1 in the cytosol, nuclear NOTCH1-PKC0 complex may still help hnRNPU to associate with ifng mRNA, however, PCMT1 function could be impaired due to loss of cytosolic NOTCH1-PKC0 complex.

Collectively, we report elevated pPKC0 in a mouse model of AA as well as in human PBMCs of treatment-naïve AA patients. Therapeutic targeting of PKC0 during the peak of disease significantly prolonged survival of AA mice. As with all animal models, these results need to be extrapolated to human disease with care. Detailed analyses of pPKC0 levels in AA patients before and after immunosuppressive treatment will confirm the clinical relevancy of our observations. Overall, we provide compelling evidence that PKC0 collaborates with NOTCH1IC in activated CD8+ T cells to contribute to AA pathogenesis through ifng mRNA stability, export, and translation. Moreover, inhibiting PKC0 with rottlerin robustly destabilized ifng mRNA processing and reduced
CD8$^+$ T cell activation, thus suggests further investigating PKCθ as a therapeutic target in treating aplastic anemia may be warranted.
Figure 1.9 Clinical applications of nanotherapeutic agents in autoimmune diseases.
Currently, there are five different immune-targeted therapeutics modified by nanocarriers to treat several autoimmune diseases including multiple sclerosis and rheumatoid arthritis.

Figure 2.1 Validating a flow cytometric assay for measuring pPKCθ in murine and human T cells.
Flow cytometric detection of phosphorylated PKCθ was validated using conventional immunoblotting methods. Wild-type splenic T cells were treated either with DMSO or the dose-dependent PKCθ inhibitor, rottlerin, (3 μM). Cells were left unstimulated (naïve) or stimulated with anti-CD3ε plus anti-CD28 for 48 hours. (a) Samples were divided and whole cell lysates from half of the samples were probed with anti-pPKCθ, (pPKCθ; Thr538). Blots were stripped and reprobed with anti-β-actin as a loading control. (b) The other half of corresponding samples were stained, intracellularly, for pPKCθ (Thr538), and its expression was quantified using flow cytometry. (c, d) Peripheral blood mononuclear cells (PBMCs) from healthy controls or from patients with AA were pre-treated either with DMSO or rottlerin (3μM) then stimulated for 72 hours with anti-CD3ε and anti-CD28. We evaluated NOTCH1IC levels in (c) CD4+ and (d) CD8+ T cells using flow cytometry. (e) We used ELISA to quantify IFN-γ secretion in supernatants of cultures treated as in c and d. n=3-6; Data are the mean ± SEM and represent three independent experiments; *p < 0.05, **p < 0.01, ***p < 0.001; (c-e) one-way ANOVA with Tukey’s post-test applied.
Figure 2.2 T cells from AA mice and human express elevated pPKCθ. F1 hybrid mice were irradiated only (γIR controls) or induced with AA (BMF) and harvested 17 days after disease induction. pPKCθ expression was determined in CD4+ and CD8+ T cells isolated from (a, b) bone marrow and (c, d) spleen via flow cytometry. In addition, we assessed pPKCθ expression in (e) CD4+ and (f) CD8+ T cells from healthy controls and from patients with AA who had not received prior IST. n=3-6; Data are the mean ± SEM of three independent experiments. *p < 0.05, **p < 0.01; unpaired, two-tailed student’s t test applied.
Figure 2.3 PKCθ regulates NOTCH1IC and IFN-γ expression. We used flow cytometry to assess NOTCH1IC expression in WT (a) CD4+ and (c) CD8+ T cells treated with DMSO or rotterlin and in (b) CD4+ and (d) CD8+ T cells from PKCθ−/− mice stimulated 48 hours with anti-CD3ε and anti-CD28. IFN-γ expression in DMSO- or rotterlin-treated (e) CD4+ and (g) CD8+ T cells or in (f) CD4+ and (h) CD8+ T cells from PKCθ−/− mice was also quantified using flow cytometry 48 hours after stimulation with anti-CD3ε and anti-CD28. Data are the mean ± SEM of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001; unpaired, two-tailed student’s t test applied.
Figure 2.4 PKCθ in CD8+ T cells is necessary to induce BMF in AA mice. AA was induced in the F1 hybrid offspring of C57BL/6 x BALB/c mating pairs by transferring splenocytes from age- and gender-matched WT or PKCθ-/- donor mice; AA mice were sacrificed 17 days or 31 days later, as noted. Disease severity was assessed in mice, by measuring (a) bone marrow cellularity, (b) Representative hematoxylin and eosin staining of bone marrow sternum collected from one mouse each, given only irradiation (left panel; γIR control), or given splenocytes from WT (middle panel; WT BMF) or from PKCθ-/- donor mice (right panel, PKCθ-/- BMF). Scale bar represents approximately 100 μm, (c) weight change, and (d) CD4+ and CD8+ T cell infiltration into the bone marrow. Circulating (e) IFN-γ and (f) TNF cytokine levels were assessed using cytometric bead array via flow cytometry. (g) Kaplan-Meier survival estimates of mice induced with WT (n=7) or PKCθ-/- splenocytes (n=5). Mean or Mean ± SEM are depicted and represent at least three independent experiments; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; (a-f) one-way ANOVA with Tukey’s post-test or (g) log rank test applied.
Figure 2.5 White and red blood cell counts in PKC0+/− and Rottlerin-treated AA mice. AA was induced in the F1 hybrid offspring of C57BL/6 x BALB/c mating pairs by transferring splenocytes from age- and gender-matched WT or PKC0+/− donor mice; AA mice were sacrificed 17 days or 31 days later, as noted. The number of (a) white blood cells and (b) red blood cells were measured. Also, WT splenocytes were treated either with DMSO or rottlerin (20mg/kg/day) beginning 10 days after disease induction and continuing for 7 days, at which time treatment was discontinued. Some mice were sacrificed 17 days after BMF was induced (the day DMSO or rottlerin treatment was discontinued). The number of (c) white and (d) red blood cells were counted in these mice.
Figure 2.6 PKCδ splenocytes do not expand in recipient mice and CD8+ T cells require PKCδ to induce AA in mice. AA mice were induced either with (a) WT-GFP+ or (b) PKCδ−/−-GFP+ splenocytes and sacrificed 3, 6, 9, 12, 13, 15, or 17 days later. At each time point, percent GFP+ T cells was quantified in the spleen, lymph nodes (cervical, axillary, mesenteric, inguinal), peripheral blood and bone marrow of AA mice; n=3-11. (c) Schematic of add-back combinations of WT cells that did not express GFP (WT) and GFP-expressing PKCδ−/− cells (KO) used to determine in which cellular subset PKCδ is required to mediate BMF. We evaluated disease severity in AA mice infused with combinations of WT and PKCδ−/− cells, including (d) weight change, peripheral (e) white, (f) red blood cell counts, (g) BM cellularity, (h) CD4+, and (i) CD8+ T cells into the bone marrow.
Figure 2.7 Rottlerin treatment attenuates AA in mice when administered at time of BMF induction. (a) F1 progeny were administered rottlerin (10mg/kg/day) for five days then CD4+ T cells were isolated and stimulated for 48 hours ex vivo with antibodies specific for CD3ε and CD28. Whole cell lysates were generated, proteins separated by SDS PAGE, and immunoblotted with antibodies specific for pPKCθ, NOTCH1IC, and β-actin. WT splenocytes were used to induce AA in mice, which were then treated either with DMSO or rottlerin (10mg/kg/day) beginning 1 hour after disease induction and continuing for 17 days, at which time treatment was discontinued. (b) Disease severity was assessed in mice by evaluating bone marrow cellularity. (c) Representative hematoxylin and eosin staining of bone marrow sternum collected from one mouse each on day +17 and receiving only irradiation and DMSO, as vehicle control (top left panel; γIR+DMSO), irradiation and rottlerin (top right panel, γIR+Rottlerin), or receiving splenocytes from WT donor mice and treated either with DMSO (bottom left panel; BMF+DMSO) or with rottlerin (bottom right panel; BMF+Rottlerin). Scale bar represents approximately 100 µm, (d) weight change, and peripheral (e) white and (f) red blood cell counts were measured in DMSO- and rottlerin-treated AA mice. We used flow cytometry to quantify (g) total bone marrow-infiltrating T cells and (h) CD4+ and CD8+ T cell infiltration into the bone marrow as well as circulating (i) IFN-γ and (j) TNF in DMSO- and rottlerin-treated AA mice. (k) We analyzed percentages of CD4+ and CD8+ T cells in spleens of AA mice receiving only irradiation (γIR+DMSO) and in mice which received rottlerin (BMF+Rottlerin); n=6-8. Data represent the mean ± SEM. *p <0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; One-way ANOVA with Tukey’s post-test applied.
at least three independent experiments; *p < 0.05, **p < 0.01, ***p < 0.001; (a) marrow. We also determined circulating approximately 100 µl of BMF+Rottlerin). Scale bar represents approximately 100 µm. (c) weight change, and (d) CD4+ and CD8+ T cell infiltration into the bone marrow. We also determined circulating (e) IFN-γ and (f) TNF in DMSO- and rottlerin-treated AA mice; n=2-6. The percent-positive cells and protein expression levels of (g) Total PKCθ, (h) pPKCθ (Thr538), (i) NOTCH1, (j) T-BET, and (k) IFN-γ in bone marrow-infiltrating CD8+ T cells were measured via flow cytometry (n=4-5), (l) Kaplan-Meier survival estimates of mice induced with WT splenocytes and treated with DMSO (n=5) or rottlerin (n=9). Data are the mean ± SEM and represent at least three independent experiments; *p < 0.05, **p < 0.01, ***p < 0.001; (a-f) one-way ANOVA with Tukey’s post-test, (g-k) unpaired, two-tailed student’s t test, and (l) log rank test applied.

Figure 2.8 Rottlerin treatment inhibits PKCθ signaling in vivo and attenuates AA in mice. WT splenocytes were used to induce AA in mice, which were then treated either with DMSO or rottlerin (20mg/kg/day) beginning 10 days after disease induction and continuing for 7 days, at which time treatment was discontinued. Some mice were sacrificed 17 days after BMF was induced (the day DMSO or rottlerin treatment was discontinued). Disease severity was assessed in mice by measuring (a) bone marrow cellularity, (b) Representative hematoxylin and eosin staining of bone marrow sternum collected from one mouse each on day +17 and given only irradiation and DMSO, as vehicle control (top left panel; γIR+DMSO), irradiation and rottlerin (top right panel; γIR+Rottlerin), or given splenocytes from WT donor mice and treated either with DMSO (bottom left panel; BMF+DMSO) or with rottlerin (bottom right panel; BMF+Rottlerin). Scale bar represents approximately 100 µm. (c) weight change, and (d) CD4+ and CD8+ T cell infiltration into the bone marrow. We also determined circulating (e) IFN-γ and (f) TNF in DMSO- and rottlerin-treated AA mice; n=2-6. The percent-positive cells and protein expression levels of (g) Total PKCθ, (h) pPKCθ (Thr538), (i) NOTCH1, (j) T-BET, and (k) IFN-γ in bone marrow-infiltrating CD8+ T cells were measured via flow cytometry (n=4-5), (l) Kaplan-Meier survival estimates of mice induced with WT splenocytes and treated with DMSO (n=5) or rottlerin (n=9). Data are the mean ± SEM and represent at least three independent experiments; *p < 0.05, **p < 0.01, ***p < 0.001; (a-f) one-way ANOVA with Tukey’s post-test, (g-k) unpaired, two-tailed student’s t test, and (l) log rank test applied.
Figure 2.9 Nuclear vs. cytoplasmic distribution of NOTCH1 and PKCθ in early T cell activation. CD8⁺ T cells were either left unstimulated (t=0 h) or stimulated for 2 hours with anti-CD3 and anti-CD28. Later, nuclear and cytoplasmic extracts were obtained from CD8⁺ T cells that were either treated with 3 µM Rottlerin, 5 mM LiCl or DMSO (vehicle control) as well as PKCθ⁺ CD8 T cells. They were run on SDS-PAGE and followed by immunoblotting. (a) The cytoplasmic loading control in different conditions was assessed by Tubulin and (b) the nuclear loading control was assessed by HDAC1. We probed the samples with (c) total PKCθ, (d) pPKCθ (Thr538), (e) total NOTCH1, and (f) NOTCH1IC (Val1744) specific antibodies. The adjusted densities were quantified via Image J software by normalizing to Tubulin (for cytosolic proteins) and to HDAC1 (for nuclear proteins) followed by normalization to DMSO samples. Data are the mean ± SEM and represent at least two independent experiments; ns: p>0.05, *p < 0.05, **p < 0.01, ***p < 0.001; one-way ANOVA with Tukey’s post-test applied.
**Figure 2.10 PKCθ and NOTCH1 interact in CD8+ T cells.** CD8+ T cells were harvested from bulk splenocytes in C57BL/6 WT or PKCθ−/− mice. WT CD8+ T cells were treated with 3 μM rottlerin for 30 minutes before anti-CD3 and anti-CD28 stimulation (DMSO was used as vehicle control). Later, the cells were stimulated for 24 hours. At the end of 24 hours, cytoplasmic and nuclear lysates were obtained from these cells and run on SDS-PAGE followed by immunoblotting with specific antibodies against (a) Total PKCθ, (b) Total NOTCH1, (c) pPKCθ (Thr538), and (d) NOTCH1IC (Val1744). The adjusted densities were quantified via Image J software by normalizing to Tubulin (for cytosolic proteins) and to HDAC1 (for nuclear proteins) followed by normalization to DMSO samples. (e) Also, the lysates were incubated and immunoprecipitated (IP) via DynaBeads coated with 2 μg of NOTCH1 (D1E11) antibody at 16 hours of stimulation. Later, IP fractions were immunoblotted for pPKCθ (Thr538), total PKCθ, NOTCH1, and NOTCH1IC (Val1744). IgG control was used as a loading control. (f) CD8+ T cells were stained for NOTCH1 and pPKCθ (Thr538) at 24 hours of stimulation and their colocalization was visualized via imaging flow cytometry using AMNIS IDEAS software. Colocalization similarity scores (higher colocalization score indicates more NOTCH1-pPKCθ colocalization), representative histograms indicating the frequency of CD8+ T cells colocalizing NOTCH1 and pPKCθ (Thr538), and representative images of control and rottlerin-treated CD8+ T cells are shown. Data are the mean ± SEM and represent at least two or three independent experiments; ns: p>0.05, *p < 0.05, **p < 0.01, ***p < 0.001; one-way ANOVA with Tukey’s post-test applied.
Figure 2.11 Early regulation of PKCθ and GSK3β signaling in CD8+ T cells. Nuclear and cytoplasmic extracts were obtained from CD8+ T cells that were either treated with 3 µM Rottlerin, 5 mM LiCl (GSK3β inhibition) or DMSO (vehicle control) as well as PKCθ- CD8+ T cells. The cells were either left unstimulated (t=0 h) or stimulated with anti-CD3 and anti-CD28 for 2 hours. The lysates were run on SDS-PAGE and followed by immunoblotting with (a) total GSK3β, (b) pGSK3α/β (Ser21/9), (c) total PKCθ, (d) pPKCθ (Thr538), (e) total NOTCH1, and (f) NOTCH1IC (Val1744) specific antibodies. (g) Spatiotemporal analysis of pSC35 in CD8+ T cells. The adjusted densities were quantified via Image J software by normalizing to Tubulin (for cytosolic proteins) and to HDAC1 (for nuclear proteins) followed by normalization to DMSO samples. Data are the mean ± SEM and represent at least two independent experiments; ns: p>0.05, *p < 0.05, **p < 0.01, ***p < 0.001; (a, b, g) one-way ANOVA with Tukey’s post-test, (c-f) unpaired, two-tailed student’s t test applied.
Figure 2.12 PKCθ and GSK3β activity counteract on NOTCH1 regulation. CD8+ T cells were harvested from bulk splenocytes in C57BL/6 WT or PKCθ−/− mice. WT CD8+ T cells were treated with 3 μM rottlerin or 5 mM LiCl for 30 minutes before anti-CD3 and anti-CD28 stimulation (DMSO was used as vehicle control). Later, the cells were stimulated for 24 hours. At the end of 24 hours, cytoplasmic and nuclear lysates were obtained from these cells and run on SDS-PAGE followed by immunoblotting with specific antibodies against (a) total GSK3β and (b) pGSK3α/β (Ser21/9). In addition, the levels of (e) total PKCθ, (d) pPKCθ (Thr538), (e) NOTCH1, and (f) NOTCH1IC (Val1744) after LiCl treatment were measured via western blot. Spatiotemporal distribution of RNA-binding proteins, (g) hnRNPU and (h) PCMT1 were shown in different conditions. The adjusted densities were quantified via Image J software by normalizing to Tubulin (for cytosolic proteins) and to HDAC1 (for nuclear proteins) followed by normalization to DMSO samples. Data are the mean ± SEM and represent at least two independent experiments; ns = p>0.05, *p < 0.05, **p < 0.01, ***p < 0.001; (a, b, g, h) one-way ANOVA with Tukey’s post-test applied, (c-f) unpaired, two-tailed student’s t test applied.
Figure 2.13 PKCθ regulates ifng mRNA processing through NOTCH1-associated PCMT1 and hnRNPU interactions. CD8⁺ T cells were harvested from bulk splenocytes in C57BL/6 WT or PKCθ−/− mice. WT CD8⁺ T cells were treated with 3 μM rottlerin for 30 minutes before anti-CD3 and anti-CD28 stimulation (DMSO was used as vehicle control). Later, the cells were stimulated for 24 hours. At the end of 24 hours, the whole cell lysates were immunoprecipitated (IP) with DynaBeads coated by 2 μg of Anti-PCMT1 or Anti-hnRNPU. IP fractions were run on SDS-PAGE followed by immunoblotting with specific antibodies. The association of PCMT1 and hnRNPU with target proteins were compared in (a) WT DMSO vs. PKCθ−/− lysates and (b) WT DMSO and WT Rottlerin lysates. (c) Mature ifng mRNA bound to hnRNPU fraction in WT and PKCθ−/− CD8⁺ T cells was assessed via RNA immunoprecipitation followed by RT-PCR using primer pair #1 (see section 2.2.15). (d) Mature ifng mRNA bound to hnRNPU was assessed via RT-PCR using primer pair #2 amplifying 3’UTR ARE sequence, the binding region of hnRNPU. Arrow indicates expected amplicon size, (e) Total ifng mRNA present in DMSO-treated, rottlerin-treated, and PKCθ−/− CD8⁺ T cells amplified by two different primer sets via RT-PCR. Arrow indicates expected amplicon size, (f) Mature ifng mRNA bound to PCMT1 and hnRNPU was assessed via RT-PCR, (g) Quantitative analysis of ifng mRNA bound to PCMT1 and hnRNPU in DMSO-treated, rottlerin-treated, and PKCθ−/− CD8⁺ T cells was performed via RNA-IP qPCR. Data are the mean ± SEM and represent at least two independent experiments; ns: p>0.05, *p < 0.05, **p < 0.01, ***p < 0.001; one-way ANOVA with Tukey’s post-test applied.
Figure 2.14 PKCδ regulates hnRNPU that selectively binds to *ifng* mRNA. (a) UTR and exon regions were highlighted in different colors on mouse *ifng* mRNA sequence. Also, 3'UTR ARE sequence was italicized and colored as purple. Primer pair #1 and #2 binding sites were underlined and bolded. Each exon and intron lengths were noted to predict potential intron retention based on the length of amplicon on agarose gel electrophoresis. (b) *Ifng* mRNA weakly bound to hnRNPLL was shown in WT and PKCδ−/− CD8+ T cells upon T cell activation (t=24h). To look at the selectivity, (c) *fasl* mRNA and (d) *notch1* mRNA bound to either hnRNPLL or hnRNPU were also amplified via RT-PCR (IgG control was used as negative control for RNA-IP experiment). M indicates marker for DNA length. Data represent the two independent experiments (n=2).
CHAPTER 3

INTRACELLULAR DELIVERY OF ANTI-pPKCθ (Thr538) VIA PROTEIN TRANSDUCTION DOMAIN MIMICS FOR IMMUNOMODULATION

3.1 Introduction

Protein kinase C enzymes comprise 3 subfamilies and 10 kinase isoforms that are structurally and functionally related (Isakov and Altman, 2012; Inoue et al., 1977). Different isoforms are activated either by proteolysis or translocation to the plasma membrane, where they associate with protein partners to mediate biological functions (Pfeifhofer-Obermair et al., 2012; Ho et al., 2001; Rosse et al., 2010). Among the PKC enzymes, protein kinase C-theta (PKCθ) exhibits a selective pattern of tissue distribution with a predominant expression in T lymphocytes, platelets, and skeletal muscle. It translocates to the center of the immunological synapse (IS) in activated CD4 T cells following the integration of T cell receptor (TCR) and CD28 costimulatory signals (Yokosuka et al., 2008). The full activation of PKCθ involves two steps: diacylglycerol (DAG) binding to its C1 domain and subsequent Threonine 538 (Thr538) phosphorylation within its activation loop (Wang et al., 2012; Kong et al., 2011; Seco et al., 2012; Chuang et al., 2011). PKCθ regulates multiple transcription factors including NF-κB, AP-1, and NFAT which, individually and combined, initiate signals that are critical for T cell activation, proliferation, and differentiation (Pfeifhofer et al., 2003; Marsland et al., 2004; Kwon et al., 2012; Zanin-Zhorov et al., 2010; Gupta et al., 2008). Recently, it has been suggested that PKCθ also translocates into the nucleus and
associates with a chromatin-bound complex to regulate microRNA and T cell-specific gene expression programs (Sutcliffe et al., 2011). Immunological studies show that distinct PKC isoforms use unique mechanisms to regulate various different functions and, thus, are attractive therapeutic targets for modulating T cell-mediated adaptive immune responses (Baier and Wagner, 2009; Zanin-Zhorov et al., 2011).

Monoclonal antibodies have emerged as potential therapeutics for many diseases such as cancer, infection, and autoimmune disorders due to their unequalled target specificity. Furthermore, advancements in genetic engineering have paved the way for “humanizing” mouse monoclonal antibodies, creating versions for clinical use that are promising due to their greater safety and selectivity. However, targets of these antibody-based biologics are currently limited to cell surface or extracellular proteins because of their inability to pass through the cellular membrane (Imai and Takaoka, 2006; Chan and Carter, 2010; Beck et al., 2010; Torchilin, 2009).

Intracellular delivery of biologically active molecules remains a significant challenge. In some cases, these therapeutics can be taken up via receptor-mediated endocytosis. However, cellular entry via endocytic pathway poses its own hurdles, including escape from endosomes and avoiding lysosomal degradation (Guillard et al., 2015). Therefore, how these macromolecules are designed and delivered are quite important. A novel approach to deliver such biologics involves using cell-penetrating peptides (CPPs), also known as protein transduction domains (PTDs), which are short sequences of peptides capable of translocating across the cell membrane. The first PTD identified was a short sequence of amino acids, consisting of the arginine-rich residues 48-60 of the HIV-1 TAT protein. Since the discovery of TAT, many cationic PTDs have
been reported including R9, penetratin, VP22, transportan, pVEC, and Pep-1 (Sgolastra et al., 2014). Although all are capable of crossing cellular membranes, synthesizing these peptides is challenging due to their structural complexities and most requires covalent attachment to their cargoes for delivery. Recent studies demonstrate that incorporating key features of PTDs into simpler, tunable scaffolds improves uptake for a broad range of cell types. Mimics of PTDs within these scaffolds facilitate fine-tuning the chemical composition of novel delivery agents for application-specific needs. For instance, successful design of polymeric mimics of PTDs, also called protein transduction domain mimics (PTDMs), provides an easy, synthetic platform to deliver biological cargo such as siRNA and proteins with superior efficiency (deRonde and Tew, 2015; Tezgel et al., 2012).

Herein, we describe a PTDM capable of delivering an antibody that recognizes and modulates the activity of the intracellular protein, phosphorylated PKCθ (Thr538), via its delivery into hPBMCs. Successful \textit{ex vivo} transport of antibodies into human immune cells lays the foundation to further develop this platform as a potential clinical modality, especially in the area of immunotherapy.

3.2 Materials and Methods

3.2.1 Materials

Anti-human phospho PKCθ (Thr538, Monoclonal Rabbit IgG, Clone: F4H4L1) was purchased from Invitrogen (Carlsbad, CA). Human PBMCs were obtained from StemCell Technologies, Inc. (Vancouver, BC, Canada). Antibodies specific for human CD25, CD4, CD45, CD8, NOTCH1, T-BET, IFNγ, and mouse CD45, and compatible
with flow cytometry were purchased from eBioscience, Inc. (San Diego, CA). Flow
cytometric data were acquired using an LSR II Flow Cytometer, LSRFortessa™ 5 laser
(Becton Dickinson, Canaan, CT) and analyzed using DIVA 7.0 software (Becton
Dickinson) or FlowJo (Treestar, Ashland, OR).

3.2.2 Synthesis of MePh₁₃-b-dG₅ (P₁₃D₅)

We have recently described the synthesis of PTDMs. P₁₃D₅ was obtained by
ring-opening metathesis polymerization (ROMP) using the Grubbs’ third generation
catalyst in dichloromethane. The final product was purified by dialysis against RO water
and obtained by lyophilization.

3.2.3 Characterization of MePh₁₃-b-dG₅ (P₁₃D₅)

The molecular weight of P₁₃D₅ was assessed by gel permeation chromatography.
After P₁₃D₅ was lyophilized, a sample was analyzed by GPC after calibration using a poly
(methyl methacrylate; PMMA) standard. The molecular weight was determined, and
polydispersity index was calculated. To confirm the chemical composition, proton
nuclear magnetic resonance spectroscopy (¹H-NMR) was performed. The sample was
diluted in deuterated acetonitrile (CD₃CN).

3.2.4 Dynamic Light Scattering (DLS)

To characterize the complexation of PTDM and antibody, 1 µM of P₁₃D₅ and 25
nM of anti-pPKCθ were complexed in PBS (phosphate buffered saline, pH 7.2) at a
specific ratio (P₁₃D₅: Anti-pPKCθ :: 40:1). It was incubated for 30 min at room
temperature (RT). Later, the complex was loaded into a spectrophotometric cuvette. The size of the complex was measured with a Malvern Zetasizer Nano ZSP instrument (Malvern Instruments, Ltd.)

3.2.5 Native Polyacrylamide Gel Electrophoresis and Silver Staining

4-20% Mini-PROTEAN TGX Precast protein gels were used to run P13D5: Anti-pPKCθ complex along with P13D5 only and Anti-pPKCθ only. Each was loaded as 250 µl into individual gels with 7 cm well size and run for 35 min at 200 V in 1X Running buffer (25 mM Tris, 192 mM Glycine, pH 7.2) without the addition of sodium dodecyl sulfate (SDS) for native conditions. The electrodes were swapped since the charge of the complex is positive. Later, the gels were stained with 0.1% silver nitrate and 0.08% formalin (37%). They were imaged using a Syngene G-box gel documentation system (Syngene, A Division of Synoptics, Ltd.).

3.2.6 P13D5: Anti-pPKCθ Complex Delivery into Human Peripheral Mononuclear Blood Cells (hPBMCs)

1 µM of P13D5 and 25 nM of anti-pPKCθ were complexed in PBS (phosphate buffered saline, pH 7.2) at a specific ratio (P13D5: Anti-pPKCθ :: 40:1) and AbDeliverIN™:Anti-pPKCθ was complexed in PBS at the ratio recommended by the manufacturer’s protocol. The complexes were incubated for 30 min at RT. hPBMCs were treated with the complexes for 4 hours at 37°C. Cells were then harvested and washed with PBS. Later, cells were thoroughly washed twice with 20 U/mL Heparin in PBS for 5 minutes on ice to remove surface-bound complexes outside cellular membrane. Pellets
were resuspended in fresh RPMI complete media (10% fetal bovine serum, 100 U/mL penicillin-streptomycin, 1 mM sodium pyruvate, 2 mM L-Glutamine) seeded into 5 µg/mL anti-CD3ε- plus 2.5 µg/mL anti-CD28-coated tissue culture wells and stimulated for 24 hours at 37°C. After stimulation, cells were harvested and washed with PBS. They were fixed and permeabilized (Foxp3 staining kit, eBioscience, according to manufacturer’s recommendation) for 30 min at 4°C, then stained with Alexa Fluor488-labeled anti-rabbit IgG (Cell Signaling Technology, Inc.) for 30 min at 4°C. Labeled cells were washed and analyzed by flow cytometry to measure delivery efficiency.

3.2.7 Cellular Viability Assay

hPBMCs were stained with 7-Aminoactinomycin D (7-AAD, eBioscience) for 15 min at RT, then pelleted by centrifugation for 5 min. Cells were resuspended in 0.2% BSA in PBS and analyzed by flow cytometry.

3.2.8 Flow Cytometric Analyses of Marker Expression

hPBMCs were treated with DMSO or 3 µM Rottlerin for 30 min or with P13D5: Anti-pPKC0 for 4 hours at 37°C. Following treatment, cells were washed with PBS and resuspended in fresh RPMI complete media. Later, P13D5: Anti-pPKC0-treated cells were thoroughly washed twice with 20 U/mL Heparin in PBS for 5 minutes on ice to remove non-internalized complexes. Cells were stimulated by seeding into anti-CD3ε- plus anti-CD28-coated wells and incubated at 37°C. Some cells were stimulated for 24 hours prior to treatment. At 24, 48, and 72 hours after treatment, cells were harvested and washed with PBS. Then, hPBMCs were stained with antibodies specific for surface (CD25,
CD69) or intracellular (NOTCH1IC, T-BET) proteins and analyzed by flow cytometry.

3.2.9 Cell Proliferation Assay

hPBMCs were treated with DMSO or 3 µM Rottlerin for 30 min or with P13D5: Anti-pPKCθ for 4 hours at 37°C. Following treatment, cells were washed with PBS and resuspended in fresh RPMI complete media. Later, P13D5: Anti-pPKCθ-treated cells were thoroughly washed twice with 20 U/mL Heparin in PBS for 5 minutes on ice. After washing with Heparin, cells were resuspended in pre-warmed PBS + 0.1% BSA and cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) dye using the Cell Trace™ CFSE Cell Proliferation Kit (Thermo Scientific, Inc.) following the manufacturer’s protocol. Cells were stimulated by seeding into anti-CD3ε- plus anti-CD28-coated wells and incubated at 37°C for 7 days. Cell proliferation was measured daily by flow cytometric analysis.

3.2.10 Enzyme Linked Immunosorbent Assay (ELISA) for Cytokine Determination

hPBMC culture supernatants were collected at designated timepoints and analyzed for cytokine secretion. 96-well Maxisorp plates were coated overnight at 4°C with the appropriate capture antibody (anti-human IFNγ or anti-human IL-2; BD Biosciences, San Diego, CA). Non-specific protein binding was prevented by blocking wells with 10% FBS in PBS for 3 hours at RT. Culture supernatants and standards were diluted appropriately and added to wells. The plate was incubated overnight at 4°C, with continuous rocking. Biotinylated detection antibodies were added to wells followed by TMB substrate reagents (BD Biosciences) at a 1:1 ratio. Color development was
monitored, and the reaction was terminated by the addition of stop solution (2 N H₂SO₄). Absorbance was read at 450 nm using a microplate reader. Cytokine concentrations were determined relative to the standard curves generated.

3.2.11 Nuclear vs. Cytosolic Protein Extraction

3x10⁶ cells/mL hPBMCs were used for each sample. Cells were harvested 24 hours after anti-CD3ε plus anti-CD28 stimulation. Nuclear and cytosolic protein extracts were generated using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Inc., Agawam, MA). Protein concentration was determined by BCA Assay and 40 µg (cytoplasmic extract) or 50 µg (nuclear extract) of protein was separated by 8% SDS-PAGE. Total PKCθ was detected using rabbit polyclonal anti-PKCθ (C-18) (Santa Cruz Biotechnology, Inc., Dallas, TX).

3.2.12 Immunoblotting

2x10⁶ cells/mL hPBMCs were used for each sample. Cells were harvested 24 hours after anti-CD3ε plus anti-CD28 stimulation, pelleted, and resuspended in RIPA lysis buffer containing protease and phosphatase inhibitors (25mM Tris-HCl pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, Halt™ Protease and Phosphatase Inhibitor Cocktail). The suspension was centrifuged for 10 min at 4°C. The supernatants were collected as protein lysates, BCA assay was performed to determine protein concentration, and 30 µg of protein was loaded for immunoblotting. CARMA1 Ser652 phosphorylation was detected with anti-phospho-CARD11 (Ser652; Cell Signaling
Technology, Inc., Danvers, MA). PKCθ Ser676 phosphorylation was detected with anti-phospho-PRKCQ (Ser676; LifeSpan Biosciences, Inc., Burlington, NC), and intracellular NOTCH1 levels were detected with anti-cleaved NOTCH1 (Val1744; Cell Signaling Technology, Inc.).

3.2.13 In vitro Human Th1 Cell Differentiation Assay

hPBMCs were sorted for CD4+ T cells using FACSia II flow sorter. Then, the cells were treated with DMSO or 3 µM Rottlerin for 30 min or with P13D5: Anti-pPKCθ for 4 hours at 37°C. Following treatment, cells were washed once with PBS and twice with 20 U/mL Heparin in PBS for 5 minutes on ice. For Th1 differentiation, they were resuspended in human Th1 differentiation media (provided in CellXVivo™ human Th1 cell differentiation kit, R&D systems). The cells were plated on human anti-CD3-coated wells. Th1-polarized cells were incubated for 5 days. After 5 days of incubation for Th1 cells, the cells were washed with RPMI complete media and restimulated for 1 hour in the media with 50 ng/mL PMA and 1µg/ml Ionomycin. Following the restimulation, they were incubated with Monensin for 3 hours before cytokine analysis via flow cytometry.

3.2.14 Ex vivo Delivery of P13D5: Anti-pPKCθ Complex into hPBMCs Subsequently Transferred into NSG Mice

Animal protocols were approved by the Institutional Animal Care and use Committee of the University of Massachusetts Amherst. NOD.Cg-Prkd<sup>scid</sup> Il2rg<sup>im1Wj/J</sup>/SzJ (NSG) mice were purchased from the Jackson Laboratory (Bar Harbor,
ME). Mice were conditioned with 2 Gy of total body irradiation using a $^{137}$Cs source then rested for 4-6 hours. hPBMCs were treated with DMSO or 3 µM Rottlerin for 30 min or with P$_{13}$D$_{5}$: Anti-pPKCθ for 4 hours at 37°C. 10x10$^6$ treated hPBMCs in 150 µl of PBS were injected into mice via the tail vein. Body weight and disease symptoms were observed daily. Bone marrow, spleen, and peripheral blood were collected on day 17 to assess percent engraftment of hPBMCs (% positive human CD45 / (% positive human CD45 cells + % positive mouse CD45 cells)) and infiltration of human CD4$^+$ and CD8$^+$ T cells. Also, the human CD4$^+$ and CD8$^+$ T cells were analyzed for CD25, pPKCθ (Thr538), NOTCH1IC, and T-BET expression.

### 3.2.15 Assessment of GvHD

Some mice were assigned to survival studies to assess whether treating hPBMCs, *ex vivo*, with P$_{13}$D$_{5}$: Anti-pPKCθ had a durable effect on the function of the transferred hPBMCs, *in vivo*. The severity of GvHD was assessed using a standardized scoring system, as previously described, and which included five different criteria (weight loss, posture, activity, fur texture, and skin integrity) (Cooke et al., 1996). Mice were evaluated on daily basis and graded from 0 (the least severe) to 2 (the most severe) for each criterion. Clinical score was generated by adding grades for five criteria. When a clinical score of “8” was reached, mice were removed from the study and humanely euthanized. The day of removal from the study was recorded as the day of lethal GvHD induction.
3.2.16 Cytometric Bead Array

IL-2 and IFNγ cytokine levels in plasma collected from NSG mice were determined by using human IFNγ and IL-2 Flex Sets (BD Biosciences). Data were acquired using a Fortessa 5 Laser Flow Cytometer (BD Biosciences) and analyzed using FCAP array software (Soft Flow Inc.).

3.3 Results

3.3.1 PTDM design and characterization

For this study, we utilized a single PTDM, MePh13-b-dG5 (P13D5), which has 13 repeats of a hydrophobic moiety and 5 repeats of a guanidinium monomer on a polyoxanorbornene di-ester polymer scaffold (Figure 3.1a, b). The choice of P13D5 was based on our previous work that showed that increasing the number of phenyl groups increased the protein delivery efficiency (Tew, Minter, unpublished). P13D5 was synthesized using ROMP, allowing for controlled, facile synthesis of the block copolymer (Figure 3.2a). The polymer structure was characterized by 1H-NMR and its molecular weight was determined by gel permeation chromatography (Figure 3.2b, c). Later, P13D5 was complexed with Anti-pPKC0 and the complex formation was shown both by dynamic light scattering and native gel electrophoresis. The size and polydispersity index (PDI) of P13D5: Anti-pPKC0 complex was measured via dynamic light scattering. The high PDI for P13D5 only and Anti-pPKC0 only samples showed that there was an aggregation when present alone in solution. The low PDI and narrow size distribution (narrow peak around 1 µm size, Figure 3.2d) for the P13D5: Anti-pPKC0
mixture indicated that two components formed complexes (Figure 3.1c). We confirmed the complex formation using native gel electrophoresis. The $P_{13}D_5$: Anti-pPKCθ complex could be visualized on the native gel with an apparent molecular weight between 1,000 – 1,200 kDa (Figure 3.1d). Anti-pPKCθ only could be detected as free antibody, migrating at approximately 150 kDa, and as aggregates with two different sizes of ~ 480 kDa and ~1,200 kDa. These results confirmed that no free antibody could be detected following complexing with $P_{13}D_5$ at the ratios used in this study.

3.3.2 Anti-pPKCθ (Thr538) delivery into hPBMCs

The antibody that we complexed to $P_{13}D_5$ specifically recognizes human phosphorylated PKCθ (Thr538). We incubated the $P_{13}D_5$: Anti-pPKCθ complex with hPBMCs then assessed the uptake efficiency and cellular toxicity. For comparison, we also tested anti-pPKCθ uptake with a commercially available antibody delivery reagent, AbDeliverITM. To determine the amount of antibody delivered intracellularly, we subsequently permeabilized the hPBMCs then stained them with a fluorescently-labeled secondary antibody (Figure 3.3a).

Using flow cytometric analysis, we detected robust fluorescence intensity in cells incubated with $P_{13}D_5$: Anti-pPKCθ complexes and stained with the fluorescent secondary antibody. Approximately 60% of hPBMCs stained positively for Anti-pPKCθ (Figure 3.3b, c) with an average MFI of 4000 a.u., indicating highly-efficient Anti-pPKCθ delivery (Figure 3.3d). This is in stark contrast to hPBMCs that were incubated with $P_{13}D_5$ alone, anti-pPKCθ alone, or AbDeliverITM: Anti-pPKCθ complexes, prior to staining with fluorescent secondary antibody (Figure 3.3b-d). Moreover, when we
measured the amount of antibody present in the cells over time, we observed that Anti-pPKCθ could be detected for up to 72 hours (Figure 3.4a). Cell viability for P13D5: Anti-pPKCθ-treated PBMCs remained above 80%, suggesting the complex had minimal toxicity (Figure 3.3e).

3.3.3 P13D5: Anti-pPKCθ delivery into ‘unstimulated’ hPBMCs greatly reduces their activation potential

To determine whether intracellular anti-pPKCθ delivery neutralizes the actions of pPKCθ, we further analyzed cell proliferation, protein expression levels of signature T cell activation and differentiation molecules via flow cytometry, with and without P13D5: Anti-pPKCθ treatment. We delivered P13D5: Anti-pPKCθ to hPBMCs 4 hours before the cells were stimulated with plate-bound anti-CD3ε plus anti-CD28. Pre-treating hPBMCs prior to stimulation did not affect cell viability or cellular proliferation (Figure 3.4b, d), but resulted in a significant impact on biological function. Specifically, expression of the high-affinity IL-2 receptor, CD25, the earliest inducible cell surface glycoprotein, CD69, the intracellular, signaling-competent form of the NOTCH1 transmembrane receptor, NOTCH1IC, and the Th1 transcriptional regulator, T-BET were all significantly reduced compared to levels in DMSO-treated cells (Figure 3.5a-f, 3.6a, b). Rottlerin treatment was included as positive control since it is known to prevent Thr538 phosphorylation of PKCθ (Lopez-Huertas et al., 2011). As shown in Figure 3.5, pretreating hPBMCs with P13D5: Anti-pPKCθ resulted in similar low levels of expression of all the assayed proteins, as did treatment with rottlerin.
Furthermore, expression of CD25, NOTCH1IC, and T-BET all remained significantly lower in P13D5: Anti-pPKC0-treated cells, compared to DMSO controls, even at 72 hours after treatment. Compared with DMSO controls, CD69 levels were downregulated in P13D5: Anti-pPKC0-treated cells 24 hours after treatment and were not upregulated for 72 hours (Figure 3.6a, b). To assess the functional impact on PKCθ activity of delivered P13D5: Anti-pPKC0, we also analyzed expression of two important pro-inflammatory cytokines produced by activated T cells, IFNγ and IL-2, using standard ELISA techniques. We observed that IFNγ and IL-2, both, were significantly lower after P13D5: Anti-pPKC0 treatment during the 24 to 72 hours of cell culture period, showing reduced levels that were comparable to those of rottlerin-treated cells (Figure 3.5h, i). In addition, we asked whether P13D5: Anti-pPKC0 treatment was reversible. To test this, we incubated hPBMCs with P13D5: Anti-pPKC0 for 7 days and measured IFNγ and IL-2 levels via cytometric bead array analyses. We observed that IFNγ levels were similar by Day 5 after treatment whereas IL-2 levels were similar by Day 6 after treatment suggesting the early inhibitory effects of Anti-pPKC0 treatment were reversible within 5-6 days of treatment (Figure 3.7a, b). Taken together, these results indicated that P13D5: Anti-pPKC0 treatment of T cells prior to delivery of T cell receptor and costimulatory signals greatly reduced their activation potential.

3.3.4 P13D5: Anti-pPKC0 delivery into ‘activated’ hPBMCs diminishes expression of downstream activation markers

In humans, PKC0 in activated T cells contributes to the pathology of various aberrant immune conditions and elevated levels of phosphorylated PKC0 (Thr538) have
been observed both in activated CD4$^+$ and CD8$^+$ T cells (Solomou et al., 2006). Therefore, we asked what effect delivering P$_{13}$D$_5$: Anti-pPKC$\theta$ has on cells which have already been activated and show increased levels of phosphorylated PKC$\theta$ (Thr538). In Figure 3.8, we clearly demonstrate that when hPBMCs were stimulated for 24 hours with anti-CD3$\varepsilon$ plus anti-CD28, then incubated with P$_{13}$D$_5$:Anti-pPKC$\theta$, levels of CD25, NOTCH1$^{IC}$, and T-BET were significantly lower following an additional 24 hours of culture (48 hours from time of stimulation), compared to DMSO-treated cells (Figure 3.8a-g). Interestingly, P$_{13}$D$_5$: Anti-pPKC$\theta$-treated cells did not upregulate these markers, even up to 72 hours after treatment (96 hours after stimulation). This was not due to toxicity of the treatment since cellular viability in P$_{13}$D$_5$: Anti-pPKC$\theta$-treated cells remained similar to DMSO-treated cells (Figure 3.4c). Additionally, CD69 levels were not upregulated in P$_{13}$D$_5$: Anti-pPKC$\theta$-treated cells for 72 hours (Figure 3.6c, d). Finally, stimulated cells treated with P$_{13}$D$_5$: Anti-pPKC$\theta$ produced significantly lower amounts of IFN$\gamma$ and IL-2 compared to DMSO-treated controls and, again, at levels comparable to those of rotterin-treated samples (Figure 3.8h, i).

We also asked whether anti-pPKC$\theta$ or P$_{13}$D$_5$, individually, exerted any biological effect on T cells. hPBMCs were treated either with anti-pPKC$\theta$ alone or with P$_{13}$D$_5$ alone, and levels of NOTCH1$^{IC}$ and T-BET were measured after 24 hours of stimulation. When compared to DMSO-treated cells, we found there were no significant differences in NOTCH1$^{IC}$ (Figure 3.9a, b) or T-BET (Figure 3.9c, d) levels in anti-pPKC$\theta$-only-treated or in P$_{13}$D$_5$-only-treated cells after 24 hours. These observations suggest complex formation between antibody and P$_{13}$D$_5$ is necessary for intracellular anti-pPKC$\theta$ delivery (as shown in Figure 3.3b-d) and modulation of PKC$\theta$ function.
3.3.5 P13D5: Anti-pPKCθ delivery alters the activity and localization of PKCθ in hPBMCs

The PKCθ signaling pathway coordinates important signaling events to direct Th1 cell functions (Solomou et al., 2006; Anderson et al., 2006; Sutcliffe et al., 2012). Following its phosphorylation on Thr538, PKCθ is autophosphorylated at serine residues to become fully activated (Wang et al., 2012). pPKCθ will phosphorylate an intracellular scaffold protein, CARMA1, to facilitate formation of the macromolecular signaling aggregate known as the CARMA1/BCL10/MALT1 (CBM) complex. Assembly of the CBM components precedes, and is thought to be necessary for, liberation of NF-κB transcriptional regulators from their cytosolic inhibitory complexes (Matsumoto et al., 2005; Cartwright et al., 2011). Moreover, it has been reported that pPKCθ is also able to translocate into the nucleus where it can associate with a chromatin-bound complex to regulate IL2 gene expression (Sutcliffe et al., 2011, 2012). Having demonstrated that P13D5: Anti-pPKCθ delivery modulated biological functions, we asked whether this might be due to its interfering with specific downstream actions of PKCθ.

To address this question, we evaluated PKCθ autophosphorylation at Ser676 residue, CARMA1 phosphorylation, NOTCH1IC levels, as well as nuclear localization of PKCθ. Compared to control-treated cells, hPBMCs treated with P13D5: Anti-pPKCθ showed reduced Ser676 autophosphorylation (Figure 3.10a) and decreased levels of CARMA1 phosphorylation (Figure 3.10b). In addition, the level of cleaved NOTCH1IC was diminished following anti-pPKCθ delivery (Figure 3.10c). Moreover, when we assessed the accumulation of total PKCθ in the nucleus, we noted that P13D5: Anti-pPKCθ-treated cells exhibited less total PKCθ in the nucleus than did DMSO-treated
cells (Figure 3.10d). However, there was no change in cytosolic levels of total PKCθ (Figure 3.11). Thus, not only does P13D5: Anti-pPKCθ delivery into hPBMCs reduce the activation potential of unstimulated cells for up to 72 hours after treatment, it also reduces the ability of stimulated cells to sustain increased expression of various markers of activation. Our mechanistic studies suggest this may result from the combination of reduced CARMA1 phosphorylation and impaired nuclear translocation of PKCθ in P13D5: Anti-pPKCθ-treated cells.

3.3.6 *Ex vivo* delivery of P13D5: Anti-pPKCθ into hPBMCs provides a survival benefit in a lymphocyte transfer, humanized mouse model of graft-versus-host disease (GvHD)

Our *in vitro* experiments demonstrated that P13D5: Anti-pPKCθ delivered into hPBMCs effectively reduced the functional activity of PKCθ up to 72 hours in culture. However, it was unclear how durable the effects of our *ex vivo* treatment would be if these treated cells were used in proof-of-principle, lymphocyte transfer experiments in a “humanized” model of GvHD, induced when hPBMCs are transferred into the NOD-*scid-il2rgnull* (NSG) strain of mice. In this graft (hPBMCs)-*versus*-host (NSG mouse) model, transferred lymphocytes acutely target the bone marrow of recipient mice resulting in lethal immune-mediated bone marrow failure within approximately 20 days. Th1 cells play an important role in the progression of GvHD (Fu et al., 2015). Moreover, PKCθ and NOTCH1 signaling are required for alloreactivity and GvHD induction (Valenzuela et al., 2009; Zhang et al., 2011). To evaluate the long-term effects of P13D5: Anti-pPKCθ treatment on cells, we induced GvHD in three cohorts of mice. In one cohort, we induced
GvHD by transferring DMSO-treated hPBMCs; in a second cohort we induced GvHD with P13D5: Anti-pPKCτ-treated hPBMCs; in the third cohort we induced GvHD using hPBMCs pre-treated with rottlerin. We harvested mice on day 17 after GvHD induction to determine how well the transferred cells engrafted and expanded in target tissues such as the spleen and bone marrow, as well as in peripheral blood (Figure 3.12a). Mice that received P13D5: Anti-pPKCτ-treated cells showed a similar degree of hPBMC expansion as DMSO-treated control cells, indicating P13D5: Anti-pPKCτ treatment did not affect cellular viability in vivo, following cell transfer (Figure 3.12b). Additionally, we determined that infiltration of human CD4+ and CD8+ T cells into the target tissues were also similar in mice receiving DMSO-treated or P13D5: Anti-pPKCτ-treated hPBMCs (Figure 3.12c). To determine whether the effects of Anti-pPKCτ treatment were consistent with in vitro results, we measured levels of pPKCτ, T-BET, NOTCH1IC, and CD25 in CD4+ T cells infiltrating the bone marrow, in P13D5: Anti-pPKCτ-treated hPBMCs, although the differences were not significant compared to DMSO or to rottlerin-treated hPBMCs (Figure 3.12d-i, 3.13a, b). Furthermore, we did not see any upregulation in CD25 or NOTCH1IC levels, as measured by flow cytometry (Figure 3.13c-f). We also measured IL-2 and IFNγ levels in the plasma of mice from all three cohorts. Although IL-2 levels were similar in all animals evaluated (Figure 3.13g), IFNγ levels were significantly lower in mice receiving P13D5: Anti-pPKCτ-treated hPBMCs, compared to mice receiving DMSO-treated control cells (Figure 3.12j). We next asked whether delivering P13D5: Anti-pPKCτ to hPBMCs prior to transferring them into recipient mice would affect GvHD severity and survival. Remarkably, GvHD clinical scores were significantly reduced (Figure 3.12k) and Kaplan-Maier analysis revealed a
significant survival benefit when \( \text{P}_{13}\text{D}_5 \): Anti-pPKC\( \theta \) was delivered to hPBMCs prior to transfer, compared to mice that received DMSO-treated cells (Figure 3.12l).

Two of three mice that received hPBMCs pre-treated with rottlerin did not show overt signs of disease. However, no human cells were detected in the peripheral blood of these mice, when they were removed from the study on day +70 (data not shown), suggesting GvHD was not induced in those mice since rottlerin treatment also had cytotoxic effects on hPBMCs. These observations are consistent with other data from our lab supporting the notion that PKC\( \theta \) plays a critical role in T cell activation, proliferation, and survival. Collectively, our data demonstrate that we can modulate PKC\( \theta \) activity in hPBMCs, \textit{ex vivo}, using anti-pPKC\( \theta \) delivery by \( \text{P}_{13}\text{D}_5 \). Furthermore, our \textit{in vivo} proof-of-principle experiments suggest the effects of \( \text{P}_{13}\text{D}_5 \): Anti-pPKC\( \theta \) delivery are durable and may constitute the basis of a novel therapeutic strategy that targets the actions of intracellular proteins to alter disease progression.

3.4 Discussion

To the best of our knowledge, this is the first study to demonstrate intracellular delivery of a functional antibody into human PBMCs using PTDMs. The efficiency of anti-pPKC\( \theta \) delivery by \( \text{P}_{13}\text{D}_5 \) was significantly higher than that of a commercial antibody delivery reagent and did not show cellular toxicity. We observed significant PKC\( \theta \) neutralization in cells treated with \( \text{P}_{13}\text{D}_5 \): Anti-pPKC\( \theta \), whether before or after stimulation, and the inhibitory effects persisted up to 72 hours in culture. Activation markers such as CD25 and CD69 were expressed to a much lower extent compared to control samples. Furthermore, expression of NOTCH1\textsuperscript{IC} and T-BET were significantly
diminished in \( \text{P}_{13}\text{D}_5 \) : Anti-pPKC\( \theta \)-treated cells, suggesting distal signaling events important to T cell activation were also affected (Fu et al., 2015). This was further evidenced by impaired CARMA1 phosphorylation, reduced nuclear translocation of PKC\( \theta \), and significantly less production of pro-inflammatory cytokines, such as IFN\( \gamma \) and IL-2. Moreover, \textit{ex vivo} \( \text{P}_{13}\text{D}_5 \): Anti-pPKC\( \theta \) delivery into hPBMCs, showed durable effects on PKC\( \theta \) signaling in a humanized model of GvHD, and provided a significant survival benefit that was not observed when DMSO-treated cells were used to induce disease.

Protein transduction domain mimics (PTDMs) represent a promising platform for cargo delivery into immune cells as a means of modulating their responses. Due to their tunable characteristics, these polymeric structures can be easily designed in order to increase their delivery efficiency, especially for hard-to-transfect cells such as hPBMCs (deRonde and Tew, 2015; Tezgel et al., 2012). Current strategies for intracellular antibody delivery show low efficiency, high toxicity, non-specific effects, or reduced endosomal escape. As a result, some biologics which show potential \textit{in vitro} are considerably less effective when used \textit{in vivo} (Beck et al., 2010; Chan and Carter, 2010; Brekke and Sandlie, 2003). Our current strategy, using PTDMs, provides an improved platform for antibody delivery enabling specific targeting of intracellular proteins.

Over the past two decades, the Food and Drug Administration (FDA) has approved an increasing number of therapeutic antibodies for clinical use. For example, antibodies specific for CD20, IL-6, or TNF have been used to treat various aberrant immunological conditions. However, there remain concerns about safety and efficacy when targeting extracellular or cell surface molecules that continue to pose significant
drawbacks (Chan and Carter, 2010; Reichert, 2012). Depleting healthy as well as abnormal cells or dampening global immune responses can increase patient risk of infection. Additionally, humanized antibodies may be processed as foreign antigens through the endocytic pathway to initiate CD4+ T cell-dependent humoral responses, which can further diminish their clinical efficacy (Chan and Carter, 2010; Torchilin, 2009). By contrast, our delivery strategy provides very specific targeting of an intracellular molecule, resulting in the precise blockade of a particular cellular response.

Understanding the details of immune function lays the foundation for specifically modulating T cell responses. PKCθ is not necessary for anti-viral responses following allogeneic bone marrow transplantation, strengthening the possibility that inhibiting PKCθ may selectively suppress allograft rejection and GvHD without disrupting anti-viral or anti-tumor immunity (Chuang et al., 2011; Valenzuela et al., 2009; Manicassamy et al., 2008; Isakov, 2012; Kwon et al., 2010; Gruber et al., 2009b). We used a known pharmacological inhibitor, rottlerin, as a positive control to inhibit Thr538 phosphorylation and, thereby, PKCθ actions in T cell signaling. Our intracellular antibody delivery decreased PKCθ signaling as robustly as rottlerin, but with less toxicity. Extensive efforts have been made to develop isozyme-selective PKCθ inhibitors; however, the high degree of homology shared by PKC family members makes this challenging (Evenou et al., 2009; Lamba and Ghosh, 2012; Chand et al., 2012; Mochly-Rosen et al., 2012; Altman and Kong, 2014). For instance, enzastaurin can inhibit both PKCβ and PKCθ. Therefore, using enzastaurin to target PKCθ, could also shut down PKCθ-related pathways, likely causing undesired off-target effects (Bronk et al., 2012). Several chemical compounds such as aminopyrimidine, pyridine carbonitrile, and thieno-
(2,3-b) pyridine-5-carbonitriles (2-alkenyl and 2-phenyl) derivatives are considered to be more selective for PKCθ than for other PKC family members. Preclinical studies have shown that these compounds decreased IL-2 production in activated T cells derived from wild-type mice but had reduced effect on activated T cells from PKCθ knockout mice (Hage-Sleiman et al., 2015). Overall, the difficulties encountered creating PKCθ-specific inhibitors highlight the challenges of rational drug design for this target. The PTDM-mediated antibody delivery approach described here may represent a specific and nontoxic means of modulating the function of intracellular targets.

The biological response to neutralizing PKCθ activity supports a novel targeting strategy for PKCθ in the context of T cell activation and differentiation. Cytosolic and nuclear PKCθ may exert differential effects on T cell fate. PKCθ and NOTCH1 physically associate in the cytoplasm and have substantial roles during T cell activation and differentiation (Shin et al., 2014; Roderick et al., 2013). Our studies suggest that modulating PKCθ localization, within a narrow window following stimulation, resulted in significantly altered T cell fate. This effect persisted for several weeks, as demonstrated by the attenuated disease severity and prolonged survival in a humanized model of GvHD. The delivery strategy employed here will enable us to further investigate molecular events mediated by PKCθ and will advance our understanding of specific mechanisms of immune response.

In conclusion, this study is the first to describe a successful strategy for routine and effective intracellular antibody delivery by PTDMs and demonstrates its powerful application by targeting PKCθ in the context of T cell immunomodulation. Fine-tuning this approach to specifically inhibit other cellular proteins will lay the foundation for
“drugging the undruggable”. This may further open the door to modulating intracellular targets with the effectiveness of small molecule inhibitors but coupled with the greater specificity that antibodies provide.
Figure 3.1 PTDM design and characterization. (a) Chemical structure of MePh$_{13}$-$b$-$dG_5$ (P$_{13}D_5$), with the hydrophobic/aromatic moiety shown in green and cationic/guanidinium moiety in blue. (b) Cartoon representation of the MePh$_{13}$-$b$-$dG_5$ with the same coloring scheme. (c) Size and polydispersity index measurements for P$_{13}D_5$: Anti-pPKC$\theta$ complex by dynamic light scattering. (d) Native gel electrophoresis demonstrating complexation between P$_{13}D_5$ and Anti-pPKC$\theta$. 

<table>
<thead>
<tr>
<th>Sample</th>
<th>Size (d,nm)</th>
<th>Polydispersity Index (PDI)</th>
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<tr>
<td>P$_{13}D_5$ only</td>
<td>95.11±10.8</td>
<td>0.535±0.02</td>
</tr>
<tr>
<td>Anti-pPKC$\theta$ only</td>
<td>8.71±0.3</td>
<td>0.677±0.07</td>
</tr>
<tr>
<td>P$_{13}D_5$/Anti-pPKC$\theta$ Complex</td>
<td>1291.50±31.5</td>
<td>0.400±0.07</td>
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Figure 3.2 Synthesis and characterization of $P_{13}D_5$. (a) $P_{13}D_5$ was synthesized using ring-opening metathesis polymerization. (b) $P_{13}D_5$ structural characterization was determined via $^1$H-NMR. (c) Molecular weight of $P_{13}D_5$ was determined by gel permeation chromatography using tetrahydrofuran (THF) grade. (d) Complexation of $P_{13}D_5$ and Anti-pPKCθ was shown using dynamic light scattering. Upon complexation, the particle size of the complex increased to approximately 1 µm.
Figure 3.3 Anti-pPKCθ (Thr538) delivery into hPBMCs. (a) Schematic of Anti-pPKCθ delivery experiment. Human PBMCs were incubated with anti-pPKCθ only, P₁₃D₅ only, P₁₃D₅: Anti-pPKCθ, or AbDeliverIN™: Anti-pPKCθ for 4 hours. hPBMCs were subjected to a heparin wash to remove surface-bound proteins, stimulated for 24 hours with anti-CD3ε + anti-CD28, fixed and permeabilized, then incubated with AlexaFluor488-conjugated anti-rabbit secondary antibody. (b) Histogram showing Anti-pPKCθ uptake under different treatment conditions as in (a), above. (c) Percent hPBMCs staining positively for Anti-pPKCθ, following treatment as in (a). (d) Median fluorescent intensity (MFI) of AlexaFluor488-conjugated anti-rabbit secondary antibody in hPBMCs following treatment as in (a), above. (e) Viability of hPBMCs following treatment as in (a). Data represent the mean ± SEM of three independent experiments. **p<0.01; ***p<0.001, calculated using an unpaired, two-tailed student t test.
Figure 3.4 The presence of anti-pPKCθ and its effect on cellular viability. We used flow cytometry to detect how long Anti-pPKCθ could be detected in the cell after its delivery. (a) We could detect intracellular Anti-pPKCθ up to 72 h after it was delivered into hPBMCs, using an AlexaFlour488-conjugated secondary antibody and flow cytometry. We used 7-Aminoactinomycin (7-AAD) staining to determine cellular viability at 24, 48 and 72 hours after treatment for (b) hPBMCs treated before stimulation or (c) hPBMCs treated after stimulation for 24 hours with plate-bound anti-CD3ε plus anti-CD28. Data represent the mean ± SEM of three independent experiments. ns p>0.05; *p<0.05; **p<0.01, ***p<0.001 calculated using an unpaired, two-tailed student t test. (d) Cell proliferation assay was performed using CFSE labeling. CFSE intensity was measured up to 7 days after the antibody delivery in DMSO-, Rottlerin-, and P13D3: Anti-pPKCθ-treated hPBMCs.
Figure 3.5 PiD5: Anti-pPKCθ delivery into ‘unstimulated’ hPBMCs greatly reduces their activation potential. (a) Schematic of experimental design. hPBMCs were treated with DMSO (vehicle control), Rottlerin, or PiD5: Anti-pPKCθ for 4 hours at 37°C, then stimulated with plate-bound anti-CD3ε plus anti-CD28. Samples were analyzed at 24, 48, and 72 hours after stimulation, using flow cytometry. Percent positive cells, as well as MFI, were assessed for several markers of activation, as indicated. (b, c) CD25; (d, e) NOTCH1IC; (f, g) T-BET. Cytokine levels of (h) IFNγ and (i) IL-2 were determined by ELISA. Data represent the mean ± SEM of three independent experiments. nd (not detected); ns p>0.05; *p<0.05; **p<0.01; ***p<0.001, calculated using an unpaired, two-tailed student t test.
Figure 3.6 CD69 expression in P13D3: Anti-pPKC0-treated hPBMCs. We used flow cytometric approaches to assess CD69 expression 24, 48 and 72 hours after treatment. (a) Percent CD69 positive and (b) MFI of CD69 expression in hPBMCs treated before stimulation. (c) Percent CD69 positive and (d) MFI of CD69 expression in hPBMCs treated after stimulation for 24 hours with plate-bound anti-CD3ε plus anti-CD28. Data represent the mean ± SEM of three independent experiments. ns p>0.05; *p<0.05; **p<0.01 calculated using an unpaired, two-tailed student t test.
Figure 3.7 Reversibility of P13D5: Anti-pPKCθ treatment. We measured T cell functions up to 7 days after treatment in order to assess whether the treatment is reversible. The cells were treated either with DMSO or Rottlerin for 30 min or P13D5: Anti-pPKCθ for 4 hours at 37°C. Later, the cells were stimulated by plate-bound anti-CD3ε plus anti-CD28 until the time of analysis. (a) IFNγ levels and (b) IL-2 levels present in supernatants were measured by cytometric bead array. Data represent the mean ± SEM of three independent experiments. ***p<0.001 calculated using an unpaired, two-tailed student t test.
Figure 3.8 

**P<sub>13</sub>D<sub>5</sub>:** Anti-pPKCθ delivery into ‘activated’ hPBMCs diminishes expression of downstream activation markers. 

**Figure legend:** (a) Schematic of experimental design. hPBMCs were stimulated with plate-bound anti-CD3ε plus anti-CD28. After 24 hours of stimulation, cells were treated with DMSO (vehicle control), Rottlerin, or P<sub>13</sub>D<sub>5</sub>: Anti-pPKCθ complex for 4 hours at 37°C. Samples were analyzed 24, 48, and 72 hours after treatment, which corresponded to 48, 72, and 96 hours after stimulation, respectively, using flow cytometry. Percent positive cells, as well as MFI, were assessed for several markers of activation, as indicated. (b, c) CD25, (d, e) NOTCH1/C, (f, g) T-BET. Cytokine levels of (h) IFNγ and (i) IL-2 were determined by ELISA. Data represent the mean ± SEM of three independent experiments. *p<0.05; **p<0.01; ***p<0.001 calculated using an unpaired, two-tailed student t test.
Figure 3.9 Effects of P1 D5-only or Anti-pPKC0-only treatment on hPBMCs. Unstimulated hPBMCs were treated with DMSO (vehicle control), rottlerin, or P1 D5: Anti-pPKC0 for 4 hours at 37°C. We used flow cytometry to measure expression of (a, b) NOTCH1IC and (c, d) T-BET 24 hours after stimulation with anti-CD3ε plus anti-CD28. Data represent the mean ± SEM of three independent experiments. ns $p>0.05$; *$p<0.05$; **$p<0.01$ calculated using an unpaired, two-tailed student $t$ test.
Figure 3.10 P13D5: Anti-pPKCθ delivery alters the activity and localization of PKCθ in hPBMCs. hPBMCs were treated with DMSO (vehicle control), Rottlerin, or P13D5: Anti-pPKCθ for 4 hours at 37°C then stimulated with plate-bound anti-CD3ε plus anti-CD28 for 24 hours. (a) Autophosphorylation of PKCθ (Ser676), (b) phosphorylation of CARMA1 (Ser652), and (c) expression of NOTCH1IC was determined by immunoblotting proteins isolated from whole cell lysates. (d) Nuclear localization of total PKCθ was determined by immunoblotting proteins isolated from nuclear fractions. (e) Contour plots for human Th1 cells differentiated in vitro, (f) Percent of Th1-polarized cells, (g) MFI of T-BET expression, and (h) MFI of IFNγ in Th1-polarized cells. Data in (a-d) are representative of three independent replicates. Data in (f-h) represent the mean ± SEM of three independent experiments. *p<0.05; **p<0.01 calculated using an unpaired, two-tailed student t test.
Figure 3.11 Effects of P13D5: Anti-pPKCθ on total PKCθ expression. Unstimulated hPBMCs were treated with DMSO (vehicle control), rottlerin, or P13D5: Anti-pPKCθ for 4 hours at 37°C. We determined expression of total PKCθ in treated hPBMCs by immunoblotting proteins isolated from whole cell lysates at 24 hours after stimulation with plate-bound anti-CD3ε plus anti-CD28. Data are representative of three independent replicates.
Figure 3.12 *Ex vivo* delivery of P3D5: Anti-pPKC0 into hPBMCs provides a survival benefit in a lymphocyte transfer, humanized mouse model of *graft-versus-host disease* (GvHD). (a) Schematic of experimental approach. hPBMCs were treated with DMSO (vehicle control), Rottlerin, or P3D5: Anti-pPKC0 for 4 hours at 37°C. Graft-vs-host responses were induced in NSG mice by transferring 10^6 cells treated as above, *via* tail vein injection. On day +17 after disease induction, mice were humanely sacrificed and (b) percent engraftment of human CD45 cells in bone marrow (BM), peripheral blood (PB), and spleen was determined using flow cytometry. (c) Infiltration of CD4^+ and CD8^+ T cells into BM, PB, and spleen was also assessed using flow cytometry. (d-f) MFI of pPKC0 (Thr538) expression in CD4^+ T cells infiltrating BM, PB, and spleen. (g-i) MFI of T-BET expression in CD4^+ T cells infiltrating BM, PB, and spleen. (j) IFNγ levels in plasma as determined by cytometric bead array analysis. (k) GvHD clinical scores were calculated as described in Materials and Methods (n=12). (l) Kaplan-Maier analysis was used to determine the survival benefit of *ex vivo* treatment modalities. Mice receiving hPBMCs pretreated with Rottlerin were removed from the study on day +70. n=12 mice for each *ex vivo* treatment of hPBMCs. Data shown in (b-j) represent the mean ± SEM of three independent experiments. *p<0.05; **p<0.01; ***p<0.001 calculated using an unpaired, two-tailed student *t* test.
Characterization of CD4+ T cells, treated ex vivo, and used to induce GvHD. Bone marrow, peripheral blood, and spleen were collected from 3 NSG mice per cohort. Subsequently, the tissues were processed and CD4+ T cells were stained for several markers of GvHD. (a) Percent of pPKC\(\theta\) (Thr538) positive cells, (b) Percent of T-BET positive cells, (c) percent of CD25 positive cells, (d) MFI of CD25 expression, (e) Percent of NOTCH1 positive cells, and (f) MFI of NOTCH1 expression were determined by flow cytometry. (g) IL-2 levels in plasma were determined by cytokine bead array assay.
CHAPTER 4
TARGETING PKCθ VIA CELL-PENETRATING ANTIBODY Generates
Super-Suppressive FOXP3-PD-1+ IFNγ iTREGS Preventing Graft-Versus-Host Disease

4.1 Introduction

Naïve CD4 T cells differentiate into different T helper subsets upon acquiring certain signals in peripheral tissues. Regulatory T cells (Tregs) are a subset of differentiated T helper cells that play critical role in immunosuppression (Ohkura et al., 2013). In humans, Tregs are characterized in vivo as CD4+CD25+CD127-FOXP3+ cells that consistently confer suppressive properties across species in multiple disease models (Hori et al., 2003; Khattri et al., 2003; Sakaguchi et al., 2010; Brunkow et al., 2001; Fontenot et al., 2005; Liu et al., 2006; Simonetta et al., 2010). Treg function is very critical for suppression of autoimmune responses, allotransplant tolerance, tumor and microbial immunity, graft rejection, and suppression of graft-versus-host disease (GvHD) in mice and humans (Fontenot et al., 2005; Ohkura et al., 2013; Ganguly et al., 2014; Lee et al., 2015; Komanduri and Champlin, 2011; Lu et al., 2012; Pankratz et al., 2014). In autoimmunity, Tregs are negatively regulated by inflammatory cytokine milieu and their function is inhibited (Shevach, 2009). Early studies showed that appropriate localization to secondary lymphoid organs and subsequent expansion are necessary for Treg suppressive function in vivo (Nguyen et al., 2007). Tregs suppress alloresponsive T cells via cell-cell contact inhibition through multiple co-inhibitory receptors. Several studies demonstrated that suppressive Tregs highly express
surface molecules, such as neuropilin-1 (NRP1), PD-1, LAG-3, and CTLA-4, that competitively prevent the contact of antigen presenting cell (APC) with naïve T cell (Raimondi et al., 2006; Delgoffe et al., 2013; Do et al., 2016; Matheu et al., 2015). It was shown that Tregs can modify the immune synapse (IS), that is established between the naïve T cell and the antigen-loaded dendritic cell (DC), by inhibiting the recruitment of protein kinase C-theta (PKCθ) to the IS (i.e. contact site) between T cell and the peptide-loaded DC. Active PKCθ phosphorylated at threonine 538 translocates to the IS and renders the activation signal from T cell receptor (TCR) with costimulatory signal, CD28, thereby affecting transcriptional activity of immune-responsive genes (Isakov and Altman, 2002; Gupta et al., 2008; Sun et al., 2000; Sumoza-Toledo et al., 2006; Sedwick and Altman, 2004; Barouch-Bentov et al., 2005; Manicassamy et al., 2006; Manicassamy and Sun, 2007). Interestingly, PKCθ is the only PKC isoform translocating to the IS and regulatory T cells sequester their PKCθ away from the IS (Boschelli, 2009; Roybal and Wülfing, 2010). In effector T cells, PKCθ promotes T cell activation, whereas it inhibits regulatory T cell function (Roybal and Wülfing, 2010; Gruber et al., 2009a; Sun et al., 2000). Inhibition of PKCθ with a small molecule inhibitor as well as siRNA in Treg cells enhances suppressive function and can restore impaired function in Tregs from rheumatoid arthritis and block the autoimmune response in a mouse model of colitis (Zhang et al., 2013; Zanin-Zhorov et al., 2010). Therefore, inhibition of PKCθ in Tregs may be a valuable compartment in Treg adoptive immunotherapy to treat autoimmunity and graft-versus-host disease (GvHD) (Zanin-Zhorov et al., 2010).

The initial evidence for requirement of PKCθ in alloresponses came from the study where alloreactive T cells injected into PKCθ-deficient mice exerted significantly
diminished local T cell response compared to WT mice similarly challenged (Sun et al., 2000). Also, PKCθ-deficient mice survived bone marrow transplantation (BMT) procedure and did not develop GvHD, whereas majority of WT mice died from GVHD while protecting anti-viral and anti-tumor immunity (Sun, 2012). These studies reinforced the idea that inhibiting PKCθ actions would be beneficial for immunotherapy. However, PKCθ is located in the intracellular environment, thereby limiting antibody targeting strategies and most of existing small molecule PKCθ inhibitors have toxic side effects and less efficiency due to less penetration into T cells (Isakov, 2012; Mochly-Rosen et al., 2012). Recently we developed and utilized highly specific, successful strategy for routine and effective intracellular antibody delivery by cell-penetrating peptide mimics and demonstrated its powerful application by targeting activated PKCθ (pPKCθ-Thr538) in the context of T cell immunomodulation. *Ex vivo* manipulation of T cells via intracellular αpPKCθ delivery altered their cell fate and transfer of these cells into humanized NSG model did not induce GvHD as severe as non-manipulated T cells. This effect persisted for several weeks, as demonstrated by the attenuated disease severity and prolonged survival in a humanized model of GvHD (Ozay et al., 2016).

It was shown that adoptive transfer of induced Tregs (iTregs) generated *in vitro* in allogeneic mouse BMT model for acute GvHD was very beneficial for suppressing the immune response (Nguyen et al., 2007). In humans, cellular immunotherapy by adoptive transfer of iTregs has become the most realistic and efficacious option for clinical use (Ganguly et al., 2014). The first trial of adoptive iTreg therapy in humans exerted promising outcomes in preventing graft-versus-host disease (GvHD) upon allogeneic stem cell transplantation, thereby offering great promise for treating autoimmune diseases.
and allograft rejection (Joffre et al., 2004; Taylor et al., 2004; Raimondi et al., 2006; Fehérvári and Sakaguchi, 2004; Walsh et al., 2004; Bluestone, 2005; Wood and Sakaguchi, 2003). Here, we report that targeting active PKCθ via a cell-penetrating antibody (intracellularly delivered αpPKCθ-Thr538 via cell-penetrating peptide mimics) favors iTreg differentiation and expansion. Moreover, αpPKCθ-treated iTregs significantly increase their suppression ability \textit{in vitro} as characterized by overexpression of co-inhibitory receptors, PD-1 and LAG-3. Also, these super-suppressive iTregs could still be detected 17 days after their administration into humanized mice and appeared to be highly efficacious in preventing GvHD \textit{in vivo}. Therefore, both \textit{in vitro} expansion and \textit{ex vivo} manipulation to augment the function of iTregs via intracellular αpPKCθ delivery represent very a promising strategy to prevent GvHD.

4.2 Materials and Methods

4.2.1 Animals

All animal studies were approved by, and conducted under the oversight of, the Institutional Animal Care and Use Committee of the University of Massachusetts, Amherst. Seven-week old female NOD.Cg-Prkdc<sup>scid</sup> II2rg<sup>im1Wj</sup>/SzJ (NSG) mice, were purchased from the Jackson Laboratories (Bar Harbor, ME). Upon arrival, these mice were rested for one week prior to use. Mice were housed under pathogen-free conditions in micro-isolator cages and received acidified water (pH 3.0) supplemented with two types of antibiotics (trimethoprim + sulfamethoxazole) throughout the duration of the experimental procedures.
4.2.2 Antibodies and Reagents

Antibodies used in this study were acquired from: (1) BioLegend: CD3ε (Purified, Clone: UCHT1), CD28 (Purified, Clone: CD28.2), CD4 (BV711, Clone: RPA-T4), CD8 (APC/Cy7, Clone: RPA-T8), CD25 (Biotin, Clone: BC96) CD25 (AF700, Clone: BC96), CD25 (PE/Cy7, Clone: BC96), CD45RA (BV510, Clone: HI100), CD45RO (PE, Clone: UCHL1), CD127 (Biotin, Clone: A019D5), CD127 (AF700, Clone: A019D5), CTLA-4 (APC, Clone: BNI3), FOXP3 (AF488, Clone: 150D), LAG-3 (BV421, Clone: 11C3C65), NRP1 (PerCP/Cy5.5, Clone: 12C2), PD-1 (APC/Fire750, Clone: EH12.2H7), (2) eBioscience: CD4/CD8 cocktail (FITC/PE, Clones: RPA-T4, RPA-T8), human CD45 (PE, Clone: 2D1), NOTCH1 (PE, Clone: mN1A), pSTAT3 Tyr705 (PE, Clone: LUVNKL), pSTAT5 Tyr694 (PE, Clone: SRBCZX), (3) BD Biosciences: mouse CD45 (FITC, Clone: 30-F11), IFNγ (APC, Clone: B27), (4) GeneTex: NOTCH1 (FITC, Clone: mN1A), (5) Cell Signaling Technology: Histone H3 (Unconjugated, anti-mouse, Clone: 96C10), pPKCθ (Unconjugated, anti-rabbit, Thr538), Total PKCθ (Unconjugated, Clone: E1I7Y), (6) Sigma: α-Tubulin (Unconjugated, anti-mouse, Clone: B-5-1-2), (7) Life Technologies: Phospho-PKC theta (Thr538) (Unconjugated, Clone: F4H4L1), F(ab’)2-Goat anti-rabbit IgG (H+L) secondary antibody (Qdot625, polyclonal). For nuclear staining, DRAQ5™ was obtained from Thermo Scientific. Live/dead staining was performed utilizing either Zombie aqua or Zombie violet fixable viability kit purchased from BioLegend. For in vitro suppression assay, cells were tracked via labeling with CytoTell™ UltraGreen or CytoTell™ Red650 purchased from AAT Bioquest, Inc.
4.2.3 Human iTreg differentiation coupled with intracellular P\textsubscript{13}Ds:

\textit{αpPKCθ delivery}

1 \(\mu\)M of P\textsubscript{13}D\textsubscript{5} and 25 nM of αpPKC\textsubscript{θ} (Thr538, Clone: F4H4L1) were complexed in PBS (phosphate buffered saline, pH 7.2) at a specific ratio (P\textsubscript{13}D\textsubscript{5}: αpPKC\textsubscript{θ} = 40:1). The PTDM: antibody complex was incubated for 30 min at RT. Meanwhile, CD4\textsuperscript{+} T cells were isolated from human PBMCs (purchased from StemCell Technologies, Inc.) via MojoSort\textsuperscript{TM} Human T Cell Isolation Kit (BioLegend). Isolated human CD4\textsuperscript{+} T cells were then treated with the PTDM: antibody complex for 4 hours at 37°C (some cells were treated with DMSO as vehicle control). Cells were harvested and washed with PBS. Later, cells were thoroughly washed twice with 20 U/mL heparin in PBS for 5 minutes on ice to remove surface-bound complexes outside cellular membrane. For iTreg differentiation, CellXVivo\textsuperscript{TM} Human Treg Differentiation Kit (R&D Systems) was used and iTreg Differentiation Media was prepared using X-VIVO\textsuperscript{TM} 15 Chemically Defined, Serum-free Hematopoietic Cell Medium according to manufacturer’s instructions. Treated cell pellets were then resuspended in iTreg differentiation media and seeded onto 5 \(\mu\)g/mL of anti-CD3\textepsilon- plus 2.5 \(\mu\)g/mL of anti-CD28-coated tissue culture wells and stimulated for 5 days at 37°C.

4.2.4 Immunoblotting

iTreg cells were harvested on day 5 of differentiation. Nuclear and cytosolic extracts were prepared by using NE-PER\textsuperscript{TM} Nuclear and Cytosolic Extraction Kit (Thermo Scientific). 1X SDS Laemmli Buffer was added into the samples for running on
8% SDS-PAGE for western blot. The blots were probed with anti-pPKCθ (Thr538) and anti-total PKCθ for further analysis. Anti-α-Tubulin was probed for cytosolic loading control and anti-histone H3 was probed for nuclear loading control.

4.2.5 Protein subcellular localization via AMNIS imaging flow cytometry

For in vitro analysis, iTreg cells were harvested on day 5 of differentiation. For in vivo analysis, bone marrow, spleen, and peripheral blood were collected on day +17 and single cell suspensions were prepared from each organ. Cells were surface-stained for CD4 BV711 and CD25 PECy7. Each sample was then fixed and permeabilized according to the manufacturer’s directions using the foxp3 staining buffer kit (BD Biosciences, Billerica, MA) and stained for foxp3 af488, pPKCθ (Thr538) followed by Qdot625-labeled secondary antibody, pSTAT3 (Tyr705) PE, or pSTAT5 (Tyr694) PE. Nuclei were stained using the cell-permeable draq5tm fluorescent probe (ThermoFisher Scientific, Waltham, MA). The cells were visualized and quantified using an ImageStream®X mark II imaging flow cytometer (EMD Millipore, Billerica, MA). Subcellular localizations and nuclear similarity scores of FOXP3, pPKCθ (Thr538), pSTAT3, and pSTAT5 proteins were determined using nuclear localization wizard on the ideas® software upon masking of nuclear and non-nuclear regions to quantify proteins localized in and out of the nucleus.
4.2.6 *In vitro* suppression assay

On day 0, human CD4\(^+\) T cells were plated on Anti-CD3+Anti-CD28-coated wells and differentiated for 5 days in iTreg differentiation media. On day 5, iTreg cells (suppressors) were loaded with a cell tracker dye, Red650 (APC fluorescence). On the other hand, total hPBMCs (responders) were thawed and stimulated in solution with Anti-CD3+Anti-CD28 crosslinked by mouse IgG. After the soluble stimulation, the cells were loaded with another cell tracker dye, UltraGreen (FITC fluorescence). Upon the loading, responders were seeded on tissue culture plate and suppressors were added onto the responders in various ratio. Cells were co-cultured for 4 days to look at proliferation of responders. On day 9, percent of *in vitro* suppression efficiency of the suppressors was calculated as Suppression (%) = 100% - FITC-negative cells (%).

4.2.7 Surface vs. intracellular expression of co-inhibitory receptors

iTreg cells were harvested on day 5 of differentiation. Cells were stained with Zombie Aqua viability dye. For surface only expression (Staining 1), cells were directly stained for LAG-3 BV421, PD-1 APC/Fire750, CTLA-4 APC or NRP1 PerCP/Cy5.5. For surface+intracellular expression (Staining 2), cells were fixed and permeabilized according to the manufacturer’s directions using the Foxp3 Staining Buffer Kit followed by staining with those fluorescently-conjugated co-inhibitory receptor antibodies. Samples were run on BD LSRFortessa\textsuperscript{TM} Flow Cytometer (Becton Dickinson) and median fluorescent intensities (MFI) for each protein was calculated. For intracellular only expression, MFI of Staining 1 was subtracted from MFI of Staining 2.
4.2.8  **Quantitative Real Time PCR (Q-PCR)**

Total RNA was isolated from samples via Quick-RNA Isolation Kit (Zymo Research) according to the manufacturer’s protocol. 1 µg of total RNA was reverse transcribed to cDNA using dNTPs (New England Biolabs, Inc.), M-MuLV reverse transcription buffer (New England Biolabs), oligo-DT (Promega), RNase inhibitor (Promega), and M-MuLV reverse transcription (New England Biolabs, Inc.) on a Mastercycler gradient Thermal Cycler (Eppendorf). Q-PCR primers used in this study were listed in Table 4.1. Q-PCR was performed in duplicate with 2x SYBR Green qPCR Master Mix (BioTool) using the RealPlex² system (Eppendorf). Q-PCR conditions were as follows: 95°C for 1 min, 95°C for 25 s, 62°C for 25 s (40 cycles), 95°C for 1 min, 62°C for 1 min, and 95°C for 30 s. Relative gene expression was determined using the ΔΔCt method. The results are presented as the fold expression in gene expression normalized to the housekeeping gene β-actin for cells and relative to Tconv + DMSO sample for *in vitro* experiments and Naïve + DMSO for *in vivo* experiments.

4.2.9  **In vivo suppression analysis via adoptive transfer of iTregs in humanized GvHD model**

hPBMCs from a healthy donor were used to isolate total CD4⁺ T cells and subsequently treated with P_{13}D₅: αPKCθ complex. They were differentiated for 5 days into iTregs as previously described. On day 4, total hPBMCs from the same donor were thawed and rested overnight in fresh RPMI complete media (10% fetal bovine serum, 100
U/mL penicillin-streptomycin, 1 mM sodium pyruvate, 2 mM L-Glutamine) at 37°C in 5% CO₂ incubator. On day 5, NOD.Cg-Prkdc<sup>scid</sup> Il2rg<sup>tm1WjI</sup>/SzJ (NSG) mice were conditioned with 2 Gy of total body irradiation using a ¹³⁷Cs source then rested for 4-6 hours. 10x10<sup>6</sup> of total hPBMCs was mixed with 3.3x10<sup>6</sup> of iTreg cells and adoptively transferred into irradiated NSG mice via the tail vein. Body weight and disease symptoms were observed daily. On day 17, some animals were sacrificed for tissue analysis. After CO₂ asphyxiation, peripheral blood was obtained via cardiac puncture. Sterna and spleens were collected for histology. BM cells were recovered from the tibias and femurs of both legs by flushing the bones with complete RPMI media. Splenocytes were isolated by manipulation through a 40 µm filter. Red blood cells were lysed in ACK lysis buffer, and the remaining white blood cells were enumerated using Trypan Blue exclusion. White and red cell counts were performed using scil Vet ABC™ Hematology Analyzer (scil Animal Care Company GmbH). Bone marrow, spleen, and peripheral blood were assessed for percent engraftment of hPBMCs (% positive human CD45 / (% positive human CD45 cells + % positive mouse CD45 cells)) and infiltration of human CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Also, the human CD4<sup>+</sup> T cells were analyzed for CD25, CD127, FOXP3, CTLA-4, LAG-3, NRP1, PD-1, pPKC0 (Thr538), and pSTAT5 (Tyr694) expression.

4.2.10 GvHD Clinical Scoring

GvHD severity was assessed using a standardized scoring system, as previously described and which included five different criteria (weight loss, posture, activity, fur texture, and skin integrity) (Ozay et al., 2016). Mice were weighed, evaluated daily, and graded from 0 (the least severe) to 2 (the most severe) for each criterion, beginning on
day +12 after disease induction. Daily clinical scores were generated by adding grades for five criteria. When a clinical score of “8” was reached, mice were removed from the study and humanely euthanized. The day of removal from the study was recorded as the day of lethal GvHD induction.

4.2.11 Magnetic sorting of *ex vivo* iTregs for mRNA analysis

Spleen and bone marrow were collected from NSG mice on day 17 post GvHD induction. Bone marrow cells were recovered from the tibias and femurs. Splenocytes were isolated by manipulation through a 40 µm filter. Red blood cells were lysed in ACK lysis buffer, and the remaining white blood cells were enumerated using Trypan Blue exclusion. Afterwards, cells were incubated with human CD4 T lymphocyte enrichment cocktail (BD Biosciences) followed by an incubation with BD IMag™ Streptavidin Particles Plus (BD Biosciences) to deplete non-CD4 T cell fraction. Biotinylated anti-CD127 antibody and biotinylated anti-CD25 antibody followed by an incubation with BD IMag™ Streptavidin Particles Plus were sequentially used to obtain iTreg cell fraction (negative fraction from anti-CD127 incubation and positive fraction from anti-CD25 fraction) and naïve T cell fraction (positive fraction from anti-CD127 incubation and negative fraction from anti-CD25 incubation). After cells were isolated, total RNA isolation procedure was followed.
4.2.12 Histology

Sterna and spleens harvested on day +17 were fixed overnight in 10% NBF (VWR), decalcified 48 h (Cal-Rite; Richard Allen Scientific), preserved in 70% ethanol at 4°C until processed, paraffin-embedded, sectioned, and stained with hematoxylin and eosin.

4.2.13 LEGENDPlex™ Bead-based Immunoassay

Peripheral blood for cytokine analysis was obtained in heparin-coated syringes on day +17 from animals via cardiac puncture, immediately following humane euthanasia. The LEGENDPlex™ Human Th1/Th2 panel (8-plex; BioLegend) was used to determine the levels of IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IFN-γ and TNF-α. Data were acquired on BD LSRFortessa™ Flow Cytometer and analyzed using LEGENDPlex™ Software, Version 7.0 (BioLegend).

4.2.14 Statistical Analysis

The results are shown are the mean ± SEM; all in vitro experimental replicates were repeated at least three times. All in vivo experimental replicates were repeated in three separate experiments. Unpaired, two-tailed Student’s t test using (Prism5; GraphPad Software, San Diego, CA) was used for statistical comparison of two groups, with Welch’s correction applied when variances were significantly different. Survival benefit was determined using Kaplan–Meier analysis with an applied log-rank test. P values of ≤0.05 were considered significantly different.
4.3 Results

4.3.1 Generation of CD4^+CD25^hiFOXP3^hi iTregs in vitro via cell-penetrating αPKCθ

Previously, we showed that complexation of cell penetrating peptide mimic, P_{13}D_{5}, with PKCθ neutralizing antibody (αPKCθ-Thr538) outperformed successful, efficient intracellular delivery into T cells. We discovered that delivering αPKCθ into T cells significantly reduced downstream PKCθ signaling and its nuclear translocation as well as attenuated Th1 differentiation (Ozay et al., 2016). Therefore, we investigated the effect of αPKCθ delivery in iTreg differentiation. We isolated CD4 T cells from hPBMCs and treated them with P_{13}D_{5}: αPKCθ complex (cell-penetrating αPKCθ) prior to iTreg differentiation in vitro. Later, CD4 T cells were cultured in iTreg-polarizing conditions in the presence of CD3 and CD28 stimulation for 5 days (Figure 4.1a). We found that there was greater percentage of CD4^+CD25^+FOXP3^+ iTregs (iTreg+αPKCθ) upon αPKCθ delivery as compared to non-treated cells (iTreg+DMSO, vehicle control) (Figure 4.1b). Both DMSO- and αPKCθ-treated iTregs were able to upregulate CD25 expression significantly more than non-polarized T cells (Tconv+DMSO or Tconv+αPKCθ) (Figure 4.1c). Interestingly, when we gated CD4^+CD25^hi T cells, αPKCθ delivery further enhanced the percentage of FOXP3-expressing CD4^+CD25^hi iTregs under iTreg-polarizing conditions, whereas most of DMSO-treated iTregs remained as FOXP3-negative (Figure 4.1d, e). Moreover, CD45RA and CD45RO expression can be used to identify iTreg populations (Walter et
al., 2016; Booth et al., 2010). We found that there was no significant difference in total CD45, CD45RA, and CD45RO expressions between DMSO- and αpPKCθ-treated iTregs (Figure 4.2a-c). In αpPKCθ-treated iTregs, we observed an increase in CD25 expression and significantly higher amount of FOXP3 expression as compared to DMSO-treated iTregs although FOXP3 gene expression did not differ between the treatments (Figure 4.1f, g, 4.2d). In addition, αpPKCθ-treated iTregs were able to upregulate their IL10 expression as it is an important mediator of Treg function (Figure 4.2e). These results demonstrate that although iTreg-polarizing conditions with CD3 and CD28 stimulation significantly converted CD4 T cells into CD4+CD25hi T cells, only αpPKCθ-treated iTregs further enhanced the percentage of FOXP3-expressing CD4+CD25hi T cells with higher FOXP3 protein expression. Thus, cell-penetrating αpPKCθ delivery prior to iTreg differentiation leads to generate higher percentage of CD4+CD25hiFOXP3hi iTregs in vitro.

4.3.2 Nuclear vs. cytosolic PKCθ in iTreg+αpPKCθ cells

Next, we investigated whether αpPKCθ-treated iTregs inhibited PKCθ function as well as other phosphorylation-dependent signals that are important for iTreg function. Functional PKCθ is phosphorylated from Thr538 and pPKCθ (Thr538) is important to inhibit Treg function (He et al., 2014; Hage-Sleiman et al., 2015; Zanin-Zhorov et al., 2010). We observed similar percent pPKCθ-positive iTregs, however, significantly less pPKCθ expression in αpPKCθ-treated iTregs than DMSO-treated iTregs (Figure 4.3a, b). In addition, there was no significant PRKCQ gene (encoding PKCθ) expression upon
αpPKC0 delivery (Figure 4.4a). We also determined the localization of PKC0 upon αpPKC0 delivery in iTregs. As previously shown, αpPKC0 delivery sequestered PKC0 in the cytosol of both Tconvs and iTregs suggesting the inhibition of nuclear translocation of PKC0. However, we detected a different band than full, 82 kDa PKC0 protein (upper band) with lower molecular weight (lower band). We saw a significant decrease in the upper band and increase in the lower band in the cytosol of αpPKC0-treated iTregs (Figure 4.3c, d). Interestingly, only lower band showed up in the nucleus of iTregs and this was missing in αpPKC0-treated iTregs (Figure 4.3c, e). In addition to phosphorylation of PKC0, STAT3 and STAT5 in iTregs are translocated into the nucleus upon phosphorylation and regulate Treg-specific gene expression (Mahmud et al., 2013; Pallandre et al., 2007). We did not observe any significant change in both pSTAT3 (Tyr705) and pSTAT5 (Tyr694) expressions in both iTreg treatments (Figure 4.4b, c). We also measured STAT5A gene expression since STAT5 is shown to be more critical for Treg activity. We also did not see any significant change in the gene expression (Figure 4.4d). These results suggested that αpPKC0-treated iTregs diminished their PKC0 levels both in the cytosol and nucleus while generating a different PKC0 isoform with lower molecular weight. This isoform, which seemed to be particularly localized in the nucleus, may have an important function in iTregs.

4.3.3 Nuclear localization of functional Treg markers in iTreg+αpPKC0 cells

FOXP3 is the master transcription factor for Tregs. It induces multiple Treg-specific gene transcription programs with the help of enhancers such as STAT proteins
(especially STAT5). Since we observed that αpPKC0-treated iTregs had higher FOXP3 expression, we asked the question whether they also increased nuclear localization of FOXP3 and STAT5 regarding the high Treg functionality. In addition, there was a small fraction of nuclear total PKCθ in αpPKC0-treated iTregs as shown in Figure 4.3c. We both visualized and quantified nuclearly-localized FOXP3, pSTAT5 (Tyr694), pSTAT3 (Tyr705) as well as pPKCθ (Thr538) via imaging flow cytometry with higher sensitivity than western blot. We observed that αpPKC0-treated iTregs significantly increased nuclear localization of FOXP3 while reducing nuclear localization of active pPKCθ (Thr538) (Figure 4.5a, b). αpPKCθ delivery did not affect FOXP3 translocation in Tconvs and slightly reduced nuclear translocation of pPKCθ (Thr538) (Figure 4.6a, b). Furthermore, nuclear translocation of both pSTAT3 (Tyr705) and pSTAT5 (Tyr694) significantly increased in αpPKC0-treated iTregs while not differing in αpPKC0-treated Tconvs (Figure 4.5c, d, 4.6c, d). These results indicate that αpPKC0-treated iTregs may enhance Treg functionality by increasing nuclear FOXP3, pSTAT3, and pSTAT5 while reducing nuclear pPKCθ simultaneously.

4.3.4 Super-suppressive iTreg+αpPKCθ cells positive for high surface LAG-3 and PD-1 expression

It was of a great importance whether αpPKC0-treated iTregs exhibited enhance functionality since they sequestered PKCθ in the cytosol and had higher nuclear FOXP3 and pSTAT5 (Tyr694). Tregs function as immunosuppressive cells by inhibiting effector T cell activation. We tested their suppression ability in vitro by measuring the inhibition
of effector T cell proliferation when they mixed in co-culture. We differentiated iTregs for 5 days with and without αpPKC0 delivery as previously described. On day 5, differentiated iTregs (suppressors, T_{sup}) were co-cultured with stimulated hPBMCs (responders, T_{res}) for four more days in three different ratios to measure Treg suppression potency. Suppressors and responders were also labeled with different cell tracker dyes in order to track their proliferation (Figure 4.7a). Strikingly, αpPKC0-treated iTregs exhibited significantly better suppression of responder T cell proliferation when mixed in 1:1 and 1:10 ratio (Figure 4.7, 4.8a). Also, αpPKC0-treated iTregs were able to proliferate better than DMSO-treated iTregs (Figure 4.8b). Interestingly, αpPKC0-treated iTregs appeared to preserve their Treg-ness as they showed significantly higher percentage of CD4^{+}CD25^{hi}FOXP3^{hi} iTreg population in the co-culture (Figure 4.7c).

Immunosuppressive ability of Tregs is mediated through co-inhibitory receptors expressed on the surface of the cell, hence, these receptors on Tregs bind to co-inhibitory ligands expressed on effector T cell surface. NRP1, PD-1, LAG-3, and CTLA-4 are the main co-inhibitory receptors expressed in Tregs that increase contact time and lead decreased cytokine production in effector T cells (Thaventhiran, 2013; Park et al., 2012; Zhang and Vignali, 2016; Okazaki et al., 2011; Gonçalves-Lopes et al., 2016; Delgoffe et al., 2013; Overacre and Vignali, 2016; Radziewicz et al., 2009; Sarris et al., 2008; Mizui and Kikutani, 2008; Yokosuka et al., 2012). However, these receptors are effective in cell-to-cell suppression when they are expressed on surface (Wang et al., 2001b; Woo et al., 2010; Raimondi et al., 2006). For instance, freshly isolated Tregs retain PD-1 in intracellular compartments and upon TCR signaling they translocate PD-1 to the cell surface and become more suppressive (Raimondi et al., 2006). Therefore, we measured
both intracellular and surface expression of the co-inhibitory receptors upon αpPKC0 delivery in iTregs. Interestingly, αpPKC0-treated iTregs had significantly more surface LAG-3 and less intracellular LAG-3 as compared to DMSO-treated iTregs despite the similarity in LAG3 gene expression (Figure 4.7c, d, 4.8c). The most striking difference observed was elevated surface PD-1 expression on αpPKC0-treated iTregs. Both the protein and the gene expression of PD-1 were significantly increased as compared to DMSO-treated iTregs (Figure 4.7f, g). Conversely, there was significantly less intracellular PD-1 in αpPKC0-treated iTregs (Figure 4.8d). On the other hand, we did not observe any difference in surface and intracellular CTLA-4 levels as well as CTLA4 gene expression (Figure 4.8e-g). In addition, surface NRP1 levels were similar in both iTregs, whereas αpPKC0-treated iTregs significantly reduced their intracellular NRP1 (Figure 4.7h, i). NRP1 gene expression levels were also comparable (Figure 4.8J). These data revealed that αpPKC0-treated iTregs acquire higher surface LAG-3 and PD-1 expressions and become more potent in effector T cell suppression in vitro.

4.3.5 Highly efficacious iTreg+αpPKC0 cells in vivo in humanized GvHD model

We next studied in vivo suppression efficiency of αpPKC0-treated iTregs in a disease model. Previously, we successfully tested the inhibitory effect of pPKC0 via cell-penetrating antibody in the humanized mouse model of GvHD (Ozay et al., 2016). The understanding of the pathophysiology of GvHD to develop novel treatment regimens has been supported by studies in animal models (Boieri et al., 2016). Humanized GvHD mouse models have contributed significantly to the understanding of healthy and diseased
human hematopoiesis and have led to the translation of promising therapeutic compounds into clinical applications (Theocharides et al., 2016). Especially, adoptive immunotherapy with Tregs in the GvHD model represents a viable strategy to understand T cell biology and Treg-mediated suppression, thereby facilitating the translation into human clinical use (Trzonkowski et al., 2015; Hahn et al., 2015). It was shown that presence of iTregs correlated with attenuated GvHD response and provided long-term graft tolerance without the need for drug-induced immunosuppression (Roncarolo and Battaglia, 2007; Spence et al., 2015; Tang and Bluestone, 2013; Rosa Bacchetta, Mike Bigler et al., 1994). In order to evaluate potential translational efficacy of αpPKCθ-treated iTregs along with their in vivo suppression ability, we utilized our humanized GvHD model together with adoptive iTreg transfer. In this graft (hPBMCs)-versus-host (NSG mouse) model, transferred lymphocytes acutely target the bone marrow of recipient mice resulting in lethal immune-mediated bone marrow failure in three weeks. The disease shows its critical symptoms at peak around day 17 according to our previous findings (Ozay et al., 2016). iTregs, with or without αpPKCθ delivery, were differentiated from the same donor whose PBMCs induced GvHD in this model. Later, we adoptively transferred differentiated iTregs simultaneously with hPBMCs into the mice via tail vein. We used 3:1 ratio of hPBMC to iTreg for the adoptive transfer based on our in vitro suppression data (Figure 4.9a). On day 17, we analyzed bone marrow (BM) cellularity as an indication of GvHD suppression. As expected, GvHD control showed significantly reduced BM cellularity. However, adoptive transfer of both DMSO-treated iTreg and, more profoundly, αpPKCθ-treated iTregs preserved BM cellularity (Figure 4.9b). Moreover, the number of red and white blood cells as well as other blood
counts appeared to increase upon iTreg administration (Figure 4.9c, 4.10a). These results proved the clinical benefit of iTreg transfer as a means of in vivo suppression of autoreactive T cells in humanized GvHD model.

Next, we looked at the human hematopoietic engraftment, human CD4, CD8 infiltrations into bone marrow, spleen, and peripheral blood in these mice by quantifying CD45-, CD4-, and CD8-expressing populations. Our gating strategy to analyze these population was shown in Figure 4.10b. We observed that percentage of human CD45-positive cells in the bone marrow was significantly diminished upon adoptive transfer of iTregs as compared to GvHD control, whereas CD45-positive cells remained similar in the spleen and peripheral blood (Figure 4.9d, 4.10c). In addition, there were significantly less CD4 and CD8 infiltrations into bone marrow upon adoptive iTreg transfer suggesting the impaired migration ability of autoreactive T cells by virtue of iTreg immunosuppression (Figure 4.9e, 4.10d). We also observed significant decrease in circulating CD4 T cells only upon αpPKC0-treated iTreg transfer (4.9e, 4.10d). Nevertheless, we did not see any significant change in circulating CD8 T cells, CD4 and CD8 T cells in the spleen (Figure 4.9e, 4.10d). Since autoreactive CD4 and CD8 infiltrations into bone marrow exhibit severe, hypoplastic BM and pancytopenia, the disease severity was also seen on H&E-stained sterna (bone marrow) of humanized GvHD control (Figure 4.9f). While spleen histology among the cohorts did not differ, adoptive transfer of DMSO-treated iTregs mildly affected GvHD progression and, strikingly, adoptive transfer of αpPKC0-treated iTregs significantly ameliorated GvHD as high bone marrow cellularity was observed (Figure 4.9f). We next asked whether
administering αpPKC0-treated iTregs would affect GvHD severity and survival. Cumulative GvHD clinical score was significantly diminished upon αpPKC0-treated iTreg transfer (Figure 4.9g). Remarkably, adoptive transfer of αpPKC0-treated iTregs significantly extended survival of humanized GvHD mice as the median survival increased up to 78 days while the median survival of mice administered with DMSO-treated iTregs was 38 days (Figure 4.9h). Since GvHD was mediated by Th1 response as a result of impaired Th1/Th2 balance, we measured Th1 and Th2 cytokines in the circulation. We saw a significant increase in IL-2, IL-4, IL-10, IL-13, and TNFα levels upon transfer of αpPKC0-treated iTregs implying more Th2 response than Th1 response with anti-inflammatory environment (Figure 4.10e). Unexpectedly, we detected significantly higher IFNγ levels in mice transferred with αpPKC0-treated iTregs than the ones transferred with DMSO-treated iTregs (Figure 4.9i). Overall, these data demonstrated that αpPKC0-treated iTregs were highly efficacious cells for in vivo suppression in the humanized GvHD model, thus, showed promising advantage in clinical cell-based therapy for GvHD.

4.3.6 A unique population of FOXP3^{hi}PD-1^{hi}IFNγ^{hi} iTregs upon intracellular αpPKC0 delivery

αpPKC0-treated iTregs provided superior benefit for preventing GvHD in the humanized model. However, we analyzed whether these cells would be detected by day 17. Accumulating evidences indicated that in vitro expanded Tregs have unstable FOXP3 expression, therefore they may lose their Treg properties under proinflammatory conditions, which characterize GvHD milieu, in vivo (Beres and Drobyski, 2013). To
establish better correlation of iTregs and low risk of GvHD upon adoptive transfer, we characterized iTregs based on CD4, CD25, CD127, and FOXP3 expression as it was shown in previous reports (Z Fang, Hua Z, Changying L, Jianmin W, 2013; Bremm et al., 2011; Trzonkowski et al., 2009). First, we gated CD4+CD127-LO population from the live cells. Later, we looked at CD25+FOXP3+ population within this gate. Intriguingly, in the BM, we saw significantly higher percentage and number of CD4+CD127-LOCD25+FOXP3+ iTregs in mice transferred with αpPKC0-treated iTregs than in mice with DMSO-treated iTregs (Figure 4.11a-c). On the other hand, the percentages for iTregs did not appear to be different in peripheral blood and spleen (Figure 4.12a-c). We found these mice transferred with αpPKC0-treated iTregs had significantly more FOXP3hi expressing population as compared to the mice transferred with DMSO-treated iTregs in their BM although FOXP3 protein expression was not significantly different (Figure 4.11d). Also, we observed FOXP3hi-expressing iTreg population in the circulation, but not in the spleen, in mice transferred with αpPKC0-treated iTregs (Figure 4.13a-b). Interestingly, CD4+CD127-LOCD25+FOXP3+ BM iTregs from the mice with αpPKC0-treated iTregs had significantly lower percentages of pPKC0-expressing iTregs, albeit no difference in pPKC0 protein expression, than iTregs in mice transferred with DMSO-treated iTregs (Figure 4.11e). Similar to FOXP3 results, we observed lower pPKC0-expressing iTreg population in the circulation, but not in the spleen, in mice transferred with αpPKC0-treated iTregs (Figure 4.13a, b). Nuclear localization of pPKC0 in these BM iTregs in mice transferred with αpPKC0-treated iTregs was significantly diminished as well while we did not observe any change in nuclear localization in these cells in peripheral blood and spleen (Figure 4.11f, 4.12d, e). Interestingly, nuclear
localization of FOXP3 in BM iTregs was significantly decreased in the mice transferred with \( \alpha \)pPKC\( \theta \)-treated iTregs (Figure 4.12f). On the contrary, we found higher nuclear localization of FOXP3 in splenic iTregs from the mice transferred with \( \alpha \)pPKC\( \theta \)-treated iTregs while circulating iTregs had similar percentages of nuclear FOXP3 in both the cohorts (Figure 4.12g, h). In consistent with \textit{in vitro} data, we saw significantly more PD-1\textsuperscript{hi}-expressing BM iTreg population in the mice transferred with \( \alpha \)pPKC\( \theta \)-treated iTregs than in the mice transferred with DMSO-treated iTregs although total percentage of PD-1-positive cells seemed comparable between the cohorts. PD-1 protein expression appeared to be higher in BM iTregs from the mice transferred with \( \alpha \)pPKC\( \theta \)-treated iTregs despite it did not reach significance (Figure 4.11g). On the other hand, we did not observe any difference in PD-1-expressing iTregs and their PD-1 protein expression in the peripheral blood and spleen of the mice from both cohorts (Figure 4.13a, b). We also measured CD25, pSTAT5 (Tyr694), LAG-3, and NRP1 protein expression levels in iTregs from BM, peripheral blood, and spleen in both cohorts, however, we did not observe any stark differences (Figure 4.13c-e). These results suggested that \( \alpha \)pPKC\( \theta \)-treated iTregs preserved higher iTreg population in GvHD mice and exerted stable FOXP3 expression \textit{in vivo} together with retaining the characteristics of super-suppressive iTregs (lower nuclear pPKC\( \theta \) and higher PD-1) differentiated \textit{in vitro}.

In addition to protein expression, we also investigated the transcriptional changes by looking at gene expression in these iTregs. We magnetically sorted CD4\(^{+}\)CD127\(^{-}\)CD25\(^{+}\) T cells, defined as iTregs, as well as CD4\(^{+}\)CD127\(^{+}\)CD25\(^{-}\) T cells, defined as naïve T cells from BM and spleen. Strikingly, BM iTregs, but not naïve T cells, from the mice transferred with \( \alpha \)pPKC\( \theta \)-treated iTregs exhibited higher level, although it did not reach
significance, of $FOXP3$ transcript (Figure 4.11h). In splenic iTregs, we did not observe any significant change in $FOXP3$ transcript levels between the cohorts (Figure 4.13f). More interestingly, we observed significantly higher levels of $PDCD1$ transcripts in both naïve T cells and iTregs from the BM of the mice transferred with αpPKCθ-treated iTregs relative to the mice transferred with DMSO-treated iTregs (Figure 4.11i). Splenic cells did not show any difference in $PDCD1$ mRNA levels (Figure 4.13g). Surprisingly, we found that BM iTregs from the mice transferred with αpPKCθ-treated iTregs had elevated $IFNG$ mRNA expression than the BM iTregs from the mice transferred with DMSO-treated iTregs (Figure 4.11j). On the other hand, we saw an opposite trend in $IFNG$ mRNA expression in splenic iTregs from the mice transferred with αpPKCθ-treated iTregs (Figure 4.13h).

These results suggested the elevated IFNγ concentration in the plasma in these mice with αpPKCθ-treated iTregs may be linked to IFNγ-expressing iTregs that were modulated upon αpPKCθ delivery. In order to examine the effect of αpPKCθ delivery on IFNγ expression in iTregs, we set up an in vitro differentiation experiment and measured both the protein and gene expression levels of IFNγ in the differentiated iTregs. Remarkably, there were significantly higher percentage of IFNγ-expressing iTregs with enhanced IFNγ production both at the protein and gene level upon intracellular αpPKCθ delivery (Figure 4.11k). These results demonstrated that αpPKCθ delivery in iTregs reprogrammed their differentiation in vitro and resulted in a unique iTreg population co-expressing higher FOXP3, PD-1, and IFNγ with superior suppression ability. More importantly, these in vitro generated αpPKCθ-treated iTregs preserved their phenotype
and function in vivo reinforcing the idea of their promising clinical efficacy as cell-based therapy.

4.4 Discussion

In our previous study, we demonstrated for the first time that PKCθ actions could be specifically inhibited by αpPKCθ delivery via cell-penetrating peptide mimics against Thr538 phosphorylation site and this led to sequestration of PKCθ in the cytosol and diminished T cell activity. Moreover, this delivery strategy could be used to manipulate T cells ex vivo prior to their transfer into a humanized mouse model of GvHD. Ex vivo delivery of αpPKCθ into T cells prior to the transfer showed reduced clinical severity and provided a significant survival benefit in the GvHD model (Ozay et al., 2016). In this report, we demonstrated that targeting phosphorylated PKCθ via a cell-penetrating antibody (intracellularly delivered αpPKCθ-Thr538 via cell-penetrating peptide mimics) favored iTreg differentiation and expansion in vitro. αpPKCθ-treated iTregs significantly enhanced their suppression ability in vitro as characterized by overexpression of co-inhibitory receptors, PD-1 and LAG-3. In addition, these super-suppressive iTregs were stable in vivo even after 17 days of their transfer into humanized GvHD mice and appeared to be highly efficacious in preventing GvHD in vivo. Overall, inhibiting PKCθ via a cell-penetrating antibody modulated iTreg differentiation resulting in a unique population that co-expresses higher FOXP3, PD-1, and IFNγ.
The use of therapeutic antibodies in targeting aberrant signaling pathways has been efficacious as a biotherapy in clinic since the antibodies are very specific and well tolerated by patients. However, clinical applications are currently limited to cell surface or extracellular targets and have limitations in tissue penetration (Beck et al., 2010; Chan and Carter, 2010; Brekke and Sandlie, 2003; Torchilin, 2009). Current FDA-approved immunosuppressive antibodies have the disadvantage of targeting common pathways such as calcium signaling (Anti-integrin αIIbβ3: Abciximab, Anti-CD20: Rituximab), T cell stimulation (Anti-CD3: OKT3-muromonab), T cell costimulation (Anti-PD-1: Nivolumab, Pembroliumab, Anti-CTLA-4: Ipilimumab), IL-2 signaling (Anti-CD25: Basiliximab, Daclizumab) or global T cell deletion (Anti-thymocyte globulin: ATG, Anti-CD52: Alemtuzumab) (Nurden et al., 2004; Stroopinsky et al., 2012; Benekli et al., 2006; Barbee et al., 2015; Martin-Liberal et al., 2015; Bowyer et al., 2016; Hsieh et al., 2017; Chae et al., 2017; Wartewig et al., 2017; Brennan et al., 2006; Kandus et al., 2010; Ontaneda and Cohen, 2013; Tang and Bluestone, 2013). Thus, there is an unmet need in targeting intracellular molecules via therapeutic antibodies with higher penetration in order to avoid off-target effects with higher safety (Beck et al., 2010; Chan and Carter, 2010; Brekke and Sandlie, 2003; Daugherty and Mrsny, 2006). Our strategy shows the strength of the use of cell-penetrating peptide mimics to deliver therapeutic antibodies successfully into T cells. Cell-penetrating antibodies allow us to target any intracellular molecule with greater safety, thereby facilitating exploration of novel targets and manipulating specific molecular pathways in the cell.
Besides therapeutic antibodies, cell-based therapies are currently the main focus of biomedical sciences since they allow developing personalized medicine to minimize side effects and provide long-term prevention of immunological diseases (Tang and Bluestone, 2013; Fischbach et al., 2013). In particular, T cell therapy has shown remarkable efficacy in treating recurrent cancers, GvHD, and type I diabetes. T cells are highly specific, adaptable, ‘smart’ therapeutic agents that selectively target tissues by tuning their activities in response to tissue microenvironment (Tang and Bluestone, 2013; Rosenberg and Restifo, 2015). The most pioneering example of cell-based therapies that have superior clinical efficacy in cancer immunotherapy is use of chimeric antigen receptor (CAR)-engineered T cells. Ex vivo engineered CAR T cells have shown promising results in treating chronic lymphoid leukemia (Fischbach et al., 2013; Singh et al., 2016). In addition to CAR T cell therapy, the possibility that Tregs might be used for the treatment of T cell-mediated diseases has recently gained momentum. The very first mouse study for Treg-based immunotherapy was treating GvHD in BMT model. (Wood and Sakaguchi, 2003; Sela et al., 2011; Heinrichs et al., 2016; Komanduri and Champlin, 2011; Hahn et al., 2015). Freshly isolated Tregs together with bone marrow allograft has been shown to ameliorate GvHD and has assisted successful engraftment (Pilat et al., 2010; Parmar and Shpall, 2016; Ganguly et al., 2014; Trzonkowski et al., 2015). GvHD in BMT was selected as the first setting for human clinical trials to test the feasibility and safety of the Treg therapy (Rosa Bacchetta, Mike Bigler et al., 1994; Roncarolo and Battaglia, 2007; Blazar et al., 2012; Tang and Bluestone, 2013). Upon administration of Tregs in vivo, they can exert their suppressive function by inhibiting effector T cell activation, cytokine production, and migration. Also, they can downregulate dendritic cell
maturation in a cell contact-dependent manner, inhibit monocyte and macrophage survival through FasL signaling, and restrain neutrophil activity by promoting their apoptosis (Misra et al., 2004; Venet et al., 2006; Taams et al., 2005; Lewkowicz et al., 2006). Thus, Treg therapy conveys various benefits including specific immunosuppression due to antigen specificity, long-lasting regulation, customization designed for each patient with very low side effects (Roncarolo and Battaglia, 2007; Abou-El-Enein et al., 2017). However, there are several obstacles that limit Treg-based immunotherapy. First of all, Tregs must be collected from patient’s peripheral blood and need to be expanded in vitro. Number of circulating Tregs in humans is approximately around 0.25x10^9. In mice, 30x10^6 of Tregs provided long-term graft survival when combined with the deletion of donor-reactive T cells from the hosts. This number would be estimated around 30x10^9 of cells for a possible human GvHD therapy (Tang and Bluestone, 2013). Therefore, it is clear that ex vivo expansion is required to increase the number of Tregs. Secondly, they need to be phenotypically characterized as immunosuppressive. Recent studies showed that instead of isolating and expanding natural Tregs taken from patients ex vivo, in vitro conversion of CD4^+CD25^- T cells into iTregs cultured with several biologics (such as TGF-β, IL-2, all-trans retinoic acid, DNA methyltransferase (DNMT) inhibitors, histone deacetylase (HDAC) inhibitors butyrate, rapamycin etc.) showed greater immunosuppressive capacity, making iTregs more attractive for immunotherapy (Singer et al., 2014; Riley et al., 2009; Lu et al., 2014; Ohkura et al., 2013; Kukreja et al., 2002; Lindley et al., 2005; Curotto de Lafaille and Lafaille, 2009; Chen et al., 2003; Barrat et al., 2002; Benson et al., 2007). In our study, we found that upon intracellular αpPKCθ delivery, we could enhance the suppressive
capacity of iTregs that can also expand efficiently in vitro. αpPKC0-treated iTregs can express higher amounts of nuclear FOXP3, nuclear pSTAT5 (Tyr694), surface PD-1, and surface LAG-3 that contribute to their immunosuppressive function as compared to DMSO-treated iTregs. This strategy appears to be highly efficient for in vitro iTreg generation.

Another obstacle in successful Treg-based immunotherapy is Treg instability and plasticity in vitro (Singer et al., 2014; Ohkura et al., 2013; Li et al., 2014b). Tregs need to sustain their FOXP3 expression upon adoptive transfer in vivo (Sela et al., 2011). It is a concern whether iTreg cells induced in vitro can sustain their FOXP3 expression and suppressive function upon transfer. Also, Tregs can exert their immunosuppressive function once they reach to target organs (Shi et al., 2012; Booth et al., 2010). In our study, we utilized humanized GvHD model for which the target organ for the disease was bone marrow. Upon hPBMC transfer, autoreactive donor T cells migrate to the bone marrow and destroy hematopoietic stem cells as a result of GvHD. Our results demonstrated that mice transferred with αpPKC0-treated iTregs showed greater infiltration of iTregs into bone marrow with sustained FOXP3 expression and thus provided greater benefit for preventing GvHD in the long term. Furthermore, iTregs from the mice transferred with αpPKC0-treated iTregs possessed very similar immunophenotyping as in vitro differentiated, αpPKC0-treated iTregs suggesting the stability of these cells expanded in vitro.
Previously, we showed that inhibiting PKC\(\theta\) actions via cell-penetrating antibody was beneficial for preventing GvHD in humanized model as PKC\(\theta\) has been implicated as a driver protein for aberrant T cell activation in the context of GvHD progression (Ozay et al., 2016; Solomou et al., 2006; Valenzuela et al., 2009). PKC\(\theta\) is phosphorylated from Thr538 for its complete activation and, later, it translocates to both the nucleus and immunological synapse (Brezar et al., 2015; Bi et al., 2001). There are many studies suggesting PKC\(\theta\) inhibition as a novel therapy for T cell-mediated diseases (Hage-Sleiman et al., 2015; Cywin et al., 2007; Chand et al., 2012; Sun et al., 2000; Mochly-Rosen et al., 2012). PKC\(\theta\) has become an interesting target since it has opposing effects in effector T cells and Tregs. It was shown that inhibiting PKC\(\theta\) function in Tregs enhanced their suppressive function both \textit{in vitro} and \textit{in vivo} (Zanin-Zhorov et al., 2010; Sun, 2012). However, these studies utilized small molecule inhibitors that lacked the specific targeting of PKC\(\theta\) actions and other PKC family members (such as PKC\(\alpha\) and PKC\(\delta\)) could be affected by those inhibitors. Considering the greater specificity of antibodies, we showed that we could target PKC\(\theta\) in iTregs via highly specific cell-penetrating antibody to target its action. As a proof-of-principle, we observed reduction in phosphorylation of PKC\(\theta\) and enhanced suppressive function in iTregs. However, the molecular mechanism of action still remains unclear how inhibiting PKC\(\theta\) affected transcriptional changes as well as co-inhibitory receptor expression on the cell surface. Several studies showed that PKC\(\theta\) is located in close proximity with PD-1 in the cytosol (Sheppard et al., 2004; Yokosuka et al., 2012). We saw that there was a significant reduction in the cytosolic PKC\(\theta\) and higher surface PD-1 expression. Further studies will elucidate the potential connection between cytosolic PKC\(\theta\) and PD-1 signaling as a
means of enhanced iTreg suppressive function. Interestingly, we observed that nuclear PKC0 was completely sequestered in the cytosol upon αpPKC0 delivery. Nuclear PKC0 has been shown to interact with RNA polymerase II, histone kinases, histone deacetylases, and chromatin modifiers (Sutcliffe and Rao, 2011; Sutcliffe et al., 2012, 2011; Li et al., 2016; McCuaig et al., 2015) to regulate gene expression. Therefore, further studies are required to illuminate the critical role of nuclear PKC0 in the context of transcriptional and post-transcriptional control of Treg differentiation program.

An interesting characteristic that αpPKC0-treated iTregs exhibited in this study was their high IFNγ expression both in vitro and in vivo. IFNγ acts as a paradoxical cytokine in immune responses and inflammatory processes. It can promote Th1 function and T cell migration to the site of inflammation and initiate proinflammatory signaling events. On the contrary, the inflammation induced by IFNγ-producing Th1 cells triggers the immune system to call for a controlling mechanism (Wang et al., 2006). This leads to activation of multiple cellular and molecular events leading to peripheral conversion of CD4+CD25− T cells to CD4+CD25+ Tregs for the purpose of regulating overt inflammation. IFNγ has been demonstrated to enhance T cell migration to the central nervous system and promotes Th1-driven experimental autoimmune encephalomyelitis (EAE) as well as experimental autoimmune uveitis (Wang et al., 2006; Horwitz et al., 1997; OLSSON, 1995; Ferber et al., 1996; Willenborg et al., 1996; Krakowski and Owens, 1996; Caspi et al., 1994). Unexpectedly, blocking of IFNγ did not prevent but exacerbated the severity and pathogenesis of the disease (Ferber et al., 1996; Willenborg et al., 1996; Krakowski and Owens, 1996; Caspi et al., 1994; Jones et al., 1997; Chu et
al., 2000; Tran et al., 2000). It was found that IFNγ was promoted the conversion of CD4+CD25− T cells into CD4+CD25+ Tregs that ultimately suppressed the autoimmune response (Wang et al., 2006). Another study showed the conversion of CD4+CD25− T cells into FOXP3-expressing Tregs by copolymer-I (COP-I) treatment. COP-I-mediated conversion was IFNγ-mediated as recombinant IFNγ treatment further enhanced the number of FOXP3-expressing iTregs and T cells of IFNγ-knockout mice treated by COP-I were failed to induce FOXP3 expression both in vitro and in vivo (Hong et al., 2005). Furthermore, STAT1, an important mediator in IFNγ signaling, was found to be critical in the induction of CD4+CD25+ Tregs (Nishibori et al., 2004). Because we observed higher levels of IFNγ in the plasma from the mice transferred with αpPKC0-treated iTregs and more CD4+CD25+FOXP3+ iTregs in vivo and αpPKC0-treated iTregs differentiated in vitro showed significantly higher IFNγ production, it is possible that IFNγ may act as both an exogenous and endogenous factor to promote iTreg differentiation and enhance their function.

It has been argued that the source of IFNγ was critical to drive either proinflammatory or anti-inflammatory reaction in GvHD (Wood and Sawitzki, 2006). Considering Th1 cells as the mediators of GvHD progression due to their upregulated IFNγ expression, IFNγ has been implicated as a pathogenic cytokine promoting bone marrow destruction in GvHD (Lin et al., 2014). However, functional consequences of IFNγ production by Tregs in GvHD remain unexplored. It is likely that IFNγ produced by Tregs can be advantageous to prevent allogeneic skin graft rejection (Koenecke et al., 2012; Sawitzki et al., 2005). Moreover, allogeneic donor FOXP3-expressing Tregs
appeared to express IFNγ upon BMT and prevented the development of lethal GvHD (Taylor et al., 2002; Hoffmann et al., 2002). However, donor Tregs treated with neutralizing anti-IFNγ monoclonal antibody or Tregs from IFNγ-knockout donor mice failed to prevent the lethal GvHD (Koencke et al., 2012; Lu and Waller, 2009). In healthy individuals, IFNγ-producing iTregs comprise only 0.04% of all CD4 T cells in the peripheral blood and this population showed 5-fold increase of IFNG mRNA expression upon alloantigenic stimulation after renal transplantation. These renal transplant recipients had lower numbers of activated B cells, CD4 and CD8 T cells suggesting an immunoregulatory role for IFNγ-producing Tregs. Interestingly, they found that IFNγ produced by IFNγ-positive Tregs functioned in an autocrine manner and they were still able to suppress IFNγ production by the responder cells in co-cultures (Daniel et al., 2014; Chowdary Venigalla et al., 2012). More interestingly, several studies showed that only patients with good long-term graft function had IFNγ-positive Tregs that seemed more suppressive and stable than IFNγ-negative Tregs as they had higher FOXP3 expression due to lower methylation on their Treg-specific demethylated region (TSDR) (Daniel et al., 2015; Trojan et al., 2017; Daniel et al., 2016; Trojan et al., 2016). Together with these evidences, our results support the notion that αPKC0-treated iTregs exhibited more suppressive function and provided long-term graft function due to their nature of IFNγ expression. Further studies are required to investigate how PKC0 modulates IFNγ expression in iTregs and whether IFNγ production by these iTregs upregulate PD-1 and other co-inhibitory receptor expression as well as regulating FOXP3 TSDR methylation for the purpose of potentiating Treg suppressive capacity and stability.
Our results with the supporting evidence from the literature demonstrate that modulation of PKCθ function in iTregs reprograms their cell fate and enhances their immunosuppression ability both \textit{in vitro} and \textit{in vivo}. Intracellular αpPKCθ delivery via cell-penetrating peptide mimics seems to be very promising strategy to fine-tune iTreg differentiation in favor of generating and expanding a unique suppressive population. αpPKCθ-treated iTregs co-express higher FOXP3, PD-1, LAG-3, and IFNγ implying an outstanding feature of better suppressive iTreg phenotype. More importantly, adoptive transfer of αpPKCθ-treated iTregs into humanized GvHD mice appears to be highly efficacious in preventing GvHD \textit{in vivo} and providing long-term graft function. However, it remains to be elucidated what other observations need to be made in preclinical animal models to translate this into human therapy; which method should be used to safely and efficiently expand and generate iTregs \textit{ex vivo}; whether a combined therapy with Treg transfer and other immunosuppressive drugs is necessary. Therefore, these questions will need further preclinical studies to better design highly efficacious human Treg therapy in clinic.
Figure 4.1 αPKCδ delivery generates CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>hi</sup> iTregs in vitro. (a) Schematic of in vitro iTreg differentiation protocol in the presence of cell-penetrating Anti-αPKCδ (P₃D₆: αPKCδ). (b) Representative scatter plot of iTregs based on their CD25 and FOXP3 expression. (c) Quantification of the percentage of total CD4<sup>+</sup>CD25<sup>+</sup> T cells. (d) Percent of FOXP3-negative cells within CD4<sup>+</sup>CD25<sup>+</sup> T cell gate. (e) Percent of FOXP3-positive cells within CD4<sup>+</sup>CD25<sup>+</sup> T cell gate. (f) Representative histograms and median fluorescent intensity (MFI) of CD25 expression within CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> T cell gate. (g) Representative histograms and median fluorescent intensity (MFI) of FOXP3 expression within CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> T cell gate. Data represent mean ± SEM of three independent experiments. Unpaired, two-tailed t test was used for analysis; *p < 0.05, **p < 0.01, ***p < 0.0001.
Figure 4.2 Flow cytometric and qPCR analysis of certain iTreg differentiation markers in vitro. Representative histograms, percent positive, and median fluorescent intensity (MFI) of (a) CD45, (b) CD45RA, and (c) CD45RO expression. Fold expression of (d) FOXP3 and (e) IL10 mRNAs. Data represent mean ± SEM of three independent experiments. Unpaired, two-tailed t test was used for analysis.
Figure 4.3 pPKCθ is diminished in both cytosol and nucleus in iTreg+αpPKCθ cells. (a) Representative histogram of pPKCθ-positive cells upon αpPKCθ delivery during iTreg differentiation (and non-differentiating conditions). (b) Percent pPKCθ-positive cells and MFI of pPKCθ expression. (c) Representative blots for nuclear vs. cytosolic distribution of total PKCθ in conventional T cells (Tconvs) and iTregs upon αpPKCθ delivery assessed by western blot. (d) Quantification of relative density of cytosolic PKCθ for both lower and upper band seen in (c). (e) Quantification of relative density of nuclear PKCθ. Data represent mean ± SEM three independent experiments. Unpaired, two-tailed t test was used for analysis; *p < 0.05, **p < 0.01, ***p < 0.0001.
Figure 4.4 Analysis of pPKCθ, pSTAT3, and pSTAT5 expression for in vitro iTreg differentiation. (a) Fold expression of PRKCQ mRNA. Representative histograms, percent positive, and median fluorescent intensity (MFI) of (b) pSTAT3 (Tyr705) and (c) pSTAT5 (Tyr694) expression. (d) Fold expression of STAT5A mRNA. Data represent mean ± SEM of three independent experiments. Unpaired, two-tailed t test was used for analysis.
Figure 4.5 iTreg+pPKC0 cells localize functional Treg markers to nucleus while reducing nuclear pPKC0. Nuclear localization data for FOXP3, pPKC0 (Thr538), pSTAT3 (Tyr705), and pSTAT5 (Tyr694) were assessed via AMNIS imaging flow cytometry and 1000 cells were counted to collect the data. (a) Nuclear localization score distribution of FOXP3-expressing cells (left panel), quantification of nuclear similarity scores for FOXP3 (middle panel), representative image showing nuclear FOXP3 in iTregs (right panel). (b) Nuclear localization score distribution of pPKC0-expressing cells (left panel), quantification of nuclear similarity scores for pPKC0 (middle panel), representative image showing nuclear pPKC0 in iTregs (right panel). (c) Nuclear localization score distribution of pSTAT3-expressing cells (left panel), quantification of nuclear similarity scores for pSTAT3 (middle panel), representative image showing nuclear pSTAT3 in iTregs (right panel). (d) Nuclear localization score distribution of pSTAT5-expressing cells (left panel), quantification of nuclear similarity scores for pSTAT5 (middle panel), representative image showing nuclear pSTAT5 in iTregs (right panel). Data represent mean ± SEM three independent experiments. Unpaired, two-tailed t test was used for analysis; *p < 0.05, **p < 0.01.
Figure 4.6 Nuclear localization of critical iTreg proteins in conventional T cells (Tconv). Nuclear localization score distribution histograms (upper panels) and representative image showing nuclear protein localization in Tconvs (lower panels) for (a) FOXP3, (b) pPKC\(\alpha\) (Thr538), (c) pSTAT3 (Tyr705), and (d) pSTAT5 (Tyr694). Data represent mean ± SEM three independent experiments. Unpaired, two-tailed t test was used for analysis; *p < 0.05, **p < 0.01.
Figure 4.7 iTreg+αpPKCφ cells behave as super-suppressive iTregs and substantially express suppressive receptors, LAG-3 and PD-1, on their surface. (a) Experimental setup for in vitro suppression assay with UltraGreen-labeled responder cells and Red650-labeled suppressor cells mixed in three different ratios. (b) Percent of suppression efficiency of suppressor cells. (c) Flow cytometric analysis of iTregs on Day 4 co-culture with responders. (d) Representative histogram, quantification of percent-positive, and MFI of surface LAG-3-expressing iTregs. (e) LAG3 mRNA transcript levels via qPCR. (f) Representative histogram, quantification of percent-positive, and MFI of surface PD-1-expressing iTregs. (g) PDCD1 mRNA transcript levels via qPCR. Data represent mean ± SEM three independent experiments. Unpaired, two-tailed t test was used for analysis; *p < 0.05, **p < 0.01, ***p<0.001.
Figure 4.8 In vitro proliferation profile of responder and suppressor cells and their intracellular and surface co-inhibitory receptor expressions. Proliferation of (a) UltraGreen-labeled responder cells and (b) Red650-labeled suppressor cells mixed in three different ratios. Representative histograms, quantification of percent-positive, and MFI of (c) Intracellular LAG-3, (d) Intracellular PD-1, (e) Surface CTLA-4, (f) Intracellular CTLA-4, (h) Surface NRP1, and (i) Intracellular NRP1. Fold expression of (g) CTLA4 and (j) NRP1 mRNAs. Data represent mean ± SEM three independent experiments. Unpaired, two-tailed t test was used for analysis; *p < 0.05, **p < 0.01, ***p<0.0001.
Figure 4.9 Adoptive transfer of super-suppressive iTreg+αPKCθ cells that are highly efficacious in vivo in humanized GvHD model. (a) Schematic representation of adoptive transfer of in vitro differentiated iTregs into humanized GvHD model. (b) Bone marrow (BM) cellularity on day 17. (c) Blood counts of white blood cells (WBCs) and red blood cells (RBCs). (d) Percent of human CD45-positive cells in bone marrow, peripheral blood, and spleen on day 17. (e) Percent of human CD4+ and CD8+ T cell subsets in bone marrow, peripheral blood, and spleen on day 17. (f) Histopathological analysis of spleen and sternum on day 17 via H & E staining. (g) Clinical score for GvHD. (h) Survival curve. (i) IFNγ concentration in the plasma. 5 mice were used per group. Data pooled from and represent mean ± SEM of three independent experiments. Kaplan-Meier statistical analysis was performed for survival curve. Unpaired, two-tailed t test was used for analysis; *p < 0.05, **p < 0.01, ***p < 0.0001.
A tailed t test was used for analysis; *p < 0.05, **p < 0.01, ***p<0.0001.

Figure 4.10 Cellular and cytokine profiles of adoptive iTreg transfer experiment in humanized GvHD model on day 17. (a) Platelet counts, hemoglobin (g/dL) and hematocrit (%) values. (b) Gating strategy for flow cytometry experiments for human CD4+ and CD8+ T cells in bone marrow, peripheral blood, and spleen. (c) Aggregated data for percentages of hPBMCs in bone marrow, peripheral blood, and spleen. (d) Aggregated data for human CD4+ and CD8+ T cells infiltrating into bone marrow, peripheral blood, and spleen. (e) Cytokine profile for IL-2, IL-4, IL-5, IL-10, IL-13, and TNF-α present in peripheral blood. 5 mice were used per group. Data pooled from and represent mean ± SEM of three independent experiments. Unpaired, two-tailed t test was used for analysis; *p < 0.05, **p < 0.01, ***p<0.0001.
Figure 4.11 Super-suppressive iTreg+aqPKC0 cells are long lasting in vivo and represent a unique population of FOXP3+PD-1+IFNg+ iTregs. (a) Representative scatter plot for percent of CD4+CD127hiCD25hiFOXP3hi iTregs in bone marrow on day 17. (b) Quantification of percent of CD4+CD127hiCD25hiFOXP3hi iTregs in bone marrow on day 17. (c) Number of CD4+CD127hiCD25hiFOXP3hi iTregs in bone marrow on day 17. (d) Representative histogram and MFI of FOXP3 expression in CD4+CD127hiCD25hiFOXP3hi iTregs in bone marrow on day 17. (e) Representative histogram, percent positive, and MFI of pPKC0 expression in CD4+CD127hiCD25hiFOXP3hi iTregs in bone marrow on day 17. (f) Representative histogram and representative image of nuclear pPKC0-positive CD4+CD127hiCD25hiFOXP3hi iTregs in bone marrow on day 17. (g) Representative histogram, percent positive, and MFI of PD-1 expression in CD4+CD25hiFOXP3hi iTregs in bone marrow on day 17. qPCR analysis of (h) FOXP3, (i) PDCD1, and (j) IFNG gene expression in CD4+CD25hiCD127naive and CD4+CD25hiCD127iTreg in bone marrow on day 17. (k) Representative histogram, percent positive, MFI of IFNg expression, and IFNG mRNA transcript levels in Tconv and iTregs in vitro. In vivo data pooled from 4 mice and represent mean ± SEM of three independent experiments. In vitro IFNg data represent mean ± SEM of three independent experiments. Unpaired, two-tailed t test was used for analysis; *p < 0.05, **p < 0.01, ***p<0.001.
Figure 4.12 Analysis of iTregs in peripheral blood and spleen in humanized GvHD model on day 17. (a) Representative scatter plots for percent of CD4^+CD127^−/loCD25^+FOXP3^+ iTregs in peripheral blood and spleen. Quantification of percent of CD4^+CD127^−/loCD25^+FOXP3^+ iTregs in (b) peripheral blood and (c) spleen. Nuclear localization score histograms and representative images of nuclear pPKCθ-positive CD4^+CD127^−/loCD25^+FOXP3^+ iTregs in (d) peripheral blood and (e) spleen. Nuclear localization score histograms and representative images of nuclear FOXP3-positive CD4^+CD127^−/loCD25^+FOXP3^+ iTregs in (f) bone marrow, (g) peripheral blood, and (h) spleen. 5 mice (a-c) and 3 mice (d-h) were used per group. Data pooled from and represent mean ± SEM of three independent experiments. Unpaired, two-tailed t test was used for analysis.
three independent experiments. Unpaired, two-tailed t test was used for analysis; *p < 0.05, **p < 0.01.

Figure 4.13 Immunophenotyping of iTregs in bone marrow, peripheral blood, and spleen in humanized GvHD model on day 17. Representative histograms, percent positive, and MFI of FOXP3, pPKC0 (Thr38), and PD-1 expression in CD4+CD25+FOXP3+ iTregs in (a) peripheral blood and (b) spleen. Representative histograms, percent positive, and MFI of CD25, pSTAT5 (Tyr694), LAG-3, and NRP1 expression in CD4+CD25+FOXP3+ iTregs in (c) bone marrow, (d) peripheral blood and (e) spleen. qPCR analysis of (f) FOXP3, (g) PDCD1, and (h) IFNG gene expression in CD4+CD25CD127− (Naïve) and CD4+CD25+CD127− (iTreg) cells in spleen. Data pooled from 4 mice and represent mean ± SEM of three independent experiments.
Table 4.1 List of qPCR primers used in this study

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<tr>
<th>Gene</th>
<th>Forward Primer (5'-3')</th>
<th>Reverse Primer (5'-3')</th>
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</thead>
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<tr>
<td>ACTB (Housekeeping)</td>
<td>GTTGTGCAGACGACGACG</td>
<td>GCACAGACCCCGCCCTT</td>
</tr>
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<td>CTACCTGGGCATAGGCAACG</td>
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<td>IFNG</td>
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<td>CCTACACCTCTTTGGGATGCT</td>
</tr>
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<td>AGGGAGTTTCATGGCCTCT</td>
</tr>
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<td>LAG3</td>
<td>TCACCTGTCTGAGTCTGAG</td>
<td>CACTTGGCAGTGAGGAAAGA</td>
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<td>CTATCAATAGCGAGAAACCATG</td>
<td>CTACATCAACGAGACTCC</td>
</tr>
<tr>
<td>STAT5A</td>
<td>ACATTGGAGGAGCTGCGACT</td>
<td>CCTCCAGAGACACCTGCTTC</td>
</tr>
</tbody>
</table>
CHAPTER 5

PKCθ MODULATES PCMT1 TO SWITCH RNA PROCESSING AND FOXP3
STABILITY IN REGULATORY T CELLS

5.1 Introduction

Translational ability of RNA in the immune cells is altered by various immunological signals. Such alterations are essentially mediated by RNA binding protein (RBP) assemblies that coordinate multiple cellular mechanisms such as activation, tolerance, and plasticity. It was discovered that modifications in RBP-mediated post-transcriptional regulation influence cellular reactivity during inflammatory response and autoimmunity (Kafasla et al., 2014). RBPs include two main classes of proteins: heterogeneous nuclear ribonucleoproteins (hnRNPs) that bind to splicing silencers and serine-arginine-rich (SR) proteins that bind to splicing enhancers (Black, 2003; Matlin et al., 2005; Hung et al., 2008; Kornblihtt et al., 2013; Cooper et al., 2009; Wang et al., 2013). Although these proteins are firstly discovered as spliceosome components regulating alternative splicing, they are involved in numerous other cellular processes such as transcription, chromatin dynamics, mRNA stability, mRNA nuclear export, and translation (Melton et al., 2007; Ip et al., 2007; Lee et al., 2007; Shav-Tal and Zipori, 2002). These multifunctional RBPs remain bound to mRNA in order to facilitate nucleation of other regulatory proteins for the purpose of mRNA export to cytoplasm and subsequent translation efficiency (Keene, 2007; Kornblihtt et al., 2013; Han et al., 2010).
Several studies described that activity of these proteins is tightly regulated by phosphorylation in response to cellular stimuli leading to altered activity and localization of these proteins (Allemand et al., 2005; Blaustein et al., 2005; Patel et al., 2001, 2005; Van Oordt et al., 2000).

T cell receptor (TCR)-mediated signaling pathways result in multiple changes in the cell morphology and function as a result of alternative splicing and orchestration of positively- and negatively-regulating RBPs on 3’ untranslated regions (3’UTRs) (Crabtree and Clipstone, 1994; Weiss and Littman, 1994; Kafasla et al., 2014; Ip et al., 2007; Black, 2003; Matlin et al., 2005). Many of the immunological molecules including cytokines and chemokines harbor 3’UTR regulatory elements which enable fine-tuning of the immunological response with respect to cellular requirements (Ganguly et al., 2016; Meininger et al., 2016; Uehata et al., 2013). There have been several reports about alternative splicing and RNA processing regulated in a tissue- and cell-specific fashion through specific environmental cues (Grabowski, 1998; Wang and Manley, 1997; Chalfant et al., 1998; König et al., 2012; Screaton et al., 1997; Smith et al., 1997; Wang et al., 1997; Xie and McCobb, 1998). However, molecular mechanisms underlying such differences in post-transcriptional regulation are not investigated extensively.

One such well-characterized change is the alternative splicing of surface protein, CD45 (Trowbridge et al., 1991; Trowbridge and Thomas, 1994; Lynch and Weiss, 2000; Heyd and Lynch, 2010; Oberdoerffer et al., 2008). It is shown that protein kinase C and Ras signaling induce exon skipping of CD45 gene, thereby decreasing CD45 phosphatase activity (Lynch and Weiss, 2000; Hermiston et al., 2002; Lynch, 2004; Rothrock et al.,
Different CD45 isoforms are generated by triggering multiprotein complexes of RBPs including hnRNPL (Lemaire et al., 1999; Screaton1 et al., 1995; Fu, 1995; Manley and Tacke, 1996; Preußner et al., 2012; House and Lynch, 2006). The studies in the immune system have been focused on hnRNPL, a critical nuclear RBP with four RNA recognition motifs that mediates basal splicing, mRNA stability, and nuclear export (Hui et al., 2003b; a; Oberdoerffer et al., 2008; Rossbach et al., 2009; Gaudreau et al., 2016). hnRNPL binds to CA-repeat motifs and CA-rich elements thereby repressing exon skipping (Waterston et al., 2002; House and Lynch, 2006; Rothrock et al., 2005; Guang et al., 2005). T cell activation induces posttranslational modification of hnRNPL to increase its silencing activity (Oberdoerffer et al., 2008; Gaudreau et al., 2012; Vu et al., 2013). Because hnRNPL activity is higher in resting cells, they carry either CD45RA or CD45RB form whereas activated and memory T cells mainly express the shortest isoform, CD45RO, due to low hnRNPL activity (Oberdoerffer et al., 2008). In another study, CD45 isoform expression in regulatory T cells (Tregs) was associated with FOXP3 stability and suppressive capacity as well as their tissue distribution in vivo (Booth et al., 2010). More interestingly, differential splicing and RNA processing regulated by TCR and hnRNPL are key drivers of helper versus regulatory T cell differentiation. Knockdown of hnRNPL suppressed Treg induction suggesting that hnRNPL is critical RNA regulatory protein for stimulating the Treg differentiation program (Hawse et al., 2017).

Tregs are capable of suppressing an immune response for the maintenance of self-tolerance within the immune system (Pillai et al., 2007; Vignali et al., 2008; Rudensky, 2012; Gavin et al., 2006). Several studies demonstrated that demethylation of Treg-
specific demethylated region (TSDR) on intronic sequence of FOXP3 promoter is a prerequisite for stable expression and Treg suppressive function (Overacre and Vignali, 2016; Schmidt et al., 2016; Polansky et al., 2008; Lal and Bromberg, 2009). Our previous studies demonstrated that we could modulate T cell activation via delivering a cell-penetrating antibody against functional PKCθ and utilizing intracellular αpPKCθ delivery strategy for human Treg differentiation in vitro could generate highly stable induced Tregs (iTregs) with a unique phenotype ((Ozay et al., 2016) and STM paper). Inhibition of PKCθ function and localization in iTregs resulted in superior suppressive capacity with unusual orchestration of transcriptional changes both in vitro and in vivo. This led us to investigate how these transcriptional changes were regulated by PKCθ during iTreg differentiation. An interesting, potential link between PKCθ and transcriptional diversity has been explored in T cells (McCuaig et al., 2015; Tabellini et al., 2003; Boronenkov et al., 1998). PKCθ directly phosphorylated the splicing factor, SC35, from its RNA recognition motif and serine-arginine-rich (SR) domain (McCuaig et al., 2015; Qian et al., 2011; Colwill et al., 1996; Prasad and Manley, 2003). PKCθ and SC35 colocalize with RNA polymerase II and active histone marks, therefore, enhance transcriptional elongation (McCuaig et al., 2015). Moreover, SC35 binds to exonic splicing enhancers and cooperates alternative splicing, RNA stability, mRNA export, and translation (Cazalla et al., 2002; Lin et al., 2008; Zhong et al., 2009; Kavanagh et al., 2005; Graveley and Maniatis, 1998; Chandler et al., 1997; Hammarskjold and Rekosh, 2017). Intriguingly, a FOXP3 stabilizing protein, TIP60, was shown to promote SC35 degradation via acetylation at lysine residue 52 in proximity with PKCθ phosphorylation sites implying a potential role of PKCθ regulating splicing factors in the context of Treg
suppressive function (Edmond et al., 2011; Dhuban et al., 2017). Given that PKC\(\alpha\) phosphorylates SC35 and controls epigenetic and transcriptional regulation in T cells, we hypothesize that PKC\(\alpha\) regulates alternative splicing and further RNA processing during iTreg differentiation.

In this report, we found that intracellular \(\alpha\)PKC\(\alpha\) delivery prior to iTreg differentiation switches alternative splicing and RNA processing. PKC\(\alpha\) critically modulates two key RNA regulatory factors, hnRNPL and PCMT1, thereby reprogramming mRNA splicing, stability, nuclear export, and translational control. More interestingly, we demonstrated that PCMT1 acts as a Treg instability factor by methylating FOXP3 promoter. Targeting PCMT1 via a cell-penetrating antibody revealed a novel, attractive way to modulate RNA processing in the context of stable Treg function.

### 5.2 Materials and Methods

#### 5.2.1 Animals

All animal studies were approved by, and conducted under the oversight of, the Institutional Animal Care and Use Committee of the University of Massachusetts, Amherst. Seven-week old female NOD.Cg-Prkd\(^{\text{acid}}\) Il2rg\(^{m1Wjl}\)/SzJ (NSG) mice, were purchased from the Jackson Laboratories (Bar Harbor, ME). Upon arrival, these mice were rested for one week prior to use. Mice were housed under pathogen-free conditions in micro-isolator cages and received acidified water (pH 3.0) supplemented with two
types of antibiotics (trimethoprim + sulfamethoxazole) throughout the duration of the experimental procedures.

5.2.2 Antibodies and Reagents


Flow cytometry antibodies used in this study are acquired from: (1) BioLegend: CD4 (BV711, Clone: RPA-T4), CD25 (PECy7, Clone: BC96), CD127 (AF700, Clone: A019D5), FOXP3 (AF488, Clone: 150D), (2) BD Bioscience: IFNγ (APC, Clone: B27), (3) LifeSpan Biosciences: PCMT1 (Unconjugated, anti-rabbit, polyclonal), (4) Life Technologies: F(ab’)2-Goat anti-rabbit IgG (H+L) secondary antibody (Qdot625, polyclonal). Live/dead staining was performed utilizing either Zombie aqua fixable viability kit purchased from BioLegend.
5.2.3 Human iTreg differentiation upon intracellular $P_{13}D_5$:$\alpha$PKC$\theta$ or $P_{13}D_5$:$\alpha$PCMT1 delivery

1 $\mu$M of $P_{13}D_5$ and 25 nM of $\alpha$PKC$\theta$ (Thr538, Life Technologies, Clone: F4H4L1) or 1 $\mu$M of $P_{13}D_5$ and 25 nM of $\alpha$PCMT1 (LifeSpan Biosciences, polyclonal) were complexed in PBS (phosphate buffered saline, pH 7.2) at a specific ratio (PTDM: Antibody = 40:1). The PTDM: antibody complex was incubated for 30 min at RT. Meanwhile, CD$4^+$ T cells were isolated from human PBMCs (purchased from StemCell Technologies, Inc.) via MojoSort$^\text{TM}$ Human T Cell Isolation Kit (BioLegend). Isolated human CD$4^+$ T cells were then treated with the PTDM: antibody complex for 4 hours at 37°C (some cells were treated with DMSO as vehicle control). Cells were harvested and washed with PBS. Later, cells were thoroughly washed twice with 20 U/mL heparin in PBS for 5 minutes on ice to remove surface-bound complexes outside cellular membrane. For iTreg differentiation, CellXVivo$^\text{TM}$ Human Treg Differentiation Kit (R&D Systems) was used and iTreg Differentiation Media was prepared using X-VIVO$^\text{TM}$ 15 Chemically Defined, Serum-free Hematopoietic Cell Medium according to manufacturer’s instructions. Treated cell pellets were then resuspended in iTreg differentiation media and seeded onto 5 $\mu$g/mL of anti-CD3$\varepsilon$- plus 2.5 $\mu$g/mL of anti-CD28-coated tissue culture wells and stimulated for 5 days at 37°C.

5.2.4 Immunoblotting

iTreg cells were harvested on day 5 of differentiation. Nuclear and cytosolic extracts were prepared by using NE-PER$^\text{TM}$ Nuclear and Cytosolic Extraction Kit
(Thermo Scientific). 1X SDS Laemmli Buffer was added into the samples for running on 8% SDS-PAGE for western blot. The blots were probed for RNA-binding proteins for further analysis. Anti-α-Tubulin was probed for cytosolic loading control and anti-histone H3 was probed for nuclear loading control.

5.2.5 In vivo RNA analysis of iTregs in humanized GvHD model

hPBMCs from a healthy donor were used to isolate total CD4+ T cells and subsequently treated with P13D5: αpPKC0 complex. They were differentiated for 5 days into iTregs as previously described. On day 4, total hPBMCs from the same donor were thawed and rested overnight in fresh RPMI complete media (10% fetal bovine serum, 100 U/mL penicillin-streptomycin, 1 mM sodium pyruvate, 2 mM L-Glutamine) at 37°C in 5% CO2 incubator. On day 5, NOD.Cg-Prkdc<sup>scid</sup> I2rg<sup>tm1Wjl/SzJ</sup> (NSG) mice were conditioned with 2 Gy of total body irradiation using a <sup>137</sup>Cs source then rested for 4-6 hours. 10x10<sup>6</sup> of total hPBMCs was mixed with 3.3x10<sup>6</sup> of iTreg cells and adoptively transferred into irradiated NSG mice via the tail vein. Body weight and disease symptoms were observed daily. On day 17, some animals were sacrificed for tissue analysis. Bone marrow cells were recovered from the tibias and femurs and splenocytes were isolated by manipulation through a 40 µm filter. Red blood cells were lysed in ACK lysis buffer, and the remaining white blood cells were enumerated using Trypan Blue exclusion. Afterwards, cells were incubated with human CD4 T lymphocyte enrichment cocktail (BD Biosciences) followed by an incubation with BD IMag<sup>TM</sup> Streptavidin Particles Plus (BD Biosciences) to deplete non-CD4 T cell fraction. Biotinylated anti-CD127 antibody and biotinylated anti-CD25 antibody followed by an incubation with BD IMag<sup>TM</sup>
Streptavidin Particles Plus were sequentially used to obtain iTreg cell fraction (negative fraction from anti-CD127 incubation and positive fraction from anti-CD25 fraction) and naïve T cell fraction (positive fraction from anti-CD127 incubation and negative fraction from anti-CD25 incubation). After cells were isolated, total RNA isolation procedure was followed.

5.2.6 Quantitative real time PCR (qPCR)

Total RNA was isolated Quick-RNA Isolation Kit (Zymo Research) according to the manufacturer’s protocol. 1 µg of total RNA was reverse transcribed to cDNA using dNTPs (New England Biolabs, Inc.), M-MuLV reverse transcription buffer (New England Biolabs), oligo-DT (Promega), RNase inhibitor (Promega), and M-MuLV reverse transcription (New England Biolabs, Inc.) on a Mastercycler gradient Thermal Cycler (Eppendorf). Primers for PCMT1 were designed as: forward primer (5’-GCTGAAGAAGCCCCTTATGA-3’) and reverse primer (5’-TCTTCCTCCGGGCTTTAATGA-3’). Q-PCR was performed in duplicate with 2x SYBR Green qPCR Master Mix (BioTool) using the Mx3000P system (Agilent Technologies). Q-PCR conditions were as follows: 95°C for 1 min, 95°C for 25 s, 62°C for 25 s (40 cycles), 95°C for 1 min, 62°C for 1 min, and 95°C for 30 s. Relative gene expression was determined using the ΔΔCt method. The results are presented as the fold expression in gene expression normalized to the housekeeping gene β-actin (ACTB) for cells and relative to Tconv + DMSO sample for in vitro experiments and Naïve + DMSO for in vivo experiments.
5.2.7 Reverse Transcriptase PCR (RT-PCR) for splicing and 3’UTR analyses

Total RNA was isolated Quick-RNA Isolation Kit (Zymo Research) according to the manufacturer’s protocol. 0.5 µg of total RNA was reverse transcribed to cDNA using random hexamers (Integrated DNA Technologies) with M-MuLV reverse transcriptase (New England Biolabs, Inc.) on a Mastercycler gradient Thermal Cycler (Eppendorf). Splicing primers (Table 5.1) and 3’UTR primers (Table 5.2) were specifically designed for the genes analyzed in this study. PCRs (35 cycles) were performed using Phusion High-Fidelity DNA Polymerase (New England Biolabs, Inc.) followed by resolution on 2% agarose gel. PCR conditions were: initial denaturation at 98°C (30 sec), annealing at 98°C (5 sec), 52°C (20 sec), 72°C (1 min), final extension at 72°C for 5 min. The amplicons were imaged via G-Box gel documentation system (Syngene).

5.2.8 Lambda phosphatase treatment

Cells were lysed RIPA buffer (150 mM NaCl, 1% IgeCal-CA 360, 0.1% SDS, 50 mM Tris, pH-8.0, 0.5% Sodium deoxycholate). Lysates were treated with 100 units of lambda protein phosphatase (New England Biolabs, Inc.) in the presence of 1 mM MnCl₂ for 1 h at 30°C. 1X SDS Laemmli Buffer was added into the samples and they were boiled for 5 min at 95°C. The samples were run on 8% SDS-PAGE for western blot analysis.
5.2.9 hnRNPL immunoprecipitation

Cells were harvested on day 5 of iTreg differentiation. They were lysed in immunoprecipitation lysis buffer (50 mM HEPES, pH 7.8, 250 mM NaCl, 1% NP-40, Protease + Phosphatase inhibitors). DynaBeads (Protein G) were coupled with 3 µg of anti-hnRNPL (4D11, Novus Biologicals) in the presence of 1% BSA in PBS and incubated for 2 hours at 4°C with rotation. After the incubation, the antibody-coupled DynaBeads were washed six times with 1 mL of immunoprecipitation wash buffer (Tris-HCl, pH 8.0, 200 mM NaCl, 0.1% NP-40). Later, cell lysates were incubated with antibody-coupled DynaBeads for 1 hour at 4°C using rotator. Subsequently, they were washed beads six times with 0.5 mL of immunoprecipitation wash buffer. 1X SDS Laemmlı Buffer was added into the samples for running on 8% SDS-PAGE for western blot. The blots were probed with anti-hnRNPL, anti-PKCθ, and anti-PCMT1 for further analysis.

5.2.10 RNA immunoprecipitation

After harvesting the cells on day 5 of differentiation, they were lysed in RNA immunoprecipitation lysis buffer (50mM HEPES, pH 7.8, 250mM NaCl, 1% NP-40, 1X Protease + Phosphatase inhibitors, 100 U/ml RNase inhibitor). DynaBeads (Protein G) were coupled with 3 µg of anti-hnRNPL (4D11, Novus Biologicals) or anti-IgG (control antibody) in the presence of 1% BSA in PBS and incubated for 2 hours at 4°C with rotation. After the incubation, the antibody-coupled DynaBeads were washed six times with 1 mL of immunoprecipitation wash buffer (Tris-HCl, pH 8.0, 200 mM NaCl, 0.1%
NP-40). Cell lysates were incubated with antibody-coupled DynaBeads for 1 hour at 4°C using rotator. Subsequently, they were washed beads six times with 0.5 mL of immunoprecipitation wash buffer + 100U/mL RNase inhibitor. RNA was purified via Quick-RNA Isolation Kit (Zymo Research) according to the manufacturer’s protocol to further use on RT-PCR experiments.

5.2.11 ChIP-qPCR

Cells were crosslinked with 1% formaldehyde, lysed in sodium dodecyl sulfate (SDS) lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris, pH 8.1), and sonicated with a Bioruptor Sonicator (Diagenode). Cell lysates were incubated with 2 mg anti–PCMT1 (LifeSpan Biosciences, polyclonal) or normal rabbit IgG (Santa Cruz Biotechnology, Inc.) coupled to DynaBeads at 4°C for 2 hours. Protein–DNA complexes were recovered with Dynabeads, washed, eluted with elution buffer (1% SDS, 0.1 M NaHCO₃), and reverse crosslinked overnight at 65°C. DNA was purified by proteinase K digestion and extracted with Phenol-Chloroform extraction. Aqueous phase was transferred into a fresh tube and the DNA was precipitated with 3 M sodium acetate containing 2 ml glycogen and 4 volumes of ethanol by keeping overnight at -20°C. Genes were amplified using qPCR primers designed as follows: human FOXP3: forward (5’-TGACCAAGGCTTCATCTGTG-3’), reverse (5’-GAGGAACCTCTGGAGATGTGC-3’), human IFNG: forward (5’-CTCTTGGCTTGACTGCCAGG-3’) and reverse (5’-CTCCACACTCTTTTGATGCT-3’). Q-PCR was performed in duplicate with 2x SYBR Green qPCR Master Mix (BioTool) using the Mx3000P system (Agilent Technologies). Q-PCR conditions were as follows: 95°C for 1 min, 95°C for 25 s, 62°C
for 25 s (40 cycles), 95°C for 1 min, 62°C for 1 min, and 95°C for 30 s. Relative gene expression was determined using the ΔΔCt method. The results are presented as the fold expression in gene expression normalized to the housekeeping gene β-actin (ACTB) for cells and relative to Tconv + DMSO sample.

5.2.12 Bioinformatics for RNA-binding protein motifs

Splice variants, intron-exon sequences, and 3’UTR sequences were analyzed and obtained from Ensembl. RNA-binding protein motifs for hnRNPL were analyzed via CISBP-RNA database (Ray et al., 2013). Later, 3’UTR sequences were analyzed for hnRNPL-binding sites via RBPmap (Paz et al., 2014).

5.2.13 Bisulfite Sequencing

Sodium bisulfite modification of genomic DNA was carried out using the EZ DNA Methylation Direct Kit (Zymo Research) according to the manufacturer’s protocol. Bisulfite-treated DNA was PCR amplified using the following methylation-specific primers via ZymoTaq™ DNA polymerase (Zymo Research): forward primer: 5’-TGTTTGGGGGTAGAGGATTT-3’ and reverse primer: 5’-TATCACCCCACCTAAACCAA-3’. PCR conditions were followed as: initial denaturation at 95°C for 10 min, 40 cycles of denaturation at 95°C for 30 secs + annealing at 55°C for 40 secs + extension at 72°C for 1 min, and final extension at 72°C for 7 min. Amplified DNA product was gel purified using GeneJET gel extraction kit (Thermo Scientific) and cloned into pMiniT™ 2.0 cloning vector using NEB PCR Cloning Kit (New England Biolabs, Inc.). Competent cells were transformed with the vector. 10
individual positive bacterial colonies were selected from which recombinant plasmid DNA was purified and sequenced with sanger sequencing (Genewiz).

5.3 Results

5.3.1 Modulation of splicing regulatory proteins and RNA processing upon αpPKCθ delivery in iTregs *in vitro*

First, we wanted to investigate the modulation of splicing regulatory proteins by PKCθ in iTregs. We analyzed iTregs that were differentiated *in vitro* with (iTreg+αpPKCθ) or without (iTreg+DMSO) P13D5:αpPKCθ delivery before differentiation. In order to show the iTreg differentiation program was greatly affected by PKCθ inhibition, we wanted to compare the results to non-differentiated (Tconv) cells that were treated with P13D5:αpPKCθ (Tconv+αpPKCθ) or DMSO (Tconv+DMSO). αpPKCθ-treated iTregs exhibited different pattern of splicing regulatory proteins as compared to untreated iTregs. We looked at cytosolic and nuclear distribution of Regnase-1, p-SC35, hnRNPU, HuR, hnRNPLL, and hnRNPL in αpPKCθ-treated iTregs. Most of the factors showed decrease in both nuclear and cytosolic levels (Figure 5.1a). However, only Regnase-1 and hnRNPL levels were significantly increased in the cytosol in αpPKCθ-treated iTregs. Among these, hnRNPL also showed a striking difference between Tconvs and iTregs since PKCθ inhibition led to opposite effects. We found that upon αpPKCθ delivery, hnRNPL was significantly increased in the cytosol while it was significantly decreased in the nucleus in αpPKCθ-treated iTregs suggesting that PKCθ inhibition blocks hnRNPL translocation to the nucleus in iTregs (Figure 5.2a). On the
other hand, Tconvs decreased their cytosolic and increased their nuclear hnRNPL levels (Figure 5.1a). These results suggested that nuclear hnRNPL translocation may be critical for iTreg differentiation.

Since the splicing regulators were affected by PKCθ in iTregs, we raised the question whether there were differences in alternative splicing and 3’UTR processing. The proper generation of 3’UTRs is very critical for mRNA stability since 3’UTRs include recognition motifs for stabilizing or destabilizing RBPs which control mRNA degradation and silencing. In addition, shorter 3’UTR lengths are associated with stable mRNA production and increased protein translation in a signal-dependent manner (Gruber et al., 2014). Immune cells recruit specific RBPs to the site of translation and form riboclusters. Arrangement of RBPs on the 3’UTR elements (AU-rich and CA-rich elements) in the riboclusters determine whether mRNA will be translated or directed to nonsense-mediated decay. CD45 splicing is heavily studied in T cells and is regulated by hnRNPL. Therefore, we looked at CD45 splicing in iTregs as a proof of concept. We saw inhibiting PKCθ increased RB and RO forms in both Tconvs and iTregs with respect to untreated cells (Figure 5.1b, 5.2b). In our recent study, αpPKCθ-treated iTregs showed unique characteristics of higher FOXP3, PD-1, and IFNγ expressions. Given the fact that FOXP3 and PD1 have been reported to undergo alternative splicing (Nielsen et al., 2005; Ryder et al., 2010; Smith et al., 2006), it was interesting to ask whether this treatment affected RNA processing of key iTreg genes. We analyzed splicing patterns and 3’UTR lengths of FOXP3, PDCD1, IFNG, and IFNGR1. Conventional mRNAs for those genes consist of: exon 2-3-4-5 (FOXP3), exon 1-2-3-4-5 (PDCD1), 4 exons (IFNG), and 7 exons (IFNGR1). We observed similar splicing patterns for all four genes in iTregs with
or without αpPKCθ treatment (Figure 5.2c). Also, Tconvs had similar spliced forms except FOXP3 mRNA did not have full sequence upon αpPKCθ treatment (Figure 5.1c). However, αpPKCθ delivery affected 3’UTR processing in a gene-specific manner. PDCD1 had mRNA variants with shorter 3’UTR lengths upon αpPKCθ treatment compared to untreated cells while FOXP3, IFNG, and IFNGR1 3’UTR lengths did not change after the treatment (Figure 5.1d, 5.2d). These results indicate that PKCθ can regulate splicing regulators and RNA processing in a cell- and gene-specific manner.

5.3.2 Tissue-, cell-, and gene-specific modulation of RNA processing in ex vivo-treated iTregs by αpPKCθ delivery

Previous results showed PKCθ inhibition via cell-penetrating antibody did not affect alternative splicing in iTregs in vitro. Since it is known that iTregs can behave differently in vivo due to cytokines and other cell types in tissue-specific manner and alternative splicing being regulated by external signals in T cells upon an immune response, we asked the question whether these αpPKCθ-treated iTregs would alter their alternative splicing programs in vivo. In our previous report, αpPKCθ-treated iTregs were found highly efficacious in preventing GvHD response in humanized mice model. These cells were still present on the peak day of the disease (day 17) and characterized with unique, stable gene expression pattern. In order to test whether RNA processing leads to formation of more stable mRNA variants in these iTregs, we adoptively transferred αpPKCθ-treated iTregs into our humanized GvHD mice on the day of GvHD induction and analyzed RNA processing in iTregs on day 17. We magnetically sorted the
cells from bone marrow and spleen based on their CD4, CD25, and CD127 expressions. We found that CD45 splicing was only affected in bone marrow iTregs upon αpPKCθ treatment (Figure 5.3a) and naïve T cells had similar pattern of CD45 splice variants in both tissues and treatments (Figure 5.4a). Surprisingly, we found that alternative splicing of FOXP3 and PDCD1 in bone marrow iTregs was strikingly different upon αpPKCθ treatment although we could detect similar variants in both untreated and treated iTregs in spleen (Figure 5.3b, c). We were not able to amplify FOXP3 3’ UTR in untreated iTregs in the bone marrow suggesting that those mRNAs were unstable (Figure 5.3b). Consistent with in vitro data, PDCD1 mRNA variants had shorter 3’UTR lengths in αpPKCθ-treated iTregs in the bone marrow suggesting more stable PDCD1 transcripts were generated by those iTregs (Figure 5.3c). IFNG and IFNGRI mRNA splice variants were amplified in all iTregs in spleen but in the bone marrow, only αpPKCθ-treated iTregs had strong amplification (Figure 5.3d, e). We saw that IFNG and IFNGRI mRNAs were more stable in αpPKCθ-treated iTregs since we saw the amplification of 3’UTR only in these cells but not untreated iTregs in the bone marrow (Figure 5.3c, d). In naïve T cells, we could amplify stable FOXP3 mRNA variants in bone marrow and spleen (Figure 5.4b), we could only detect stable PDCD1 in spleen (Figure 5.4c). Although we saw IFNG mRNA splice variants in naïve T cells in bone marrow (Figure 5.4d), they were lacking IFNGRI mRNA due to aberrant 3’UTR structure (Figure 5.4e). These data suggest that both alternative splicing and 3’UTR shortening of key iTreg genes were modulated by PKCθ in vivo upon an immune response in tissue-, cell-, and gene-specific manner.
5.3.3 Post-translational and post-transcriptional regulation of PCMT1 in iTregs

Given the fact that PKCθ regulates RNA maturation and stability for key iTreg genes based on the *in vitro* and *in vivo* data, we hypothesize that PKCθ may directly regulate alternative splicing of an RNA stability factor. We discovered that PCMT1 (protein L-isoaspartate (D-aspartate) methyltransferase) was tightly regulated by PKCθ in iTregs. PCMT1 has been discovered to repair damaged proteins by methylating them from the carboxyl group of L-isoaspartate or D-aspartyl residues (Misra et al., 2002). PCMT1 has been shown to regulate critical cellular processes such as RNA maturation, stability, export, histone homeostasis, and post-translational control (Enünlü et al., 2003; Yang et al., 2013; Dufu et al., 2010; MacKay et al., 2012). PCMT1 shares a lot of similarities with two other protein and RNA methyltransferases, PRMT (S-adenosylmethionine-dependent protein arginine methyltransferase) and PIMT (PRIP-interacting protein with methyltransferase domain), respectively. These enzymes were shown to methylate small nuclear RNAs (snRNAs) which, in turn, can interact with spliceosome core proteins. This snRNA-protein complex, U-snRNP, is formed in the cytosol and the methylation by those methyltransferases provides a nuclear import signal for spliceosome complex (Misra et al., 2002; Enünlü et al., 2003). Proteomic studies showed that the absence of PCMT1 diminished the amount of two crucial RNA regulators, poly(rC)-binding protein 2 (PCBP2) involved in mRNA stability and DX39B involved in mRNA export to the cytoplasm, in the whole proteome (Yang et al., 2013). Moreover, PCMT1 is a part of TREX mRNA export complex and interacts with several key RNA binding proteins such as hnRNPU, hnRNPD, hnRNPA2, and hnRNPM (Dufu et al., 2010). Among those, hnRNPU is methylated by PRMT1 implicating the change of
receiving extracellular stimuli that influences RNA maturation and transport (Herrmann et al., 2004). One can hypothesize that PCMT1 regulates splicing regulatory proteins and thus, affect alternative splicing, RNA maturation, stability, and export in iTregs. Hence, we looked at PCMT1 regulation in the context of iTreg differentiation. PCMT1 was heavily located in the cytosol but also present in the nucleus. αPKC0-treated iTregs significantly reduced cytosolic and nuclear PCMT1 protein levels while there was a trend in downregulating its gene expression (Figure 5.5a, b). We also saw that PCMT1 downregulation by αPKC0 was stronger in Tconvs as supported by qPCR and western blot results (Figure 5.6a, b). Nuclear PCMT1 in αPKC0-treated iTregs showed two separate bands raising the possibility of phosphorylation of nuclear PCMT1. We performed lambda phosphatase experiment to see whether the upper band was actually the phosphorylated form. We also included pSTAT1 (Y701) and pAKT (S473) as internal controls and they were also associated with iTreg function. We observed that pSTAT1 and pAKT signals were lost due to the phosphatase activity. Interestingly, upper band of nuclear PCMT1 was disappeared upon phosphatase treatment suggesting that nuclear PCMT1 was phosphorylated after αPKC0 treatment in iTregs (Figure 5.5c). Another interesting finding was upon αPKC0 delivery in iTregs, pSTAT1 (Y701) levels were significantly increased and pAKT (S473) levels were significantly decreased (Figure 5.5c) and this result supported the notion why these iTregs would be more suppressive as seen in previous reports.

Next, we investigated whether PKC0 regulated RNA processing of PCMT1 in iTregs. Conventional PCMT1 mRNA has 7 exons and approximately 700 bp long of 3’UTR. αPKC0-treated iTregs exerted exon skipping and created shorter mRNA
variants with long 3’UTRs (Figure 5.5d). Tconvs had similar 3’UTRs but were missing the 7-exon PCMT1 mRNA (Figure 5.6c). This implies that PCMT1 mRNA variants generated in αpPKC0-treated iTregs were unstable and not efficiently translated in vitro. We also tested the differences in iTregs in bone marrow and spleen in humanized GvHD model. PCMT1 was significantly downregulated in αpPKC0-treated iTregs in spleen whereas it was not different in αpPKC0-treated iTregs in bone marrow (Figure 5.5e). Strikingly, we detected different splice variants and shorter 3’UTR lengths in αpPKC0-treated iTregs in the bone marrow while iTregs in the spleen possessed similar patterns (Figure 5.5f). Similar to iTregs, naïve T cells had significantly reduced PCMT1 transcripts in the spleen and comparable levels in the bone marrow of mice transferred with αpPKC0-treated iTregs as compared to mice transferred with untreated iTregs (Figure 5.6d). Conventional PCMT1 mRNA variant was only detected in naïve T cells of mice transferred with untreated iTregs both in bone marrow and spleen. Interestingly, PCMT1 3’UTR was longer than expected 3’UTR length in naïve T cells of untreated iTreg-transferred mice in bone marrow. In spleen, normal 3’UTR length was predominantly observed in naïve T cells of αpPKC0-treated iTreg-transferred mice and was shorter in naïve T cells of untreated iTreg-transferred mice (Figure 5.6e). All these data demonstrated that PKC0 regulates a novel protein, PCMT1, in iTregs both post-translationally and post-transcriptionally.

### 5.3.4 Effects of αPCMT1 delivery in iTregs

Because we observed a tight regulation by PCMT1 and reduction in the protein levels in αpPKC0-treated iTregs carrying more suppressive function, we raised the
possibility of inhibiting PCMT1 actions by delivering cell-penetrating antibody against PCMT1 would somewhat mimic the blocking action of PKCθ in iTregs. We utilized our intracellular delivery strategy and delivered αPCMT1 via complexing with P13D5 before iTreg differentiation. We also compared the effects of this delivery to αpPKCθ delivery. Interestingly, we saw that there was a significant increase in the percentage of CD4⁺CD25⁺FOXP3⁺ iTregs upon αPCMT1 delivery as compared to untreated iTregs and the percentage of CD4⁺CD25⁺FOXP3⁺ iTregs after αpPKCθ delivery was comparable to the effect of αPCMT1 delivery (Figure 5.7a). In addition, FOXP3 expression was higher in both αpPKCθ- and αPCMT1-treated iTregs as larger FOXP3hi iTreg population was emerged upon those two antibody treatments (Figure 5.7b). In our previous report, we found that αpPKCθ-treated iTregs expressed significantly higher IFNγ and this could be associated with their superior suppressive function. Therefore, we wanted to look at the IFNγ expression upon αPCMT1 delivery. As expected, αpPKCθ-treated iTregs significantly elevated their IFNγ expression compared to untreated iTregs. Although the difference between untreated and αPCMT1-treated iTregs did not reach the significance, there was clearly an increase in the IFNγ production by these iTregs treated with αPCMT1 suggesting that PKCθ and PCMT1 are on the similar pathway of regulating iTreg suppressive function (Figure 5.7c).

We showed that PKCθ regulates hnRNPL localization and RNA processing of key iTreg genes and thus, hypothesized that PCMT1 may also be involved in this pathway. In order to show whether these proteins physically interact, we immunoprecipitated cytosolic and nuclear hnRNPL and looked at PKCθ and PCMT1
interactions upon αPKCθ or αPCMT1 treatment. We observed that hnRNPL and PKCθ interact both in the cytosol and the nucleus. This interaction significantly diminished in the nucleus in both αPKCθ- and αPCMT1-treated iTregs. Surprisingly, hnRNPL- PKCθ association was significantly higher in the cytosol of αPCMT1-treated iTregs while αPKCθ-treated iTregs exhibited significant loss of hnRNPL-PKCθ interaction (Figure 5.7d). Conversely, hnRNPL-PCMT1 interaction was weaker in the cytosol than their interaction in the nucleus. Upon either αPKCθ or αPCMT1 delivery, these iTregs increased hnRNPL-PCMT1 association in the cytosol and profoundly lost this interaction in the nucleus (Figure 5.7d). These results demonstrated that intracellular antibody delivery against PKCθ and PCMT1 can prevent dual interactions with hnRNPL in the cytosol and the nucleus.

Reduction in nuclear hnRNPL-PKCθ and hnRNPL-PCMT1 associations in antibody-treated iTregs would possibly affect RNA binding ability of hnRNPL. We used several bioinformatics tools to identify hnRNPL RNA binding motifs. CISBP-RNA database showed two CA-rich RNA binding motifs for hnRNPL (Figure 5.7e). CA-rich elements on 3’ UTR sequences serve as a central hub for further RNA processing events such as RNA stability and nuclear export (Iadevaia and Gerber, 2015). In general, incorrectly spliced mRNAs (with retained introns or aberrant exon skipping) can go to either nuclear RNA decay or nonsense-mediated RNA decay in the cytoplasm. Only correctly spliced, stable mRNAs are properly exported and translated (Bergeron et al., 2015). In order to investigate whether hnRNPL-PCMT1 or hnRNPL-PKCθ association is important for correctly spliced, stable mRNA export in iTregs, we isolated cytoplasmic
and nuclear RNA from iTregs followed by RNA immunoprecipitation and designed 3’UTR specific primers that amplified 3’UTR sequences containing hnRNPL binding sites. Interestingly, we found two hnRNPL binding sites on PCMT1 3’UTR.

Additionally, there were hnRNPL binding sites on the 3’UTRs of all the key iTreg genes found critical in our study (FOXP3, IFNG, IFNGR1, and PDCD1) (Figure 5.8). Strikingly, we found that hnRNPL strongly binds to PCMT1 mRNA and its stable form in the cytosol. However, both αpPKCθ- and αPCMT1-treated iTregs failed to export stable PCMT1 mRNA due to weak hnRNPL-PCMT1 mRNA association (Figure 5.7f). Moreover, only αPCMT1-treated iTregs but not αpPKCθ-treated iTregs failed to export stable FOXP3 mRNA suggesting that PCMT1 can control gene-specific mRNA export by hnRNPL (Figure 5.9a). We detected the interaction of hnRNPL-PDCD1 mRNA mainly in the nucleus, however, αPCMT1-treated iTregs enhanced their stable PDCD1 mRNA export. We did not detect any association of stable PDCD1 mRNA and hnRNPL in αpPKCθ-treated iTregs (Figure 5.9b). In contrast to PKCθ- and PCMT1-mediated tight regulation of stable PCMT1, FOXP3, and PDCD1 mRNA export by hnRNPL, we found that nuclear export of stable IFNG and IFNGR1 mRNA was not affected by αpPKCθ and αPCMT1 delivery (Figure 5.9c, d). These results revealed that stable mRNA export of PCMT1 is tightly regulated by hnRNPL association with PCMT1 and PKCθ. More importantly, both PKCθ and PCMT1 can regulate selective mRNA export of key iTreg genes in gene-specific manner.
5.3.5 PCMT1 as an iTreg instability marker

PCMT1 seems to be tightly regulated in iTregs in PKCθ-dependent manner. Targeting PCMT1 via cell-penetrating antibody generated higher percentage of iTregs expressing higher FOXP3 and IFNγ. Therefore, we asked whether PCMT1 directly regulates FOXP3 and IFNG gene expression. We performed chromatin immunoprecipitation followed by qPCR to determine direct binding of PCMT1 to FOXP3 and IFNG genes. PCMT1 was found to bind to both genes. When we treated iTregs with either αpPKCθ or αPCMT1, we observed more dramatic decrease in αPCMT1-treated iTregs than in αpPKCθ-treated iTregs in PCMT1 binding to FOXP3 gene (Figure 5.10a). However, only αpPKCθ-treated iTregs exhibited significant decrease in PCMT1 binding to IFNG gene suggesting that PKCθ is critical for PCMT1 binding to both FOXP3 and IFNG genes whereas PCMT1 is only critical for its own binding to FOXP3 gene (Figure 5.10a). FOXP3 gene expression is tightly regulated by several transcription factors binding to TSDR sequence upon demethylation. Demethylated TSDR is the most reliable marker for stable FOXP3 expression. iTregs normally are not able to demethylate their TSDR, thus, have unstable FOXP3 expression (Toker and Huehn, 2011; Li et al., 2014b). Since PCMT1 is a methyltransferase and directly binds to FOXP3 gene, it is likely to explore the role of PCMT1 on TSDR methylation. Firstly, we identified 15 CpG islands on human FOXP3 TSDR sequence and found two CpGs overlapping with STAT5 binding site (Figure 5.10b). It is known that STAT5 binding is important to maintain FOXP3 expression (Huehn et al., 2009; Ogawa et al., 2014). We performed bisulfite sequencing to determine the methylation pattern of
**FOXP3 TSDR**. As expected, untreated iTregs had very low number of demethylated CpGs. Interestingly, αpPKCθ-treated iTregs increased the number of demethylated CpG sites and this effect was significant in αPCMT1-treated iTregs (Figure 5.10c). Furthermore, we calculated the percentage of demethylated CpG on STAT5 binding region in the treated iTregs. Only αPCMT1-treated iTregs had less methylated CpG on their STAT5 binding site suggesting that inhibiting PCMT1 may increase the stability of FOXP3 expression (Figure 5.10d). In addition, we found the third and the fourth CpGs were strongly demethylated only in αPCMT1-treated iTregs further supporting the notion that PCMT1 can act as an iTreg stability marker (Figure 5.10c). Overall, we discovered that PKCθ regulates key RNA processing regulators, PCMT1 and hnRNPL, through multiple cellular mechanisms and intracellular delivery of αpPKCθ and αPCMT1 can modulate these mechanisms in favor of enhancing iTreg suppressive function. We identified, for the first time, PCMT1 as a key iTreg instability factor as it controls FOXP3 TSDR methylation.

### 5.4 Discussion

Transcriptional regulation of the immune response has been the main focus of most of the research. However, alternative splicing and RNA processing is an emerging area in the differential regulation of T cell differentiation, function, and behavior as many T cell genes have reported to produce distinct protein isoforms upon antigenic stimulation (Lynch, 2004; Martinez and Lynch, 2013; Matter et al., 2002; Lynch and Weiss, 2000; Arch et al., 1992). These processes operate together with RNA binding proteins,
transcription factors, and epigenetic modifiers (Ip et al., 2007; Martinez et al., 2012). Although numerous immune-related genes meticulously undergo alternative splicing and RNA processing, the role of differential isoform expression and RNA processing in regulatory T cell differentiation remains largely unexplored.

Our studies provide a novel, comprehensive analysis of altered RNA processing in the context of Treg differentiation and function. We applied several methods to investigate the role of PKC0 in regulating splicing regulators, RNA splicing, stability, and nuclear export. By specific targeting of PKC0 via a cell-penetrating antibody, we showed the modulation of post-transcriptional RNA processing in iTregs. PKC0 was discovered to influence RNA processing several Treg genes such as CD45, FOXP3, PDCD1, IFNG, and IFNGR1 in a cell-, and tissue-specific fashion. Furthermore, our splicing regulator analysis revealed two RNA regulatory proteins, hnRNPL and PCMT1, were controlled by PKC0 in multiple levels. PKC0 was found to regulate subcellular localization of hnRNPL and its binding to mRNA. Also, it was shown to regulate PCMT1 alternative splicing, stabilized PCMT1 mRNA through hnRNPL binding and enabled its nuclear export followed by its translation into stable protein. However, αPKC0 delivery switched its splicing pattern and destabilized and prevented the nuclear export of PCMT1 mRNA due to loss of hnRNPL binding. Translating our intracellular delivery strategy to target PCMT1 via a cell-penetrating antibody unveiled a novel way of promoting iTreg differentiation and its function. PCMT1 was found to directly bind FOXP3 gene and, more interestingly, inhibiting PCMT1 increased FOXP3 TSDR demethylation thereby enhancing FOXP3 stability and Treg maintenance. In conjunction with our recent
discovery of generating a unique, suppressive population of FOXP3$^{hi}$PD1$^{hi}$IFN$^{\gamma hi}$ iTregs upon manipulating PKC$\theta$ via intracellular $\alpha$PKC$\theta$ delivery, this study supports the notion that PKC$\theta$ directly regulates iTreg differentiation and stability through modulating pivotal RNA processing regulators.

Various PKC family members were discovered to influence mRNA splicing in many cell types (Lynch and Weiss, 2000; Revil et al., 2007; Zara et al., 2011). PKC$\alpha$, PKC$\delta$, and PKC$\theta$ have been shown to activate SC35 that play a role in co-transcriptional regulation of alternative splicing (McCuaig et al., 2015; Cataldi et al., 2009; Zara et al., 2011). As previously suggested, we discovered that PKC$\theta$ regulated cytoplasmic versus nuclear distribution of splicing regulators. Among these regulators, we focused on hnRNPL as it was uniquely altered in iTregs upon PKC$\theta$ inhibition. hnRNPL was sequestered in the cytosol upon $\alpha$PKC$\theta$ delivery unlike other splicing regulators. Consequently, we observed greater exon skipping of CD45, generating higher amount of CD45RO due to reduced nuclear hnRNPL in $\alpha$PKC$\theta$-treated iTregs compared to untreated iTregs. On the other hand, FOXP3, PDCD1, IFNG, and IFNGR1 splicing patterns in iTregs did not differ upon treatment due to lower levels of nuclear hnRNPL in vitro. This may indicate that alternative splicing of these genes is regulated by other splicing regulators. However, these $\alpha$PKC$\theta$-treated iTregs formed PDCD1 mRNAs with shorter 3’UTR lengths but did not alter 3’UTR processing of FOXP3, IFNG, and IFNGR1. These results support that $\alpha$PKC$\theta$-treated iTregs implement gene-specific 3’UTR processing and gain more stable PD1 expression in vitro. Similar results applied for the in vivo RNA dynamics when studied in the context of an immune response and
Treg differentiation. αpPKC0-treated iTregs carrying higher PD1 expression and superior suppressive function were long-lasting and increased in numbers in bone marrow and spleen upon adoptive Treg transfer in humanized GvHD mouse model. Also, other studies have claimed that Tregs can lose FOXP3 expression and take on a proinflammatory phenotype in several disease environments (Overacre and Vignali, 2016). Only αpPKC0-treated iTregs maintained their FOXP3 expression in the bone marrow since we did not amplify any stable mRNA in untreated iTregs. Interestingly, we actually saw alternative splicing of FOXP3, PDCD1, IFNG, and IFNGRI was altered only in αpPKC0-treated iTregs in tissue-specific manner. Moreover, only αpPKC0-treated iTregs but not untreated iTregs had stable mRNA production in the bone marrow while splenic iTregs did not vary in 3’UTR processing of those iTreg genes. One potential explanation of why we did not observe any change in alternative splicing in vitro but in vivo was because differential cytokine signaling provided by different neighboring cells was needed to occur to promote PKC0-mediated alternative splicing in iTregs.

Our data unraveled an unknown role of a protein methyltransferase, PCMT1, that was regulated by PKC0 signaling. We showed that PCMT1 expression was downregulated upon PKC0 inhibition. Additionally, αpPKC0 delivery resulted in phosphorylated PCMT1 in the nucleus suggesting that post-translational control of PCMT1 was affected by PKC0 signaling. Current literature suggested that PCMT1 regulates PI3K/AKT/mTOR signaling pathway. There is an increased activation of AKT/GSK3β signaling in the hippocampus of Pcmt1-knockout mice (Dung et al., 2016;
Farrar and Clarke, 2002). Also, it was found that the level of pGSK3β at serine 9 (inhibited GSK3β) was low in Pcm1+/+ mice suggesting the abundance of activated GSK3β (Farrar et al., 2005). Considering PKCθ and GSK3β have opposite effects, further studies will investigate to identify whether GSK3β phosphorylates PCMT1 and regulates its function upon PKCθ inhibition in iTregs. AKT/GSK3β pathway is very crucial since mTOR inhibition promotes regulatory T cell induction (Singh et al., 2015; Sambri et al., 2011). These suggest that inhibiting PCMT1 would promote regulatory T cell induction.

Unexpectedly, we found that pSTAT1 (Y701) levels increased in αPKCθ-treated iTregs whereas pAKT (S473) levels were decreased. One study also demonstrated enhanced IFNγ production by CD4+CD25+FOXP3+ Tregs in mice upregulated STAT1 phosphorylation and reduced AKT activation. Moreover, they found these were all in the same pathway induced by IFNγ to control skin graft rejection in vivo. In addition, IFNγ produced by Tregs also induced indoleamine 2,3-dioxygenase (IDO) production by antigen-presenting cells (Wei et al., 2010). Having αPKCθ-treated iTregs with higher IFNγ expression and more suppressive activity, this pathway may be one of the critical pathways controlling Treg suppressive function through autonomous IFNγ signaling. These cells also constitute high PD1 expression that could be somewhat related to this pathway. A downstream phosphatase of PD1 signaling, SHP-2, was shown to interact with cytosolic STAT1 and prevented its recruitment to IFNγR resulting in reduced Th1 function (Wu et al., 2012). Further studies investigating the link between PD1 and IFNγ
signaling pathways through PKC0 modulation in iTregs are needed to dissect the cellular mechanisms regulating suppressive ability and Th1-Treg plasticity.

Our data showed that PKC0 regulates PCMT1 also post-transcriptionally. αPKC0-treated iTregs had distinct PCMT1 splicing pattern and less unstable mRNA in vitro. In line with in vitro data, tissue- and cell-specific switch of PCMT1 RNA processing was observed in vivo. Since there was a robust post-translational and post-transcriptional regulation of PCMT1 by PKC0 signaling, we performed an intracellular antibody delivery experiment to target PCMT1. Surprisingly, αPCMT1 delivery also generated higher percentage of iTregs with high FOXP3 and IFNγ expressions in vitro. Moreover, PCMT1 has been found to interact with hnRNPL as PKC0. Both αPKC0 and αPCMT1 delivery inhibited their interaction with hnRNPL in the nucleus. However, there was an inverse correlation of these interactions in the cytosol. Cytosolic hnRNPL-PKC0 interaction was decreased while increasing hnRNPL-PCMT1 interaction upon αPKC0 delivery. Conversely, αPCMT1 delivery increased hnRNPL-PKC0 interaction while decreasing hnRNPL-PCMT1 interaction in the cytosol. Previous reports suggested that PCMT1 is a part of RNA nuclear export complex and associates with multiple RBPs (Dufu et al., 2010). Considering PCMT1-hnRNPL interaction in the context of RNA export, we found that PKC0 regulates hnRNPL-RNA interactions in iTregs. Interestingly, inhibiting PKC0 or PCMT1 abrogated stable PCMT1 mRNA export to the cytoplasm due to loss of hnRNPL binding suggesting that PCMT1 controls its own RNA export as well as PKC0. It was also observed that PKC0 and PCMT1 selectively regulated RNA export and hnRNPL interactions with FOXP3, PDCD1, IFNG, and IFNGR1 mRNA. It still
remains unexplored whether αpPKCθ and αPCMT1 delivery differentially influences translational control of these mRNAs in the cytosol.

Parallel findings with αpPKCθ and αPCMT1 delivery revealed the possibility of generation of more stable iTregs as a result of epigenetic regulation of FOXP3 gene and unique IFNγ expression. To date, the most reliable marker to determine Treg stability is FOXP3 TSDR demethylation (Schmidt et al., 2016). Also, IFNγ-expressing Tregs were found to exert more stable phenotype with demethylated TSDR (Daniel et al., 2014). Although there are a few studies on identifying the responsible methyltransferase for TSDR methylation, yet this requires further investigation. Here, we observed a direct binding of PCMT1 to FOXP3 and IFNG genes in iTregs and this binding could be prevented by delivering either intracellular αpPKCθ or αPCMT1 antibody. αpPKCθ delivery affected PCMT1 binding to IFNγ gene the most whereas αPCMT1 delivery affected PCMT1 binding to FOXP3 gene the most. As PCMT1 contains a domain with global methyltransferase activity, we further analyzed the methylation of FOXP3 TSDR in αPCMT1-treated iTregs. Strikingly, we observed significantly lower TSDR methylation in αPCMT1-treated iTregs and demethylated CpGs overlapped with STAT5 binding site affirming the maintenance of FOXP3 gene expression. Besides, TSDR analysis in αPCMT1-treated iTregs revealed another CpG that had stronger demethylation than the one on STAT5 binding site. When we ran PROMO algorithm to identify putative transcription factor binding sites around this CpG, we intriguingly found that this demethylated CpG was on GATA-1 transcription factor binding site and close to XBP1, TFIID, and RXR-alpha binding sites (Messeguer et al., 2002; Farré et al., 2003). GATA-1 is considered as a Treg-locking transcription factor and found to enhance
transcriptional activity of *FOXP3* expression (Fu et al., 2012; Akimova et al., 2017). Hence, our results reinforce the idea of targeting PCMT1 as a beneficial strategy for strengthening Treg stability and suppressive function.

In addition to epigenetic regulation of *FOXP3* by PCMT1, further studies will elaborate the contribution of PCMT1 to *FOXP3* stability at the post-translational level. PCMT1 regulates a histone deacetylase called SIRT1, which was shown to be important in Tregs. SIRT1 binds to and deacetylates *FOXP3* in the cytoplasm and leads to protein instability. This interaction can sterically be inhibited by a kinase called MST1 upon its phosphorylation activity. PCMT1 inhibits MST1 activity by interacting from its kinase domain and this interaction may cause MST1 methylation that could inhibit MST1 activity and enhance *FOXP3* deacetylation by SIRT1 (Li et al., 2015; Yan et al., 2013; Liang et al., 2017; Shi et al., 2017). Therefore, inhibiting PCMT1 at the protein level would also stabilize *FOXP3* protein thereby promote Treg function. Our findings justify the further evaluation of RNA regulatory proteins and PKCθ signaling in the context of Treg suppressive function. Modulation of PKCθ and PCMT1 culminates in regulated alternative splicing and RNA processing decisions in iTregs so as to generate highly stable and suppressive phenotype. Thus, it is important to further clarify the conditions of human iTreg differentiation and alterations in RNA regulatory machinery in light of Treg-based immunotherapy approaches.
Figure 5.1 Effect of αpPKCβ delivery on splicing regulators and non-differentiated T cells (Tconvs) in vitro. (a) Cytoplasmic vs. nuclear distribution of multiple splicing regulators in αpPKC0-treated Tconvs and iTregs was analyzed via western blot. Normalized densities for cytoplasmic and nuclear proteins were quantified according to tubulin and histone H3, respectively. (b) Alternative splicing analysis of CD45 in Tconvs via RT-PCR. (c) Splicing analysis of FOXP3, PDCD1, IFNG, and IFNGR1 upon PKC0 inhibition in Tconvs. (d) 3'UTR analysis of FOXP3, PDCD1, IFNG, and IFNGR1 3'UTR via RT-PCR in Tconvs. ‘←’ indicates the expected amplicon size. Data represent mean ± SEM of two or three independent experiments. Unpaired, two-tailed t test was used for analysis; ***p < 0.0001.
Figure 5.2 αPKCδ delivery modulates splicing regulatory proteins and affects RNA processing in iTregs in gene-specific manner in vitro. (a) Cytoplasmic vs. nuclear distribution of p-SC35 and hnRNPL in αPKCδ-treated iTregs was analyzed via western blot. Normalized densities for cytoplasmic and nuclear proteins were quantified according to tubulin and histone H3, respectively. (b) Alternative splicing analysis of CD45 in iTregs via RT-PCR. (c) Splicing primers were designed for several functionally important iTreg genes such as FOXP3, PDCD1, IFNG, and IFNGR1 to detect differences in multiple variants of mRNA upon PKCδ inhibition. (d) Primers were designed to assess 3'UTR processing from the last exon to close to the end of 3'UTR. FOXP3, PDCD1, IFNG, and IFNGR1 3'UTR lengths were analyzed via RT-PCR. ‘<’ indicates the expected amplicon size. Data represent mean ± SEM of two or three independent experiments. Unpaired, two-tailed t test was used for analysis; ***p < 0.0001.
Figure 5.3 RNA processing was altered in ex vivo-treated iTregs in tissue- and gene-specific manner in humanized mouse model of GVHD. hPBMCs were transferred on Day 0 together with αpPKC0-treated (or non-treated) iTregs (3:1 ratio). On Day 17, tissues were harvested and iTregs were separated via magnetic beads against CD4+, CD25+, and CD127. Total RNA was extracted from iTregs present in bone marrow and spleen on day 17. RT-PCR was performed to study alternative splicing and 3'UTR processing. (a) CD45 alternative splicing in bone marrow vs. spleen iTregs treated with αpPKC0 ex vivo. (b) FOXP3 variants and 3'UTR lengths were analyzed using specific primers in ex vivo-treated iTregs homing to bone marrow or spleen. This analysis was also done for (c) PDCD1, (d) IFNG, and (e) IFNGR1. ‘⇠’ indicates the expected amplicon size. Data represent mean ± SEM two independent experiments from 5 mice per condition.
Figure 5.4 Effect of αpPKC\(\theta\) delivery on RNA processing of naïve T cells in vivo. hPBMCs were transferred on Day 0 together with αpPKC\(\theta\)-treated (or non-treated) iTregs (3:1 ratio). On Day 17, tissues were harvested, and naïve T cells were separated via magnetic beads against CD4\(^+\), CD25\(^-\), and CD127\(^+\). Total RNA was extracted from these naïve T cells present in bone marrow and spleen on day 17. RT-PCR was performed to study alternative splicing and 3'UTR processing.

(a) CD45 alternative splicing in bone marrow vs. spleen naïve T cells. (b) FOXP3 variants and 3'UTR lengths were analyzed using specific primers in naïve T cells homing to bone marrow or spleen. This analysis was also done for (c) PDCD1, (d) IFNG, and (e) IFNGR1. ‘\(\beta\)’ indicates the expected amplicon size. Data represent mean ± SEM two independent experiments from 5 mice per condition.
Figure 5.5 PKCθ controls PCMT1 both post-transcriptionally and post-translationally in iTregs. (a) PCMT1 gene expression was quantified via qPCR. (b) PCMT1 protein in cytoplasm and nucleus were detected via western blot and quantified based on loading control densities via ImageJ software. (c) Phosphorylation of nuclear PCMT1 was confirmed via phosphatase treatment and together with known phosphorylated proteins relevant to iTreg function, STAT1 and AKT. (d) PCMT1 splicing and 3'UTR length were analyzed in vitro. (e) PCMT1 gene expression was quantified via qPCR on Day 17 in bone marrow and spleen iTregs treated with pPKCθ ex vivo on Day 0 in humanized GvHD model. (f) In vivo analysis of PCMT1 splicing and 3'UTR length were performed via RT-PCR in humanized GvHD model. *‘β’* indicates the expected amplicon size. Data represent mean ± SEM two or three independent experiments. For in vivo experiment, 4 mice per group were used. Unpaired, two-tailed t test was used for analysis; *p < 0.05.
Figure 5.6 PCMT1 splicing and 3'UTR analyses in non-differentiated T cells (Tconvs) in vitro and in vivo. (a) PCMT1 gene expression was quantified via qPCR in Tconvs. (b) PCMT1 protein in cytoplasm and nucleus were detected via western blot and quantified based on loading control densities via ImageJ software. (c) PCMT1 splicing and 3'UTR length were analyzed in vitro. (d) PCMT1 gene expression was quantified via qPCR on Day 17 in bone marrow and spleen naïve T cells in iTreg-administered humanized GvHD model. (e) In vivo analysis of PCMT1 splicing and 3'UTR length were performed via RT-PCR in humanized GvHD model. ‘ß’ indicates the expected amplicon size. Data represent mean ± SEM two or three independent experiments. For in vivo experiment, 4 mice per group were used. Unpaired, two-tailed t test was used for analysis; *p < 0.05, **p<0.01, ***p<0.001.
Figure 5.7 Inhibiting PKCθ diminishes hnRNPL association with PCMT1 at both protein and RNA levels in iTregs. (a) Percentages of CD4⁺CD25⁺FOXP3⁺ T cells upon cell-penetrating αPKCθ or αPCMT1 treatment. (b) Median fluorescent intensity of FOXP3 together with a representative histogram of FOXP3-expressing iTregs. (c) Percentage of IFNγ-positive iTregs and median fluorescent intensity of IFNγ with a representative histogram. (d) Immunoprecipitation of cytosolic vs. nuclear hnRNPL with PKCθ and PCMT1 in iTregs. Adjusted protein densities were quantified according to IgG via ImageJ software. (e) Prediction of hnRNPL RNA binding motifs via Catalog of Inferred Sequence Binding Preferences of RNA Binding Proteins (CISBP-RNA) database in human. (f) hnRNPL association with cytosolic and nuclear PCMT1 mRNA. ‘←’ indicates the expected amplicon size. Data represent mean ± SEM two or three independent experiments. Unpaired, two-tailed t test was used for analysis; *p < 0.05, **p < 0.01, ***p<0.0001.
**Figure 5.8 hnRNPL binding sites on 3'UTR sequences of iTreg genes.** 3'UTR sequences (red sequences) were determined using Ensembl. These sequences were copied and run on RBPmap to define hnRNPL binding sites (highlighted as yellow). Later, forward and reverse primers were designed to amplify these regions via RT-PCR (Primers were designed to bind black, bold sequences).
Figure 5.9 Cytoplasmic vs. nuclear mRNA association with hnRNPL in α-pPKC- or α-PCMT1-treated iTregs. hnRNPL association with cytosolic and nuclear (a) *FOXP3*, (b) *PDCD1*, (c) *IFNG*, and (d) *IFNGR1* mRNAs. ‘←’ indicates the expected amplicon size. Data represent mean ± SEM two independent experiments.
Figure 5.10 PCMT1 can be a good target to increase stability of iTregs. (a) Chromatin immunoprecipitation of PCMT1 on FOXP3 and IFNG genes in iTregs. (b) CpG islands and STAT5-binding sites on FOXP3 T cell-specific demethylated region (TSDR) in humans. (c) Bisulfite sequencing of ten different clones for FOXP3 TSDR CpG islands from each of the treatment. Percentages of non-methylated CpGs were quantified. (d) Percentages of methylated vs. demethylated CpGs on STAT5-binding sites upon αPKC0 or αPCMT1 treatment in iTregs. (e) Working model for PKC0-, hnRNPL-, and PCMT1-mediated RNA processing and iTreg stability. Data represent mean ± SEM two or three independent experiments. Unpaired, two-tailed t test was used for analysis; *p < 0.05, **p < 0.01, ***p < 0.0001.
Table 5.1 List of splicing primers

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<th>Gene</th>
<th>Forward Primer (5’-3’)</th>
<th>Reverse Primer (5’-3’)</th>
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Table 5.2 List of 3’UTR primers

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<td>PDCD1</td>
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CHAPTER 6

CYMERUS™ iPSC-MSCs SIGNIFICANTLY PROLONG SURVIVAL IN A PRECLINICAL, HUMANIZED MOUSE MODEL OF GRAFT-VERSUS-HOST DISEASE

6.1 Introduction

Hematopoietic stem cell transplantation (HSCT) can provide full hematopoietic reconstitution after myeloablative therapy commonly used to treat hematologic malignancies, solid tumors, or immune-mediated bone marrow (BM) failure diseases, such as aplastic anemia (van den Brink et al., 2015; Ratajczak and Suszynska, 2016; Dietz et al., 2016). Graft-versus-host disease (GvHD) refers to adverse sequelae following allogeneic HSCT, whereby immune-competent cells in the stem cell graft are activated and damage host tissues (Shlomchik, 2007). Acute GvHD, which occurs within 100 days of transplantation, can affect up to 80% of allogeneic HSCT recipients and remains a significant barrier to the broader use of HSCT in the clinic (Garnett et al., 2013). Preventing acute GvHD may decrease the likelihood of developing chronic GvHD, which presents after 100 days and appears to have autoimmune underpinnings (Chen et al., 2007).

T cells play a critical role in mediating GvHD pathophysiology. Followed by their activation and rapid expansion in the host, alloreactive donor T lymphocytes recognize mismatches in major and minor histocompatibility complexes, initiating GvHD in the
recipient's tissues. Skin, gut, and liver are major organs targeted by T cells during GvHD, but damage to hematopoietic tissues is also observed (Ramadan and Paczesny, 2015). T cells are fully activated by antigen-specific signals delivered through the T cell receptor, coupled with antigen non-specific co-stimulatory signals conveyed through CD28 on the T cell surface. The T cell-specific kinase, Protein Kinase C-theta (PKCθ), is phosphorylated on multiple residues, downstream of CD28 signaling. Functional PKCθ is essential for mediating GvHD responses, likely through its nuclear regulation of proinflammatory gene expression (Valenzuela et al., 2009; Sutcliffe et al., 2011). Reducing PKCθ activity in alloreactive T cells; therefore, constitutes an attractive approach to limiting GvHD.

Strategies to prevent or treat GvHD include prophylactic or therapeutic administration of immunosuppressive agents, such as cyclosporine, although prognosis is poor for patients who progress to a steroid-refractory state (Westin et al., 2011). For these patients, a second line therapy is necessary and may consist of anti-thymocyte globulin, inhibitors of tumor necrosis factor alpha signaling, mycophenolate mofetil, or inhibitors of the mTOR (mechanistic target of rapamycin) pathway. More recently, MSC-based therapies are being vigorously investigated, including products such as Temcell®, which has been approved in Japan to treat steroid-refractory GvHD (JCR Pharmaceutical Co., Ltd.) (Locatelli et al., 2017). While MSC therapy for steroid-refractory GvHD is promising, there remains a critical need both for more effective treatments for acute GvHD, in general, and for improving efficacy of MSC-based therapies, specifically (Fernández Vallone et al., 2013). A significant challenge with primary MSCs is the need for extensive ex vivo expansion and reliance on multiple donors, to generate sufficient
numbers of cells for therapeutic use, especially at a commercial scale. Inter-donor variability and diminished potency following expansion in culture have been postulated as key factors in the disappointing outcome of a Phase 3 clinical trial of a commercial BM-derived MSC product, in marked contrast to numerous successful smaller trials conducted by academic groups which involved minimally expanded BM-MSCs (Galipeau, 2013).

Induced pluripotent stem cells (iPSCs) can proliferate almost indefinitely without losing pluripotency, making it feasible to generate a nearly limitless supply of iPSCs from a single blood or tissue donation (Lei and Schaffer, 2013). Harnessing the expansion potential of iPSCs prior to differentiation, allows for producing vast numbers of iPSC-derived MSCs, without excessively expanding the MSCs themselves. This enables ongoing production of commercial quantities of minimally expanded MSCs from a single iPSC line.

Here we report the characterization and pre-clinical efficacy of the Cymerus™ MSC product, which is derived from iPSCs through the mesenchymoangioblast pathway (Vodyanik et al., 2010). Immunophenotyping and in vitro functional studies suggest these iPSC-MSCs display a typical MSC phenotype. Using a humanized mouse model of GvHD, we show that single- or dual-dose infusions of iPSC-MSCs, given under therapeutically relevant conditions, attenuate GvHD severity and provide a significant survival benefit. We also show that the immunosuppressive effects of iPSC-MSCs result from their modulating PKCθ phosphorylation and cellular localization in T cells. Finally, we assessed several pro-inflammatory molecules, whose reduced expression is highly-
correlated with clinical response to iPSC-MSC administration, and which may serve as a panel of biomarkers to monitor therapeutic response.

6.2 Materials and Methods

6.2.1 Animals

Animal studies were approved by the Institutional Animal Care and Use Committee, University of Massachusetts Amherst. Six-weeks-old female NOD.Cg-Prkdc<sup>scid</sup> Il2rg<sup>tm1Wjl/SzJ</sup> (NSG) mice, were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were rested for one week prior to use, housed under pathogen-free conditions in micro-isolator cages with acidified, antibiotic water throughout the experimental procedures.

6.2.2 Antibodies


6.2.3 iPSC-MSC licensing and immunophenotyping

iPSC-MSCs were cultured in mesenchymal serum-free expansion medium (M-SFEM); consisting of 50% human endothelial serum-free medium (Invitrogen, Waltham, MA), 50% StemLine II hematopoietic serum-free medium (Sigma-Aldrich, St. Louis, MO), 1% GlutaMax (Invitrogen), 100µM α-monothioglycerol (Sigma-Aldrich) and 10ng/mL fibroblast growth factor 2 (Peprotech, Rocky Hill, NJ). iPSC-MSCs (4.8x10⁴) were plated onto fibronectin-(Invitrogen) and collagen-(Sigma-Aldrich) coated plates on day 0. They were licensed the following day with 50 ng/mL of recombinant human IFNγ (BioLegend, San Diego, CA) for 24 and 48 hrs in M-SFEM at 37°C in 5% CO₂. At 24 and 48 hours of culture, cells were harvested and stained with antibodies specific for CD45, CD34, CD44, CD73, CD90, CD105, CD80, CD86, HLA-DR, PD-L1, and IDO. Data were acquired on a BD LSR Fortessa Flow Cytometer (Becton Dickinson, Canaan, CT) and analyzed using FlowJo (version 10.0; Treestar, Ashland, OR).
6.2.4 *In vitro* differentiation of iPSC-MSCs

iPSC-MSC cells were seeded on fibronectin- and collagen-coated 8-well chamber slides on day 0 and incubated at 37°C in 5% CO₂. Cells were then differentiated into three lineages, adipocytes, osteocytes and chondrocytes via the following procedures:

**Adipocyte differentiation:** On day 2, the culture medium was replaced with AdipoLife Differentiation Medium containing AdipoLife basal medium and DifFactor 3 (Lifeline Cell Technology, Frederick, MD). The differentiation medium was replaced every 3-4 days until day 26. On day 26, the cells were fixed and stained for lipid accumulation as per the Oil Red O Staining Kit (Lifeline Cell Technology). Images were taken at 10X using the Olympus CX40 microscope.

**Osteocyte differentiation:** The culture medium was replaced with OsteoLife Complete Osteogenesis Medium (Lifeline Cell Technology) on day 2. This medium was replaced every 3-4 days until day 21. The cells were then fixed and stained for calcium deposits using Alizarin Red Stain (Lifeline Cell Technology). Images were taken at 20X using the Olympus CX40 microscope.

**Chondrocyte differentiation:** iPSC-MSCs were covered with ChondroLife Complete Chondrogenesis Medium 2 hours after plating on day 0. The differentiation medium was replaced every 2-3 days until day 21, when the differentiation is complete. Cells were then fixed and stained for sulfated proteoglycan deposits as per the Alcian Blue Staining Kit (Lifeline Cell Technology). Images were taken at 10X using the Olympus CX40 microscope. hTERT-MSC cells (ATCC, Manassas, VA) were used as a control cell line to demonstrate differentiation and were subjected to the same methods of differentiation as described above.
6.2.5 Determining post-thaw senescence

Aliquots of iPSC-MSCs were thawed, plated on fibronectin- and collagen-coated plates and incubated at 37°C in 5% CO₂. Senescence was assayed by measuring β-galactosidase activity immediately after thawing and 24hr, 48hr and 7 days post thaw using the Senescence Cells Histochemical Staining Kit (Sigma-Aldrich, St. Louis, MO). At each time point, the number of senescent (blue) cells per 100 cells were counted. Images were taken at 10X using the Olympus CX40 microscope.

6.2.6 In vitro immunopotency assay

The degree to which the iPSC-MSCs can suppress the proliferation of CD4 T helper lymphocytes was performed as previously described (Bloom et al., 2015). Briefly, MSCs were exposed to 21 Gy of gamma irradiation, then 4x10⁵, 2x10⁵, 4x10⁴, and 2x10⁴ irradiated MSCs were plated into individual wells of a 48-well tissue culture plate. PBMCs from two individuals were separately labelled with carboxyfluorescein succinimidyl ester (CFSE). Labelled cells were plated at 4x10⁵ leukocytes per well containing iPSC-MSCs, to give titrated PBMC: iPSC-MSC ratios of 1:1, 1:0.5, 1:0.2, 1:0.1 and 1:0.05. An additional well was plated with stimulated PBMCs alone, another with iPSC-MSCs alone, and a third with a 1:0.05 ratio, without stimulation, all of which served as controls. Subsequently, T cells were stimulated with anti-human CD3ε and anti-human CD28, which were added to each well. Cells were harvested from individual wells after 4 days in culture. Cells from each well were incubated with allophycocyanin-labeled anti-human CD4. CD4 T cells were then analyzed for proliferation via CFSE
intensity using a flow cytometer. The iPSC-MSC alone control served to gate out iPSC-MSCs from co-culture wells, while the PBMC alone control served as the positive control for maximum T cell proliferation, against which the degree of iPSC-MSC-mediated suppression was measured. The non-stimulated 1:0.05 ratio well was used to generate a negative control gate against which proliferation was measured. A suppression value (Sv) was determined for iPSC-MSC: PBMC ratio on the basis of the percentage proliferation of stimulated T cells in the presence of iPSC-MSCs relative to the percentage proliferation of stimulated T cells without iPSC-MSCs. The immunopotency assay value (IPAv) for each sample was then calculated according to the following equation:

\[ S_v = 100 \left( \frac{P_{%} + M}{P_{%} - M} \times 100 \right) \]

where:
- \( P_{%} + M \) is the percentage proliferation of stimulated T cells in the presence of iPSC-MSCs
- \( P_{%} - M \) is the percentage proliferation of stimulated T cells in the absence of iPSC-MSCs
- \( n_t \) is the number of titrations

A normalized IPAv was then calculated by dividing the IPAv for each sample by the IPAv for the reference standard.

### 6.2.7 Assessing IDO and PD-L1 expression by IFNγ-licensed iPSC-MSCs

iPSC-MSCs were seeded on fibronectin- and collagen-coated plates. The next day they were licensed with 50 ng/ml of Recombinant Human IFNγ (BioLegend) for 24 and
48 hours in M-SFEM, at 37°C in 5% CO₂. Cells were split and treated as follows: RNA was isolated using the QuickRNA miniprep kit (Zymo Research, Irvine, CA) and qPCR was performed using the SYBR Green Master Mix (BioTools, Jupiter, FL) to assess indoleamine 2,3-dioxygenase (IDO) mRNA levels. β-Actin was the reference gene used for normalization. Cells were also stained with antibodies specific for surface markers, as indicated. Data were acquired on a BD LSR Fortessa Flow Cytometer (Becton Dickinson) and analyzed using FlowJo (version 10.0, Treestar).

6.2.8 Graft-vs-Host disease induction

Human PBMCs (StemCell Technologies, Vancouver, BC, Canada) were thawed and rested overnight at 37°C in 5% CO₂. NSG mice were conditioned with 2 Gy of total body irradiation from a ¹⁵⁷Cs source, then rested for 4 hours. PBMCs were washed with sterile PBS and 10x10⁶ cells in 150 µL of sterile PBS were delivered to NSG mice via the tail vein.

6.2.9 iPSC-MSC administration

iPSC-MSCs were thawed at 37°C, washed in sterile PBS, 2x10⁶ cells/mL were resuspended in 150 µL sterile PBS. For single- and dual-dose treatments, 2x10⁶ iPSC-MSCs were administered into NSG mice via tail vein injection on day +14 only, or on days +14 and +18, respectively, after GvHD induction.

6.2.10 GVHD clinical scoring
The severity of GvHD was assessed using a standardized scoring system, as previously described, and which included five different criteria (weight loss, posture, activity, fur texture, and skin integrity) (Ozay et al., 2016). Mice were weighed, evaluated daily, and graded from 0 (the least severe) to 2 (the most severe) for each criterion, beginning on day +12 after disease induction. Daily clinical scores were generated by adding grades for five criteria. When a clinical score of “8” was reached, mice were removed from the study and humanely euthanized. The day of removal from the study was recorded as the day of lethal GvHD induction.

6.2.11 Biomarker analysis

BM, spleen, and peripheral blood were collected on day +19 to determine percent PBMCs [positive human CD45 cells % / (positive human CD45 cells % + positive mouse CD45 cells %)] and infiltration of human CD4 and CD8 T cells. Human CD4 and CD8 T cells were stained with antibodies specific for CD25, pPKCθ (Thr538), NOTCH1, and T-BET. Data were acquired on a BD LSR Fortessa Flow Cytometer (Becton Dickinson) and analyzed using FACSDiva Software (version 8.0, Becton Dickinson) and FlowJo (version 10.0, Treestar).

6.2.12 LEGENDPlex™ Bead-based immunoassay

Peripheral blood for cytokine analysis was obtained on day +19 from animals via cardiac puncture, immediately following humane euthanasia. The LEGENDPlex™ Human Th1/Th2 panel (8-plex; BioLegend) was used to determine IFNγ. Data were
acquired on a BD LSR Fortessa Flow Cytometer (Becton Dickinson) and analyzed using LEGENDPlex™ Software, Version 7.0 (BioLegend).

6.2.13 Protein subcellular localization

BM, spleen, and peripheral blood were collected on day +19. Single cell suspensions were prepared, and surface stained for CD4 and CD8 T cells. Samples were fixed and permeabilized using the Foxp3 Staining Buffer Kit (BD Biosciences) and stained with fluorescently-conjugated antibodies specific for pPKCθ (Thr538), NOTCH1, and T-BET. Nuclei were stained using cell-permeable DRAQ5™ Fluorescent Probe (ThermoFisher Scientific, Waltham, MA). Cells were visualized and quantified using an ImageStream® x Mark II Imaging Flow Cytometer (EMD Millipore, Billerica, MA). Subcellular localizations of pPKCθ (Thr538), NOTCH1, and T-BET were determined using the Nuclear Localization Wizard, IDEAS® Software, upon masking of nuclear and non-nuclear regions to quantify proteins localized in and out of the nucleus.

6.2.14 Statistical analyses

Data are the mean ± SEM; all in vitro experiments were repeated at least three times. Unpaired, two-tailed Student’s t test using (Prism5; GraphPad Software, San Diego, CA) was used for statistical comparison of two groups, with Welch’s correction applied when variances were significantly different. Two-way ANOVA (Prism 5; GraphPad Software) was used for the comparison of variables, which are influenced by two different categories and followed by Bonferroni post-test. For in vivo experiments,
survival benefit was determined using Kaplan–Meier analysis with an applied log-rank test. P values of $\leq 0.05$ were considered significantly different.

6.3 Results

6.3.1 iPSC-derived MSCs phenotypically resemble native MSCs, respond to IFN$\gamma$ licensing, and dampen PBMC activation potential

MSCs are identified by a constellation of criteria: adherence to the tissue culture dish, tri-lineage differentiation potential, and expression of several distinct surface markers together with the absence of others (Dominici et al., 2006). MSCs also acquire immunosuppressive functions following exposure to proinflammatory cytokines, such as interferon gamma (IFN$\gamma$); a process referred to as IFN$\gamma$-licensing.

We characterized Cymerus™ iPSC-MSCs by examining their surface markers and whether their expression changed after exposure to IFN$\gamma$. We found that molecules expressed on iPSC-MSCs are consistent with an MSC phenotype (Figure 6.1a-i). iPSC-MSCs exhibit tri-lineage differentiation, also in agreement with their characterization as MSCs (Figure 6.2a-c) (Rebelatto et al., 2008). We next evaluated post-thaw senescence of iPSC-MSCs, because replicative senescence in cryopreserved cells, following ex vivo expansion, may reduce in vivo potency (Galipeau, 2013; Turinetto et al., 2016; de Witte et al., 2017). Our results suggest that up to one week following cell thawing, iPSC-MSCs cultured in vitro do not exhibit signs of functional senescence, as measured by $\beta$-galactosidase staining (Figure 6.2d).
In response to IFN$\gamma$ exposure, native MSCs can acquire immunosuppressive capabilities. MSCs use two well-characterized mechanisms to curtail immune cell activation: through the Programmed Cell Death Protein (PD-1)-Programmed Cell Death Protein-Ligand (PD-L1) signaling axis and through immune-modulating indoleamine 2,3-dioxygenase (IDO) (Yan et al., 2014; Shi et al., 2010). MSCs licensed by IFN$\gamma$ upregulate and can secrete soluble PD-L1, an immune checkpoint inhibitor (Davies et al., 2017).

We found that iPSC-MSCs express moderate amounts of PD-L1 that were further increased in response to IFN$\gamma$ (Figure 6.3a). At the protein level, intracellular IDO in IFN$\gamma$-licensed iPSC-MSCs, increased approximately 3-fold over baseline expression after 24 hours of exposure to IFN$\gamma$, and nearly 5-fold after 48 hours in culture with IFN$\gamma$ (Figure 6.3b). We noted very high IDO transcript levels in iPSC-MSCs cultured in the presence of IFN$\gamma$ for 24 hours, which increased further after 48 hours of IFN$\gamma$ exposure (Figure 6.3c).

We next evaluated what effects iPSC-MSCs have on human T cell proliferation and differentiation potential in vitro. We determined the suppressive capacity of the iPSC-MSCs using a previously-described immunopotency assay (Bloom et al., 2015). A normalized immunopotency assay value (IPAv) was calculated by dividing the IPAv of each sample by the IPAv of the reference standard. This allowed us to compare the level of suppression conveyed by each sample relative to the reference standard. The IPAv for products used in this study (Table 6.1) indicate that iPSC-MSCs provide a modest suppressive effect on CD4 T cell proliferation during in vitro co-culture.
After antigenic stimulation, human PBMCs upregulate signaling molecules that further facilitate their activation and differentiation potential. These include the high-affinity subunit of the IL-2 receptor, CD25, the transmembrane receptor, NOTCH1, the master transcriptional regulator of T helper type 1 (Th1) cells, T-BET (T-box expressed in T cells), and the proinflammatory cytokine, IFNγ (Osborne and Minter, 2007; Minter et al., 2005). Compared to PBMCs cultured alone, co-culturing PBMCs with iPSC-MSCs significantly reduced the percentage of cells expressing T-BET (Figure 6.3d) and IFNγ (Figure 6.3e), suggesting iPSC-MSCs reduce the potential of activated T cells to adopt a Th1 cell fate. The amount of T-BET or IFNγ produced on a per cell basis was also lower in PBMCs co-cultured with iPSC-MSCs (Figure 6.3, d and e, respectively), although this downward trend did not reach statistical significance during the short culture period. Co-culturing PBMCs with iPSC-MSCs did not alter expression of CD25 or of NOTCH1 (Figure 6.4, a and b, respectively), although NOTCH1 levels also decreased by 72 hours in co-culture.

The T cell-specific kinase, PKCθ, functions within a signal-amplifying cascade, to fully activate T cells and promote tissue destruction in GvHD (Isakov and Altman, 2012; Valenzuela et al., 2009). To assess iPSC-MSC influence on PKCθ phosphorylation, we asked whether co-culturing iPSC-MSCs with PBMCs altered pPKCθ expression. iPSC-MSCs were left unlicensed or licensed with IFNγ for 48 hours then co-cultured with stimulated PBMCs for an additional 96 hours. We observed reduced expression of pPKCθ in PBMCs co-cultured with IFNγ-licensed, but not with unlicensed, iPSC-MSCs (Figure 6.3f). In parallel, PD-1 levels also increased following co-culture with licensed, but not unlicensed iPSC-MSCs.
Collectively, these data show iPSC-MSCs phenotypically resemble native MSCs derived from BM and respond to IFNγ exposure in ways that are consistent with those of native MSCs, cultured under similar conditions. Furthermore, co-culturing with IFNγ-licensed iPSC-MSCs dampened the immune response of PBMCs, as measured by proliferation, expression of well-described activation and differentiation markers, and reduction of pro-inflammatory IFNγ, supporting the notion that iPSC-MSCs exert functional, immune-modulating actions, in vitro.

6.3.2 iPSC-MSC administration, in vivo, reduces cytokine production and weight loss in mice with GvHD

Humanized mouse models have been used to evaluate the efficacy of MSC therapy to diminish disease severity (Roemeling-van Rhijn et al., 2013). We utilized a lymphocyte transfer model of GvHD28, in which human PBMCs are transferred into transgenic NOD.Cg-Prkdc<sup>scid</sup> Il2rg<sup>tm1Wjl</sup>/SzJ (NSG) mice lacking T, B and NK cells, to investigate the therapeutic benefit of giving single or dual doses of iPSC-MSCs, under clinically-relevant conditions (supplemental Figure 6.5a).

Before defining the in vivo therapeutic activity of iPSC-MSCs, we first verified that transferred PBMCs expanded to the same extent in iPSC-MSC-treated animals as they did in untreated GvHD controls. We found no differences in percentages of circulating PBMCs collected from untreated mice and from mice that received single- or dual-dose treatments of iPSC-MSCs (Figure 6.6a). Furthermore, percentages of circulating CD4 and CD8 T cells also did not differ significantly between treated and
untreated animals (Figure 6.6, b and c, respectively). These data are consistent with *in vitro* suppression data which showed only modest effects in co-culture (Table 6.1), and with a previous report using a similar mouse model, and which demonstrated the protective effects afforded by MSCs are not due to accelerated elimination of disease-inducing PBMCs (Tobin et al., 2013).

Pro-inflammatory cytokines released into the circulation during GvHD can cause significant weight loss in patients (Pajak et al., 2008). In the pre-clinical model used here, high levels of circulating IFNγ correlate closely with disease severity (Ozay et al., 2016). We monitored IFNγ levels, as well as changes in weight on day +19, for mice that were left untreated or were treated with single- or dual-doses of iPSC-MSCs. We observed significantly lower IFNγ in mice that received dual-doses of iPSC-MSCs, compared to untreated and to single-dose treated mice (Figure 6.6d). Reduced plasma cytokines correlated with significant relief from the cachexia-associated weight loss typically observed in this Th1-mediated model (Figure 6.6e). Although administering single or dual doses of iPSC-MSCs did not affect percentages of circulating PBMCs, compared to untreated GvHD control mice, the *in vivo* capacity of transferred PBMCs to produce proinflammatory cytokines was greatly diminished in mice that received dual doses of iPSC-MSCs.

6.3.3 iPSC-MSC administration reduces BM-infiltration and expression of proinflammatory molecules in mice with GvHD

The mechanisms MSCs utilize to attenuate GvHD have not been fully elucidated, but reports suggest they influence immune cell activation and trafficking to target organs
We induced GvHD in lightly-irradiated NSG mice, harvested blood and tissue samples +19 days after GvHD induction, at the peak of disease, and evaluated target-tissue infiltration and markers of immune activation in untreated and in iPSC-MSC-treated mice. When we examined the BM, the major target of immune destruction in this model, we noted the percentages of BM-infiltrating PBMCs were significantly lower in mice given iPSC-MSCs, regardless of whether they received single or dual doses, compared to untreated controls (Figure 6.7a). We detected decreased BM-infiltration of CD4 (Figure 6.7b) and CD8 T cells (Figure 6.7c), which corresponded to higher total BM cellularity in iPSC-MSC-treated mice, compared to untreated GvHD control animals (Figure 6.7d). In contrast, percentages of human T cells in the spleen were less affected by iPSC-MSC treatment (supplemental Figure 6.8a-c). Thus, one means by which iPSC-MSCs may function in vivo in this model, is by protecting the BM from immune-mediated destruction caused by infiltrating PBMCs.

The lethal GvHD induced in this model is driven by CD4 Th1 and cytolytic CD8 T cells. We used flow cytometry to measure the expression of CD25, NOTCH1, and T-BET, to ask whether iPSC-MSC treatment attenuated this Th1-mediated response, in vivo. Except for CD25 expression on BM-infiltrating CD4 T cells, which did not differ between treatments (Figure 6.7e), there was significantly lower expression of all the pro-inflammatory markers evaluated on day +19. Compared to the levels expressed in untreated GvHD control animals, CD25 expression was lower on CD8 T cells recovered from the BM of iPSC-MSC-treated animals (Figure 6.7f). NOTCH1 (Figure 6.7g, h) and T-BET (Figure 6.7i, j), both of which are upregulated in Th1-mediated diseases, were also significantly decreased in BM CD4 and CD8 T cells from mice treated with single-
or dual-dose regimens of iPSC-MSCs (Minter et al., 2005; Roderick et al., 2013). Consistent with our \textit{in vitro} co-culture results, we observed that treating animals with single- or dual-doses of iPSC-MSCs also significantly reduced expression of pPKC\(\theta\) in CD4 and CD8 T cells recovered from the BM (Figure 6.7, k and l; respectively).

Collectively, our data suggest iPSC-MSCs decrease the activation and infiltration of proinflammatory CD4 and CD8 T cells in the BM. We conclude that the reduced expression on PBMCs of multiple markers of activation, coupled with the low level of PKC\(\theta\) phosphorylation, in BM-infiltrating CD4 and CD8 T cells, result from immune modulation mediated by iPSC-MSCs.

### 6.3.4 iPSC-MSCs attenuate GvHD severity and prolong survival in mice

We assessed disease severity in our humanized model of GvHD, using a standardized scoring system, after randomly assigning mice to one of six treatment cohorts (supplemental Figure 6.9a, b) (Ozay et al., 2016). Mice were humanely euthanized when they reached a cumulative score of “8” which was recorded as the day of lethal GvHD induction. We determined the survival benefit of administering iPSC-MSCs using Kaplan–Meier analyses with an applied log-rank test, with a \(P\) value of <0.05 considered significantly different.

Administering iPSC-MSCs significantly attenuated disease symptoms, compared to untreated mice (Figure 6.10a), and we noted further significant differences in clinical scores between single- and dual-dose treatments when we evaluated mice on days +24 and +25 post- GvHD-induction. During survival studies, single- and dual-dose treatments conferred significant survival benefits over untreated GvHD controls (\(P<0.0001\); Figure
6.10b. Animals receiving dual-dose regimens survived slightly longer (median=57 days, range=31-82 days) than single-dose-treated animals (median=48 days, range=33-60 days), although this difference was not statistically significant (P=0.0715). Utilizing this pre-clinical model of GvHD, we conclude that administering single or dual doses of iPSC-MSCs, under clinically relevant conditions, significantly attenuates disease severity and conveys a robust survival benefit.

6.3.5 iPSC-MSC treatment alters subcellular localization of pPKC\(\theta\) in BM-infiltrating T cells

One means by which protein activity can be regulated is through changes in subcellular compartmentalization (Bauer et al., 2015). Dynamic redistribution of proteins between the cytosol and the nucleus may increase, decrease, or completely alter protein-protein interactions to differentially modulate biological outcomes (Cyert, 2001). Indeed, a nuclear role for PKC\(\theta\) has also been reported in human CD4 T cells, functioning as part of a complex that facilitates transcription of proinflammatory genes, including \(IFNG\) (Sutcliffe et al., 2011). To further understand, on a molecular level, the effects of treating mice with iPSC-MSCs, we asked whether the nuclear localization of pro-inflammatory proteins was altered following iPSC-MSC administration.

Using imaging flow cytometry, we analyzed the subcellular distribution of NOTCH1, TBET, and pPKC\(\theta\), in BM-infiltrating CD4 and CD8 T cells from animals induced with GvHD and given single or dual doses of iPSC-MSC. Nuclear expression of NOTCH1 and T-BET in CD4 and CD8 T cells recovered from the BM was not significantly altered by iPSC-MSC treatment (supplemental Figure 6.11a-d). In contrast,
BM samples collected on day +19 from untreated animals with GvHD contained high percentages of nuclear pPKC0-expressing CD4 and CD8 T cells. This nuclear localization of pPKC0 was confirmed by high positive nuclear similarity scores (Figure 6.12). However, the percentages of cells expressing nuclear pPKC0 was significantly lower, both in CD4 and CD8 T cells, in BM samples from mice treated with iPSC-MSCs (Figure 6.12a, b, d, e). Furthermore, the amount of pPKC0 detected in the nucleus, was also significantly reduced following treatment with iPSC-MSCs, both in CD4 and CD8 T cells (Figure 6.12c, f).

Altogether, these data indicate that, by day +19, a high percentage of T cells found in the BM express elevated levels of nuclear pPKC0. Moreover, treating mice with iPSC-MSCs reduced the infiltration of nuclear pPKC0-expressing T cells and, further, acted to diminish the amount nuclear pPKC0 within these CD4 and CD8 T cells.

6.3.6 Pro-inflammatory molecules expressed by circulating PBMCs correlate with therapeutic response to iPSC-MSC administration

A minimally-invasive means to identify biomarkers to predict or monitor therapeutic responses to iPSC-MSC administration would be of great clinical value. Therefore, we asked whether utilizing flow cytometry to analyze expression of pro-inflammatory markers on peripheral blood CD4 and CD8 T cells, found to be significantly different on BM-infiltrating immune cells, also correlated with disease severity. We measured expression of CD25 (Figure 6.13a, b), NOTCH1 (Figure 6.13c, d), T-BET (Figure 6.13e, f), and pPKC0 (Figure 6.13g, h) in circulating PBMCs from
untreated and iPSC-MSC-treated mice, collected on day +19 after disease induction. Compared to PBMCs from untreated mice, the expression of all these pro-inflammatory molecules was significantly reduced as measured by the percent positive cells, the level of the proteins expressed, or both, following therapeutic administration of iPSC-MSCs.

Collectively, we demonstrate administering Cymerus™ iPSC-MSCs as a cell-based therapy provides relief from acute symptoms and significantly prolongs survival in a pre-clinical model of GvHD. Our data suggest iPSC-MSCs modulate PKCθ phosphorylation and subcellular localization. Furthermore, flow cytometric analysis of pro-inflammatory molecules may provide a set of biomarkers on circulating PBMCs that correlate closely with therapeutic response to iPSC-MSC administration.

6.4 Discussion

Since the first experimental use of MSCs to successfully treat steroid-resistant GvHD administering MSCs as a cell-based therapy has become an area of intensive investigation (Le Blanc et al., 2004). However, inconsistent results in the clinic underscores all we do not yet understand about the precise mechanisms of action or the longevity, post-transfer, of MSCs (Galipeau, 2013). While there are some indications cryopreserving MSCs adversely affects their immunosuppressive capacity, other reports suggest MSCs can undergo multiple passages or repeated freezing without exhibiting any diminished function (Pollock et al., 2015; François et al., 2012; Mamidi et al., 2012). Extensive ex vivo expansion has been linked to telomere shortening in cultured MSCs, but whether this contributes to functional senescence and impaired efficacy, in vivo, is not known (Bernardo et al., 2007). Additionally, in vitro studies indicate senescence can
be reversed by priming with IFNγ, but further studies are required to determine whether this phenomenon also occurs in vivo (Chinnadurai et al., 2017). One means to bypassing replicative senescence in MSCs may be to derive them from iPSCs (Sabapathy and Kumar, 2016). We show that iPSC-MSCs displayed low levels of senescence immediately after thawing, which decreased over 48 hours in culture, even without the addition of IFNγ. Furthermore, IFNγ licensing significantly increased IDO expression in iPSC-MSCs, consistent with the findings that IDO-mediated suppression is an important mechanism of immune modulation by MSCs (Chinnadurai et al., 2017). While we could not track the senescence of transferred iPSC-MSCs in vivo, the significant survival benefit noted following single- or dual-dose administration, indicate that iPSC-MSCs function for a sufficient length of time after infusion, to produce a durable immunomodulatory response.

Donor T cells traffic to secondary lymphoid organs within 24 hours after HSCT (Anderson et al., 2008). Following antigenic stimulation there, alloreactive CD4 and CD8 T cells expand and migrate to target tissues. In the humanized model of GvHD used in this study, the BM is the primary target organ, and mice die of lethal BM failure approximately three weeks after PBMC infusion. The accumulation of activated T cells in the BM of untreated mice, and the pro-inflammatory markers they express, are consistent with a Th1-mediated immune response, and one that will also drive effector functions of CD8 cytolytic T cells. This includes increased CD25 expression, upregulation of NOTCH1 and T-BET, and production of IFNγ, as well as sustained phosphorylation of the T cell-specific kinase, PKCθ, which is recruited to membrane lipid rafts following co-stimulation through the T cell receptor and CD28 (Kong et al.,
The high-affinity IL-2 receptor, CD25, is upregulated in activated T cells in a NOTCH1-dependent manner (Palaga et al., 2003; Adler et al., 2003). Furthermore, physical association NOTCH1 with PKC0 aids in assembly of the Carma1-BCL10-MALT1 supramolecular signaling complex in CD4 T cells, a pre-requisite to activating the NF-κB (nuclear factor-κB) transcriptional complex (Shin et al., 2014).

PKC0 is one of nine members of the PKC family. It is required for mediating immune destruction in GvHD, identifying it as an attractive therapeutic target (Zhang et al., 2013). However, close structural homology between PKCs make designing small molecule inhibitors to single isoforms challenging (Xu et al., 2004). Long-believed to be a cytosolic-resident kinase, a seminal study by Sutcliffe et al. (2011) revealed a nuclear function for PKC0 in CD4 T cells, including positively regulating transcription of pro-inflammatory genes such as IFNG. We utilized imaging flow cytometry to evaluate the cellular localization of pPKC0 in BM-infiltrating CD4 and CD8 T cells. We found nuclear pPKC0 highly expressed in more than 80% of CD4 and CD8 T cells in the BM of untreated mice. By contrast, both the percentage of cells expressing nuclear pPKC0, as well as the quantity of nuclear pPKC0 detected, was significantly reduced in CD4 and CD8 T cells collected from the BM of dual dose-treated animals. MSCs migrate to sites of inflammation, where the local concentration of IFNγ or TNF may be sufficient to license their immunomodulatory functions. iPSC-MSCs may be similarly recruited to the BM in this model of GvHD and exert immune suppressive effects on infiltrating T cells by reducing nuclear pPKC0 expression.
Mechanistically, it is not entirely clear how iPSC-MSCs affect cytosolic versus nuclear distribution of pPKC\(\theta\). Surface expression of PD-L1 on iPSC-MSCs has the potential to interact with its cognate receptor PD-1, on activated CD4 and CD8 T cells. This interaction negatively regulates T cell activity by recruiting the phosphatase, SHP2, to surface micro-aggregates (Yokosuka et al., 2012). Furthermore, it was recently demonstrated that SHP2 preferentially dephosphorylates CD28, within T cell receptor-CD28 signaling clusters (Hui et al., 2017). Given that PKC\(\theta\) functions downstream of CD28 engagement, one means by which iPSC-MSCs may downregulate pPKC\(\theta\) activity is through diminished CD28 signaling (Huang et al., 2002).

The immune modulator, IDO, also suppresses effector T cell functions, acting as the rate-limiting enzyme in the tryptophan catabolism pathway. IDO upregulation can suppress local tissue destruction during active GvHD (Jasperson et al., 2008). Mechanistically, limiting tryptophan availability has been shown to negatively regulate the AKT-mTOR pathway, as well as PKC\(\theta\) phosphorylation (Metz et al., 2012). Germinal center kinase-like kinase (GLK), an amino acid-sensing molecule, phosphorylates PKC\(\theta\) on threonine residue 538 following stimulation through the T cell receptor (Chuang et al., 2011). In the absence of tryptophan, its kinase activity is limited, potentially impacting PKC\(\theta\) phosphorylation. We noted robust expression of IDO by unlicensed iPSC-MSCs. This was further increased following exposure to IFN\(\gamma\) and co-culturing PBMCs with licensed iPSC-MSCs reduced PKC\(\theta\) phosphorylation. Although numerous reports have correlated high IDO expression with potent MSC-mediated immune suppression, at least one report suggests that immune suppression can proceed in
an IDO-independent manner, through PD-L1-PD1 signaling (Chinnadurai et al., 2014). Therefore, a distinct, or perhaps redundant, means by which licensed iPSC-MSCs may act to suppress T cells is through IDO-mediated regulation of tryptophan availability.

The benefits of using MSCs as a therapeutic modality are being explored for a variety of conditions, including end stage chronic heart disease, ischemic stroke, acute myocardial infarction, kidney failure, and critical limb ischemia (Butler et al., 2017; Toyoshima et al., 2017; Kanelidis et al., 2017; Urt-Filho et al., 2016; Liew and O’Brien, 2012). Developing an efficacious MSC product to treat GvHD has been an important industry focus for several years. Products such as MSC-100-IVTM (Mesoblast, LLC), its Japanese-approved counterpart, Temcell® (JCR Pharmaceutical Co.), and Multistem® (Athersys, Inc.) are being evaluated in clinical trials (Locatelli et al., 2017; Maziarz et al., 2015). Common denominators for these products are that adult MSCs are collected from donors, pooled and expanded in culture, then cryopreserved until use. Cymerus iPSC-MSCs are unique in that they are derived from mesenchymoangioblasts, which are in turn derived from iPSCs. In addition to the fact that iPSCs can undergo nearly indefinite ex vivo expansion without losing pluripotency, mesenchymoangioblasts and their progeny also have enormous ex vivo expansion potential: cultures derived from a single mesenchymoangioblast have been shown to accumulate up to $10^{22}$ MSCs in total (Vodyanik et al., 2010). Nonetheless, by primarily relying on expansion at the iPSC-level, extensive expansion of Cymerus™ iPSC-MSCs, post-differentiation, can be avoided. This approach facilitates the production of large numbers of Cymerus™ iPSC-MSCs from the same starting material, apparently without acquiring functional senescence or diminished immunosuppressive capacity in vivo. A thorough
characterization of these iPSC-MSCs reveals they mitigate signaling through the PKC0 pathway, and attenuate GvHD progression. Finally, having the ability to monitor patient response to iPSC-MSCs administration in real time, using a straight-forward, minimally-invasive flow cytometric analysis of pro-inflammatory markers expressed by circulating PBMCs, provides clinicians with valuable feedback upon which to make informed treatment decisions. Altogether, our data strongly support the use of Cymerus iPSC-MSCs as a treatment for GvHD, and its clinical efficacy is currently being studied in the context of a small, first-in-human trial for the treatment of steroid refractory GvHD.
Figure 6.1 iPSC-derived MSCs phenotypically resemble native MSCs and respond to IFNγ licensing. iPSC-MSCs were plated as described; IFNγ was added to some cells. 24 or 48 hours later, cells were harvested and stained with antibodies specific for (a) CD44, (b) CD73, (c) CD90, (d) CD105, (e) CD45, (f) CD34, (g) HLA-DR, (h) CD80, and (i) CD86. Data were acquired on a BD LSR Fortessa Flow Cytometer and analyzed using FlowJo software. Data are the mean + SEM of three independent experiments. *P < 0.05; unpaired student’s t test.
Figure 6.2 iPSC-derived MSCs exhibit tri-lineage differentiation capacity and reduced senescence \textit{in vitro}. iPSC-MSCs were seeded onto fibronectin- and collagen-coated chamber slides and grown in media to promote specific (a) adipocyte, (b) chondrocyte, or (c) osteocyte differentiation, following manufacturer’s protocols. hTERT-MSCs were grown under identical conditions as controls. Image represent results of three individual replicates. In separate experiments, iPSC-MSCs were seeded into wells of chamber slides. The percent of cells expressing (d) β-galactosidase activity 0, 24, and 48 hours, and 7 days after thawing was determined by staining with x-gal following the manufacturer’s protocol. Data are mean ± SEM of three independent experiments. *P<0.05; unpaired student’s \( t \) test.
Figure 6.3 iPSC-derived MSCs phenotypically resemble native MSCs, respond to IFNγ licensing, and dampen PBMC activation potential. iPSC-MSCs were plated as described; IFNγ was added to some cells. 24 or 48 hours later, cells were harvested and stained with antibodies specific for (a) PD-L1 or (b) IDO. Cells were permeabilized prior to IDO staining. Data were acquired on a BD LSR Fortessa Flow Cytometer and analyzed using FlowJo software. For (c) IDO gene expression, cells were harvested after 24 or 48 hours of culture with or without IFNγ. Total RNA was reverse transcribed, and IDO expression determined by quantitative real-time PCR using specific forward and reverse primers. For co-culture experiments, stimulated PBMCs were added to iPSC-MSCs and cultured an additional 24, 48, or 72 hours. PBMCs were harvested and stained with antibodies specific for (d) T-BET. Percent positive cells and amount of protein expressed, indicated by median fluorescence intensity (MFI), was determined using flow cytometry. For some cultures, golgi plug was added during the last 6 hours and (e) IFNγ levels were determined by intracellular staining and flow cytometric analysis. iPSC-MSCs were cultured without or with IFNγ for 48 hours. Stimulated PBMCs were added to iPSC-MSCs and cultured an additional 96 hours. Expression of (f) pPKCθ was determined by immunoblotting. Loading control was β-actin. Data are the mean ± SEM of three independent experiments or, for immunoblotting, are representative of two independent replicates that showed similar results. *P < 0.05; unpaired student’s t test.
Figure 6.4 PBMCs co-cultured with iPSC-MSCs show reduced proliferation and differentiation potential. iPSC-MSCs were plated as described; IFNγ was added to some cells. Stimulated human PBMCs were added to iPSC-MSCs and cultured an additional 24, 48, or 72 hours. PBMCs were harvested and stained with antibodies specific for (a) CD25 or (b) NOTCH1. Percent positive cells and amount of protein expressed, indicated by median fluorescence intensity (MFI), was determined using flow cytometry. Data are the mean ± SEM of three independent experiments. *P < 0.05; unpaired student’s t test.
Figure 6.5 iPSC-MSCs attenuate disease severity and provide a survival benefit in a pre-clinical model of GvHD. Schematic of humanized model of GvHD showing induction, treatment regimens, sample collection, and survival study.
Figure 6.6 iPSC-MSC administration, in vivo, reduces cytokine production and weight loss in mice with GvHD. The percent of (a) human CD45 (b) human CD4 and (c) human CD8 positive PBMCs, was determined by flow cytometric analysis of peripheral blood samples from single- (n=8) and dual-dose control (n=8), GvHD control (n=12), single- (n=12) and dual-dose-treated mice (n=12) harvested on day +19 after disease induction. (d) Circulating IFNγ was measured for cohorts of mice described in (a), using standard ELISA techniques. (e) The percent weight change for cohorts of mice described in (a) were also determined. Data are the mean ± SEM. *P≤0.05, **P<0.01, ***P<0.001; unpaired student’s t test.
Figure 6.7 iPSC-MSC administration reduces BM-infiltration and expression of proinflammatory molecules in mice with GvHD. The percent of (a) total human PBMCs, (b) human CD4 and (c) human CD8 T cells infiltrating the BM was determined by flow cytometric analysis of BM samples from single- (n=8) and dual-dose control (n=8), GvHD control (n=12), single- (n=12) and dual-dose-treated mice (n=12) harvested on day +19 after disease induction. (d) Total BM cellularity was assessed for each of the cohorts of mice described. Flow cytometry was used to determine expression of (e, f) CD25, (g, h) NOTCH1, (i, j) T-BET, and (k, l) pPKCθ for populations of human CD4 (e, g, i, k respectively) and human CD8 T cells (f, h, j, l respectively) retrieved from the BM of untreated and treated mice with GvHD. Data are the mean + SEM. *P≤0.05, **P<0.01, ***P<0.001; unpaired student’s t test.
Figure 6.8 iPSC-MSC administration reduces immune cell infiltration to target organs and expression of proinflammatory molecules. The percent of (a) total human PBMCs, (b) human CD4, and (c) human CD8 T cells infiltrating the spleen was determined by flow cytometric analysis of spleen samples from single-(n=8) and dual-dose control (n=8), GvHD control (n=12), single-(n=12) and dual-dose-treated mice (n=12) harvested on day +19 after disease induction. Data represent the mean ± SEM. *P≤0.05, **P<0.01, ***P<0.001; unpaired student’s t test.
Figure 6.9 Clinical scoring and treatment cohorts. (a) Criteria for determining clinical score of symptoms observed in a humanized model of GvHD. (b) Treatment cohorts and dosing strategies used to evaluate iPSC-MSCs as a treatment option utilizing a humanized model of GvHD.
Figure 6.10 iPSC-MSCs attenuate GvHD severity and prolong survival in mice. NOD.Cg-Prkdc<sup>scid</sup> I2rg<sup>tm1Wjl</sup>/SzJ (NSG) mice were conditioned with 2Gy of $\gamma$-irradiation from a $^{157}$Cs source. Control mice received only $\gamma$-irradiation (n=8), a single (n=8), or a dual dose of iPSC-MSCs (n=8) without GvHD induction. Four hours after irradiation, GvHD was induced and mice were stratified into GvHD control, single- and dual-dose groups. The GvHD control group received no treatment (n=12). For single-dose treatment (n=12), mice received 2x10<sup>6</sup> freshly thawed iPSC-MSCs on day +14 after induction; for dual-dose treatment (n=12), mice received 2x10<sup>6</sup> freshly-thawed iPSC-MSCs on day +14 and on day +18 after induction. Clinical scores were generated based on standard criteria and mice were removed from study when they reached a total score of “8”. (a) Cumulative scores of mice in all treatment groups for the first 26 days of the study are shown. (b) Survival of mice with GvHD left untreated or treated were followed until they reached a score of “8” and were removed from study. Significant differences between clinical scores were determined using two-way ANOVA and followed by Bonferroni post-test. Survival benefit of iPSC-MSC treatments were determined using Kaplan–Meier analysis with an applied log-rank test. *P≤0.05, ****P<0.0001.
Figure 6.11 iPSC-MSC-treatment alters cellular localization of pPKCδ in BM-infiltrating T cells. BM samples were collected on day +19 and single cell suspensions were prepared. Cells were surface-stained for CD4 and CD8 expression with fluorescently-conjugated antibodies, then fixed, permeabilized and stained intracellularly with fluorescently-conjugated antibodies specific for (a, b) NOTCH1 or (c, d) T-BET. Nuclei were stained using the cell-permeable DRAQ5™ Fluorescent Probe. The cells were visualized and quantified using an ImageStream® X Mark II Imaging Flow Cytometer. Subcellular localization of NOTCH1 or T-BET was determined using the Nuclear Localization Wizard and the IDEAS® Software following masking of nuclear and non-nuclear regions. Images are representative of analysis of 12 mice for each cohort analyzed. Data represent the mean ± SEM. *P<0.05, **P<0.01, ***P<0.001; unpaired student’s t test.
Figure 6.12 iPSC-MSC-treatment alters subcellular localization of pPKCθ in BM-infiltrating T cells. BM samples were collected on day +19 and single cell suspensions prepared. Cells were surface-stained for CD4 and CD8 expression with fluorescently-conjugated antibodies, fixed, permeabilized and stained intracellularly with fluorescently-conjugated antibodies specific for pPKCθ (Thr538). Nuclei were stained using cell-permeable DRAQ5™ fluorescent probe. Cells were visualized, nuclear pPKCθ was quantified, and nuclear similarity score was determined using an ImageStream®X Mark II Imaging Flow Cytometer for BM-infiltrating (a-c) CD4 and (d-f) CD8 T cells. Subcellular localization of pPKCθ was determined using the Nuclear Localization Wizard and IDEAS® Software following masking of nuclear and non-nuclear regions. Images are representative of 12 mice analyzed for each cohort. Data are the mean ± SEM. *P≤0.05, **P<0.01, ***P<0.001; unpaired student’s t test.
Figure 6.13 Pro-inflammatory molecules expressed by circulating PBMCs correlate with therapeutic response to iPSC-MSC administration. Peripheral blood samples from GvHD control (n=12), single- (n=12) and dual-dose-treated (n=12) mice were harvested on day +19 after disease induction. Flow cytometry was used to determine expression of (a, b) CD25, (c, d) NOTCH1, (e, f) T-BET, and (g, h) pPKCθ was determined for populations of human CD4 (a, c, e, g, respectively) and human CD8 T cells (b, d, f, h, respectively) in the circulation of untreated and treated mice with GvHD. Data are the mean + SEM. *P≤0.05, **P<0.01, ***P<0.001; unpaired student’s t test.
Table 6.1 Immunopotency values of iPSC-MSCs

<table>
<thead>
<tr>
<th>Sample</th>
<th>Normalized IPAv</th>
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</thead>
<tbody>
<tr>
<td>BM-MSC reference standard</td>
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</tr>
<tr>
<td>CYP-001 Lot# CYN-iPSC-MSC-P5B-FP-001</td>
<td>1.12</td>
</tr>
<tr>
<td>CYP-001 Lot# CYN-iPSC-MSC-P5B-FP-002</td>
<td>1.41</td>
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A normalized Immunopotency Assay value was determined for the iPSC-MSC lots used in this study, as described in 6.2.6 in vitro immunopotency assay section.
In this study, we investigated the molecular mechanisms driven by PKCθ in alloreactive T cells that induce acute GvHD. We established all-murine GvHD mouse model (recapitulating the pathogenesis of aplastic anemia) and humanized GvHD mouse model (recapitulating the disease symptoms in humans) to assess preclinical efficacy of PKCθ inhibition by three modes of immune-targeted therapeutics: small molecule immunotherapeutic (Rottlerin), therapeutic antibody (cell-penetrating αpPKCθ), and cell-based therapies (super-suppressive iTreg+αpPKCθ cells, Cymerus™ iPSC-MSCs). We demonstrated that specific targeting of PKCθ function by using three modes of immune-targeted therapeutics provides therapeutic benefit for the immunopathogenesis of GvHD.

We first studied the preclinical disease model of aplastic anemia (AA), which is a rare bone marrow failure disease driven by aberrant T helper type-1 (Th1) responses that destroy bone marrow stem and progenitor cells. We showed previously NOTCH1 signaling drives AA pathogenesis by regulating the Th1-associated-transcription factor, T-BET, and the proinflammatory cytokine, IFN-γ. Here we demonstrated T cells from humans and mice with AA have elevated levels of phosphorylated PKCθ, a T cell-specific kinase. Moreover, elevated pPKCθ in peripheral blood mononuclear cells collected from treatment-naïve AA patients responded to the dose-dependent PKCθ inhibitor, rottlerin, by reducing both NOTCH1IC expression and IFN-γ production.
In addition, PKCθ-deficient (PKCθ−/−) splenocytes failed to induce AA in a mouse model and, using add-back experiments, we show CD8+ T cells, specifically, require PKCθ to mediate disease. Genetically deleting PKCθ or chemically inhibiting its phosphorylation reduced NOTCH1IC and IFN-γ in stimulated T cells in the mouse model.

Figure 7.1 Models for the role of PKCθ in the context of regulating ifng mRNA stability in activated CD8 T cells
Therapeutic targeting of PKCθ during the peak of disease significantly prolonged survival of AA mice. We found that PKCθ in CD8+ T cells interacted with NOTCH1IC and together, they have been found to form RNA regulatory complexes with RNA binding proteins, hnRNPU and PCMT1. Genetic deletion or chemical inhibition of PKCθ reduced *ifng* 3’UTR stability and prevented the interaction of RNA binding proteins and *ifng* mRNA suggesting the inability of *ifng* mRNA export and inefficient translation. Collectively, these data suggested that PKCθ collaborates with NOTCH1IC to contribute to bone marrow failure pathogenesis through *ifng* mRNA stability and export and thus, identify PKCθ as a potential therapeutic target in treating aplastic anemia (Figure 7.1).

Targeting cellular proteins with antibodies, to better understand cellular signaling pathways in the context of disease modulation, is a fast-growing area of investigation. Humanized antibodies are increasingly gaining attention for their therapeutic potential, but the collection of cellular targets is limited to those secreted from cells or expressed on the cell surface. This approach leaves a wealth of intracellular proteins unexplored as putative targets for antibody binding. Protein kinase C-theta (PKCθ) is essential to T cell activation, proliferation, and differentiation, and its phosphorylation at specific residues is required for its activity. We reported on the design, synthesis, and characterization of a cell-penetrating peptide mimic (also called as PTDM in Chapter 3) capable of efficiently delivering an antibody against phosphorylated PKCθ (Thr538) into hPBMCs and altering expression of downstream indicators of T cell activation and differentiation. We used a humanized, lymphocyte transfer model of graft-versus-host disease, to evaluate the durability of PTDM: Anti-pPKCθ modulation, when delivered into hPBMCs *ex vivo*. We demonstrated that PTDM: Antibody complexes can be readily introduced with high
efficacy into hard-to-transfect human PBMCs, eliciting a biological response sufficient to alter disease progression. Thus, PTDM: Antibody delivery may represent an efficient ex vivo approach to manipulating cellular responses by targeting intracellular proteins.

Regulatory T cells (Tregs) are the immune suppressor cells which play crucial roles in immune mediated diseases. They are currently used in clinical trials to treat immune-mediated diseases and seem to be beneficial as cell-based therapy. PKCtheta (PKCθ) is shown to negatively regulate the Treg function suggesting the inhibition of PKCθ could be beneficial to augment immunosuppression by Tregs. However, it has not been clearly shown how targeting this key enzyme would affect Treg function. Previously, we showed that we could intracellularly deliver antibodies into primary immune cells via cell-penetrating peptide mimics to target intracellular molecules with high specificity. Therefore, we delivered a neutralizing PKCθ phosphorylation antibody (αPKCθ-Thr538) into human CD4+ T cells before differentiating them into iTregs. We demonstrated that targeting PKCθ via a cell-penetrating antibody (intracellularly delivered αPKCθ-Thr538 via cell-penetrating peptide mimics) inhibited its action and significantly increased the suppression ability of iTregs in vitro. Also, these super-suppressive iTregs could still be detected 17 days after their administration into mice and appeared to be highly efficacious in preventing graft-versus-host disease (GvHD) in vivo. Based on the characterization of super-suppressive iTregs via immunophenotyping, we found out that these cells expressed high levels of FOXP3, co-inhibitory molecules such as LAG-3 and PD-1 and, more interestingly, elevated levels of IFNγ. Our results indicate that highly specific inhibition of PKCθ function via cell-penetrating antibody generates
super-suppressive FOXP3^{hi}PD-1^{hi}IFNγ^{hi}iTregs that are long-lasting and highly efficacious in preventing GvHD and they are promising in effective cell-based therapy in clinic.

Protein kinase C (PKC) family members have been shown to play a pivotal role in alternative splicing and RNA processing in many cell types. T cell signaling along with cytokine-induced signals differentially regulate RNA processing to determine helper versus regulatory T cell (Treg) differentiation. PKCθ, a biochemical regulator of TCR downstream signaling, was found to phosphorylate splicing factors and affect post-transcriptional control of T cell gene expression program. Recently, we reported that delivering a cell-penetrating pPKCθ antibody prior to Treg differentiation in vitro augments their suppressive activity and stability as well as reprogramming their transcriptional signature. Here, we explored the fine-tuning of Treg differentiation program via PKCθ signaling as a means of differential RNA processing. We identified that PKCθ critically modulates two key RNA regulatory factors, hnRNPL and PCMT1, thereby switching post-transcriptional organization. More interestingly, we demonstrated that PCMT1 acts as a Treg instability factor by methylating FOXP3 promoter. Targeting PCMT1 via a cell-penetrating antibody revealed a novel, attractive way to modulate RNA processing in the context of stable Treg function (Figure 7.2).
The immune-mediated tissue destruction of graft-vs-host disease (GvHD) remains a major barrier to greater use of hematopoietic stem cell transplantation (HSCT).
Mesenchymal stem cells (MSCs) have intrinsic immunosuppressive qualities and are being actively investigated as a therapeutic strategy for treating GvHD. We characterized Cymerus™ MSCs, which are derived from adult, induced pluripotent stem cells (iPSCs), and show they display surface markers and tri-lineage differentiation consistent with MSCs isolated from bone marrow (BM). Administering iPSC-MSCs altered phosphorylation and cellular localization of the T cell-specific kinase, Protein Kinase C-theta (PKCθ), attenuated disease severity, and prolonged survival in a humanized mouse model of GvHD. Finally, we evaluated a constellation of pro-inflammatory molecules on circulating PBMCs that correlated closely with disease progression and which may serve as biomarkers to monitor therapeutic response. Altogether, our data suggest Cymerus iPSC-MSCs offer the potential for an off-the-shelf, cell-based therapy to treat GvHD.

In conclusion, this study has a significant impact on immunomodulation of T cells for the purpose of manipulating and intervening GvHD (and other immune-mediated disorders) as well as in the research field to further elucidate the novel molecular interactions of PKCθ in T cell signaling. The knowledge gathered from this study will unravel novel molecular mechanisms driven by PKCθ in the context of T cell activation. The results of this study will ultimately expand therapeutic options for various pathologies that involve PKCθ signaling.


