IMMUNOREGULATORY EFFECTS
OF VITAMIN D₃ ON MAST CELLS
DURING IMMUNOGLOBULIN E-
DEPENDENT IMMUNE RESPONSES

A thesis submitted in partial fulfilment of the PhD degree

in

School of Molecular & Biomedical Science
Faculty of Sciences
The University of Adelaide

by

Chunping (Anastasia) Yu

April 2014
# Table of contents

**IMMUNOREGULATORY EFFECTS OF VITAMIN D3 ON MAST CELLS DURING IMMUNOGLOBULIN E-DEPENDENT IMMUNE RESPONSES** ........................................ 1
Table of contents .............................................................. 2
Declaration ........................................................................... 8
Abbreviations ....................................................................... 10
Acknowledgments ................................................................. 14
Thesis summary ..................................................................... 15
CHAPTER 1 ............................................................................. 17
LITERATURE REVIEW ............................................................. 17

1.1. Mast Cells (MCs) ........................................................... 18

1.2. Basic Biology of MCs ....................................................... 18
   1.2.1. MC Ontogeny and Tissue Distribution ................. 18
   1.2.2. Homing of MCPs .................................................. 19
   1.2.3. Trafficking of Mature MCs ................................... 20
   1.2.4. MC Heterogeneity ............................................... 22
   1.2.5 MC Development and Maturation ....................... 23
   1.2.6. MC Activation .................................................... 24
   1.2.7. MC-derived Mediators ........................................ 25
   1.2.8. FceRI Signalling in MCs ....................................... 26

1.3. Biological Functions of MCs in IgE-dependent Allergic Reactions .......... 28
   1.3.1. IgE-dependent Allergic Reactions and MCs ........... 28
   1.3.2. Functions of MCs during Early-phase Allergic Reactions ... 29
   1.3.3. Functions of MCs during Late-phase Allergic Reactions ... 30
   1.3.4. Functions of MCs during Chronic Allergic Reactions ...... 31
   1.3.5. Treatment Strategies for Allergic Reactions and MCs ...... 33

1.4. Other Biological Functions of MCs ..................................... 34
   1.4.1. Roles of MCs in Homeostasis ............................... 34
   1.4.2. Roles of MCs in Parasitic Infections .................... 35
   1.4.3. Roles of MCs in Bacterial and Viral Infections ........ 36
   1.4.4. Roles of MCs in Cancers ..................................... 37
   1.4.5. Roles of MCs in Chronic Obstructive Pulmonary Diseases (COPD) .. 38
2.1.14. In Vivo Experiment Reagents ................................................................. 69

2.2. Solutions and Buffers ......................................................................................... 69

2.2.1. Tissue Culture Media and Solutions ............................................................. 69

2.2.2. Buffers and Solutions for SPE-7 Purification ............................................. 73

2.2.3. Buffers and Solutions for β-Hexosaminidase Release Assay .................... 75

2.2.4. ELISA Buffers and Solutions ......................................................................... 76

2.2.5. Flow Cytometry Buffers and Solutions ........................................................... 77

2.2.6. Histology/Immunohistology Solutions .......................................................... 77

2.2.7. Buffers and Solutions for SDS-PAGE & Western Blotting ......................... 79

2.2.8. Molecular Biology Buffers and Solutions ...................................................... 82

2.2.9. Buffers and Solutions for In Vivo Experiments ............................................ 83

2.3. Purification of Mouse α-DNP IgE (SPE-7 clone) ................................................. 84

2.3.1. Conjugation of DNP and BSA ...................................................................... 84

2.3.2. Coupling of DNP-BSA to HiTrap NHS-activated HP column ...................... 85

2.3.3. De-activation of Excess Active Groups ......................................................... 86

2.3.4. Purification of SPE-7 from Hybridoma Culture Supernatant ....................... 86

2.4. Mice .................................................................................................................. 87

2.5. General Methods ............................................................................................... 88

2.5.1. Culturing of mBMCMCs .............................................................................. 88

2.5.2. Culturing of hCBMCs .................................................................................. 88

2.5.3. Culturing of WEHI-3 hybridoma ................................................................. 89

2.5.4. Culturing of SPE-7 hybridoma ..................................................................... 89

2.5.2. Flow Cytometry ............................................................................................ 90

2.5.2.1. Surface Ag Labelling .................................................................................. 90

2.5.2.2. Intracellular Ag Labelling .......................................................................... 90

2.5.3. In Vitro Procedures ....................................................................................... 91

2.5.3.1. β-hexosaminidase Release Assay ............................................................... 91

2.5.3.2. Histamine Release Assay .......................................................................... 92

2.5.3.3. IgE + sAg Stimulation of mBMCMCs ......................................................... 93

2.5.3.4. IgE-mediated stimulation of hCBMCs ....................................................... 94

2.5.3.5. Cytokine Measurement by ELISA ............................................................. 94

2.5.3.6. VitD₃ Treatment (without stimulation) ....................................................... 95
2.5.3.7. Measurement of 1α,25(OH)2D3 ................................................................. 95
2.5.4. Molecular Biology .................................................................................... 96
  2.5.4.1. Genotyping of WT and VDR−/− Mice .................................................. 96
  2.5.4.2. RNA Extraction .................................................................................. 97
  2.5.4.3. Complementary DNA (cDNA) Synthesis ........................................ 98
  2.5.4.4. qRT-PCR .......................................................................................... 98
2.5.5. SDS-PAGE and Western Blot Analysis ................................................... 99
  2.5.5.1. Preparation of Total Cell Lysates ....................................................... 99
  2.5.5.2. SDS-PAGE ....................................................................................... 100
  2.5.5.3. Protein Transfer and Western Blot Analysis ........................................ 100
2.5.6. In Vivo and Related Procedures ............................................................. 101
  2.5.6.1. Engraftment of mBMCMCs ............................................................... 101
  2.5.6.2. PCA with Topical Application of 1α,25(OH)2D3 or 25OHD3 ............... 101
  2.5.6.3. Ear Tissue Histology and Numeration of Tissue MCs ....................... 102
  2.5.6.4. Ear Tissue Lysate Preparation and Analysis ...................................... 102
  2.5.6.5. Total RNA Extraction from Ear Tissues ............................................ 103
  2.5.6.6. Serum Preparation and Analysis ....................................................... 103
2.5.7. Histochemistry and Immunohistochemistry ......................................... 103
  2.5.7.1. Cytospin Slide Preparation ............................................................... 103
  2.5.7.2. May-Grünwald Giemsa Staining ....................................................... 104
  2.5.7.3. Kimura Staining of hCBMCs ........................................................... 104
  2.5.7.4. Toluidine Blue Staining ................................................................. 104
  2.5.7.5. Immunohistochemistry ................................................................. 104
2.5.8. Centrifugation ......................................................................................... 105
2.5.9. Statistical Analyses ................................................................................ 105
CHAPTER 3 .............................................................................................................. 107
1α,25(OH)2D3 POTENTIATES THE NEGATIVE IMMUNOREGULATORY PROPERTIES OF MAST
CELLS DURING IgE+Ag-MEDIATED ACTIVATION IN VITRO ........................................ 107
3.1. Introduction .................................................................................................. 108
3.2. Results ......................................................................................................... 110
  3.2.1. WT and VDR−/− mBMCMCs exhibit similar phenotypical and functional characteristics .......................................................... 110
3.2.2. 1α,25(OH)2D3 does not affect the efficiency of IgE preload, the expression of surface molecules, and survival of mBMCMCs................................................................................. 111

3.2.3. 1α,25(OH)2D3 reduces IgE + sAg-mediated mBMCMC degranulation in a VDR-dependent manner .............................................................................................................. 112

3.2.4. 1α,25(OH)2D3 VDR-dependently down-regulates the de-novo synthesis and secretion of pro-inflammatory cytokines but up-regulates that of the anti-inflammatory IL-10, from IgE + sAg-activated mBMCMCs ............................................................................................ 114

3.2.5. Curcumin reduces IgE + sAg-mediated cytokine production by mBMCMCs in a VDR- and non-genomic VitD3 pathway-dependent manner ...................................................................... 115

3.3. Discussion ......................................................................................................................... 116

CHAPTER 4 .............................................................................................................................. 123

MAST CELLS CAN CONVERT 25OHD3 TO 1α,25(OH)2D3 AND THUS ENABLE 25OHD3 TO IMMUNOSUPPRESS IgE + sAg-ACTIVATED MAST CELLS IN VITRO........................................... 123

4.1. Introduction ...................................................................................................................... 124

4.2. Results ............................................................................................................................... 126

4.2.1. Mouse BMCMCs constitutively express CYP27B1 in their cytosol ............................ 126

4.2.2. 25OHD3 up-regulates CYP27B1 expression in mBMCMCs in a VDR-dependent manner ............................................................................................................................................. 126

4.2.3. 25OHD3 dose-dependently induces endogenous synthesis of 1α,25(OH)2D3 by mBMCMCs ................................................................................................................................................. 127

4.2.4. 25OHD3 treatment reduces IgE + sAg-mediated degranulation and pro-inflammatory cytokine production of mBMCMCs in a dose- and VDR-dependent manner .......................................................... 128

4.3. Discussion ......................................................................................................................... 129

CHAPTER 5 .............................................................................................................................. 134

TOPICALLY APPLIED 1α,25(OH)2D3 AND 25OHD3 CAN REDUCE THE CUTANEOUS PATHOLOGY ASSOCIATED WITH IgE-MEDIATED PASSIVE CUTANEOUS ANAPHYLAXIS IN A MAST CELL VITAMIN D RECEPTOR-DEPENDENT MANNER IN VIVO .............................................................. 134

5.1. Introduction ...................................................................................................................... 135

5.2. Results ............................................................................................................................... 138

5.2.1. Topical 1α,25(OH)2D3 application alone does not affect ear thickness of WT mice 138

5.2.2. Topical 1α,25(OH)2D3 application at the site of inflammation can suppress PCA-associated ear swelling ................................................................................................................. 138

5.2.3. Topical 1α,25(OH)2D3 application reduced the extent of MC degranulation and the expression of various MC-derived mediators associated with PCA in vivo 139
5.2.4. VDR expression is required for the suppressive effect of 1α,25(OH)₂D₃ on PCA-associated cutaneous pathology .......................................................... 141

5.2.5. The PCA-suppressing effect of topical 1α,25(OH)₂D₃ treatment requires specifically VDR expression by cutaneous MCs ......................................................... 142

5.2.6. Topical 25OHD₃ can suppress PCA-associated ear swelling via similar mechanisms to 1α,25(OH)₂D₃ ....................................................................................... 145

5.2.7. The suppressive effect of topical 25OHD₃ on PCA-associated cutaneous pathology also requires MC expression of VDR ................................................................. 147

5.3. Discussion .................................................................................................................. 148

CHAPTER 6 .................................................................................................................. 155
FROM THE MOUSE TO THE HUMAN – TRANSLATIONAL ASPECTS .................................. 155
6.1. Introduction ............................................................................................................... 156
6.2. Results ....................................................................................................................... 158

6.2.1. 1α,25(OH)₂D₃ reduces IgE + α-IgE-mediated TNFα but not IL-10 production by hCBMCs .................................................................................................................... 158

6.3.2. Human CBMCs constitutively express CYP27B1, which can be up-regulated by 25OHD₃ treatment ........................................................................................................ 159

6.3.3. Human CBMCs produce endogenous 1α,25(OH)₂D₃ following 25OHD₃ treatment in a dose-dependent manner ................................................................. 159

6.3. Discussion .................................................................................................................. 160

CHAPTER 7 .................................................................................................................. 164
CONCLUDING REMARKS & FUTURE DIRECTIONS ............................................................. 164

References ..................................................................................................................... 171
Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to me (Chunping Yu) and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying according to the provisions of the Copyright Act 1968. I also give permission for the digital version of my thesis to be made available on the web, via the digital research repository of the University of Adelaide, the Library catalogue, the Australasian Digital Theses Program and also through web search engines, unless permission has been granted by the University to restrict access for any unforeseen reasons. Finally, I acknowledge that the copyright of the published works listed below resides with the copyright holder(s) of those works.

List of publications


Abbreviations

$1\alpha,25(\text{OH})_2\text{D}_3$: $1\alpha,25$-dihydroxyvitamin D$_3$

$25\text{OHD}_3$: 25-hydroxyvitamin D$_3$

6C: 6-s-cis

6T: 6-s-trans

Ab: antibody

ACD: anticoagulant citrate dextrose

Ag: antigen

APS: ammonium persulphate

ASM: airway smooth muscle

A1AT: $\alpha_1$-antitrypsin

BM: bone marrow

BSA: bovine serum albumin

BTK: Bruton’s tyrosine kinase

cAMP: cyclic AMP

CCL: CC-chemokine ligand

CHS: contact hypersensitivity

COPD: chronic obstructive pulmonary disease

CTMC: connective tissue mast cell

CYP24A1: 25-hydroxyvitamin D$_3$-24-hydroxylase

CYP27B1: 25-hydroxyvitamin D-1$\alpha$-hydroxylase enzyme

DAG: Diacylglycerol

DBP: VitD$_3$-binding protein

DC: dendritic cell

DMEM: Dulbecco’s modified eagle medium

DMSO: dimethyl sulphoxide
DNP: dinitrophenyl
DT: diphtheria toxin
ECL: enhanced chemiluminescence
EIA: enzyme immunoassay
ELISA: enzyme-linked immunosorbent assay
EMTU: epithelial-mesenchymal trophic unit
ERK: extracellular signal regulated kinase
FCS: fetal bovine serum
FceRI: high affinity IgE receptor
FDNB: 1-fluoro-2,4-dinitrobenzene
GAB: growth-factor-receptor-bound protein
GMP: granulocyte/macrophage progenitor
hCBMC: human cord-blood-derived MCs
HDC: histidine decarboxylase
HIV: human immunodeficiency virus
HMEM: Hank’s MEM
HRP: horse radish peroxidase
HS: horse serum
HSA: human serum albumin
HSC: haematopoietic stem cell
IFN: interferon
Ig: immunoglobulin
IL: interleukin
IMDM: Iscove’s modified Dulbecco’s medium
InsP₃: inositol-1,4,5-triphosphate
ITAM: immunoreceptor tyrosine-based activation motif
LN: lymph node
LT: leukotriene
MAPK: mitogen-activated protein kinase
mBMCMC: mouse bone marrow-derived cultured mast cell
MC: mast cell
MCP: mast cell progenitor
MC_Τ: tryptase positive mast cell
MC_ΤC: tryptase and chymase positive mast cell
MEK: MAPK kinase
miR: microRNA
MKP: mitogen-activated protein kinase phosphatase
MMC: mucosal mast cell
MMCP: mouse mast cell protease
MMP: matrix metalloproteinase
NEAA: non-essential amino acid
NF-κB: nuclear factor-κB
PBS: phosphate buffered saline
PCA: passive cutaneous anaphylaxis
PCR: polymerase change reaction
Pen/Strep: Penicillin/Streptomycin
PG: prostaglandin
PI3K: phosphatidylinositol-3-OH kinase
PKB (AKT): protein kinase B
PKC: protein kinase C
PL: phospholipase
PLA_2: phospholipase A_2
PLC_γ: phospholipase C_γ
PMA: phorbol-12 myristate-13 acetate
PtdIns(3,4,5)P₃: phosphatidylinositol-3,4,5-trisphosphate

PTH: parathyroid hormone

qRT-PCR: quantitative real-time PCR

RIA: radioimmunoassay

RT: room temperature

RT-PCR: real-time PCR

RXR: retinoid X receptor

sAg: specific antigen

SCF: stem cell factor

SDS-PAGE: sodium dodecyl sulfate - polyacrylamide gel electrophoresis

TCR: T cell receptor

TGF: transforming growth factor

Th: T helper

TLR: toll-like receptor

TNF: tumor necrosis factor

T_{reg}: T regulatory cell

TSLP: thymic stromal lymphopoietin

UV: ultraviolet

VDR: vitamin D receptor

VDRE: vitamin D response element

VDR_{mem}: plasma membrane-associated vitamin D receptor

VitD: vitamin D

WT: wild-type
Acknowledgments

First and foremost, I would like to thank my supervisors, Dr. Michele Grimbaldeston, Prof. Shaun McColl, and Prof. Angel Lopez, for their constant guidance and support throughout my PhD study, especially Michele for offering me such a stimulating and challenging project and for demonstrating to me personally what scientific research is really about. A big thank also goes to Shaun, for securing me a scholarship that allows me to begin my PhD study in the first place and for helping with reviewing my thesis towards the end.

I also want to thank everyone in the lab, Dr. Boris Federic, Lisa Biggs, Zhen Liu, Houng Taing and Renee Gilbey, as well as Jyotsna, Kiwi and Samantha in our neighbour lab. It was your friendship and unconditional encouragement that kept me sane and able to continuously moving forward. A special thank you must go to Boris, who has generously offered help with quite a few large-scale animal experiments. For everyone else in the Centre for Cancer Biology, I would like to express my appreciation for making me feel like a part of a big family.

Finally, I want to dedicate this thesis to my husband, Yang Lu, and all my family overseas, as without your deep understanding and unconditional love and support, I would not be able to stay focused and complete my experiments in a three-year time frame.
Thesis summary

Mast cells (MCs) can exert anti-inflammatory effects via production of interleukin (IL)-10 in a number of Immunoglobulin (Ig)E-independent immune responses. Recently, we reported that 1α,25-dihydroxyvitamin D₃ (1α,25(OH)₂D₃), the biologically active form of vitamin D₃ (VitD₃), can induce IL-10 production from mouse bone marrow-derived cultured MCs (mBMCMCs). For the current project, we further investigated if the well-recognised pro-inflammatory properties of MCs in IgE-dependent immune settings can be reduced upon 1α,25(OH)₂D₃ administration and, if so, which mechanisms are likely to be responsible. In the presence of 1α,25(OH)₂D₃, IgE + specific antigen (sAg)-stimulated mBMCMCs exhibited reduced degranulation, as well as decreased production of the pro-inflammatory cytokines, TNFα and IL-6, in a vitamin D receptor (VDR)-dependent manner. Concomitantly, 1α,25(OH)₂D₃ significantly up-regulated the production of IL-10. In addition, we demonstrated for the first time the expression of CYP27B1, the enzyme that generates 1α,25(OH)₂D₃ from its inactive precursor 25-hydroxyvitamin D₃ (25OHD₃) in both mBMCMCs and human cord-blood-derived MCs (hCBMCs). This enables mBMCMCs to produce endogenous 1α,25(OH)₂D₃ and thus granting 25OHD₃ similar VDR-dependent immunosuppressive effects to 1α,25(OH)₂D₃ on activated MCs either directly or indirectly.

By employing a mouse IgE-mediated MC-dependent passive cutaneous anaphylaxis (PCA) model as well as four mouse groups with different cutaneous MC profiles in the ears, including wild-type (WT) C57BL/6 mice, MC-deficient C57BL/6-Kitₖ-W-sh/W-sh mice and C57BL/6-Kitₖ-W-sh/W-sh mice engrafted with either WT or VDR-deficient (VDR⁻⁻)
mBMCMCs, we found that topical application of either $1\alpha,25(\text{OH})_2\text{D}_3$ or $25\text{OHD}_3$ significantly curtailed the magnitude of PCA-associated ear swelling, potentially by reducing the extent of MC degranulation and/or the secretion of various MC-derived cytokines. Notably, these PCA-suppressive effects required the presence of dermal MCs and their expression of VDR.

Taken together, data presented in this thesis provide evidence that $1\alpha,25(\text{OH})_2\text{D}_3$ and $25\text{OHD}_3$, the latter likely via its conversion to the active metabolite by MCs, can suppress IgE + sAg-mediated MC activation in a VDR-dependent manner both in vitro and in vivo. This suggests the therapeutic potential for various VitD$_3$ analogues to treat MC-dependent IgE-associated allergic disorders.