DELINEATING THE ROLE OF CALCINEURIN A ALPHA IN THE MAST CELL FCϵRI-MEDIATED IMMUNE RESPONSE

by

Edwin Leong

Submitted in partial fulfilment of the requirements

for the degree of Master of Science

at

Dalhousie University

Halifax, Nova Scotia

April 2018

Dedication Page

This is dedicated to my family, friends, and countless others who have supported me in my endeavours and believed in my aspirations.

TABLE OF CONTENTS

LIST OF TABLES viii

LIST OF FIGURES ix

ABSTRACT xi

LIST OF ABBREVIATIONS AND SYMBOLS USED..... xii

ACKNOWLEDGEMENTS xv

CHAPTER 1 – INTRODUCTION 1

1.1 Allergies 1

1.1.1 Allergic inflammation 1

1.1.2 Clinical epidemiology of allergy 1

1.1.3 Important cellular players in allergy 3

1.1.4 Phases in allergic inflammation 3

1.2 Mast cells and allergic inflammation 5

1.2.1 Mast cell characteristics 5

1.2.2 IgE – high affinity IgE receptor – (FcϵRI) mediated signaling pathway 7

1.2.3 Mast cell-secreted products 8

1.2.4 Drugs and pharmaceuticals 11

1.3 Mast cell models 14

1.4 Calcineurin 17

1.4.1 Characteristics of calcineurin 17

1.4.2 Calcineurin inhibitors as therapeutics 18

1.4.3 Calcineurin in FcϵRI-mediated immune responses 18

1.4.4 Calcineurin A – alpha isoform 19

1.5 Rationale, Objectives, Hypotheses 20

1.5.1 Rationale 20

1.5.2 Objectives 21

1.5.3 Hypotheses 21

CHAPTER 2 – MATERIALS AND METHODS 22

2.1 Animals 22

2.2 Cell isolation, cell culture, and activation 22

2.2.1 Extraction of cells from liver tissue 22

2.2.2 Mast cell monoculture 23

2.2.3 WEHI-3B cell line monoculture for IL-3 23

2.2.4 TIB-141 cell line monoculture for anti-DNP IgE 24

2.2.5 Activation of mast cells 24

2.3 Genotyping of cell cultures 25

2.3.1 DNA extraction from tissue 25

2.3.2 PCR amplification 26

2.3.3 Agarose gel electrophoresis and visualization 26

2.4 Toluidine Blue Staining 27

2.5 Flow cytometry 27

2.6 Intracellular calcium mobilization assay 28

2.7 Degranulation assay – Beta-hexosaminidase release 29

2.8 Real-time quantitative polymerase chain reaction (RT-qPCR) 30

2.8.1 RNA isolation from mast cells and cDNA synthesis 30

2.8.2 mRNA measurement using RT-qPCR 31

2.9 Enzyme-Linked ImmunoSorbent Assay (ELISA) …….. 32

2.10 Inflammatory protein array …….. 32

2.11 Gel electrophoresis and western blotting …….. 33

2.11.1 Collection of cell lysate and sample loading preparation …… 33

2.11.2 Western blotting …… 33

2.12 Electrophoretic mobility shift assay (EMSA) ……... 35

2.12.1 Collection of nuclear fraction, DNA probe labelling, and sample preparation 35

2.12.2 EMSA …… 36

2.13 Mast cell reconstitution into W^{sh} mice ……... 36

2.14 IgE-dependent passive cutaneous anaphylaxis (PCA) …… 37

2.15 IgE-dependent late phase cutaneous anaphylaxis (LPA) ...…….. 38

2.16 Statistical analyses …….. 38

CHAPTER 3 – RESULTS …….. 39

3.1 Genotyping of CnAα neonatal tissue and mast cell monocultures ……... 39

3.2 Morphological properties of CnAα wild type and deficient mast cells ……. 40

3.2.1 CnAα wild type and deficient MCs show similar morphology …… 40

3.2.2 CnAα wild type and deficient MCs display similar surface expression of CD117 and FcϵRI …… 41

3.2.3 CnAα wild type and deficient MCs display similar calcium mobilization …… 44

3.2.4: Basal granular beta-hexosaminidase concentrations are similar in CnAα wild type and deficient MCs…….. 45

3.2.5 Basal gene expression of mediators are similar in CnAα wild type and deficient MCs …….. 46

3.3 The role of CnAα in the early phase FcεRI-mediated degranulation in mast cells ……..... 47

3.3.1 CnAα deficient MCs display an impaired ability to secrete pre-formed mediators stored within granules ……. 47

3.3.2 CnAα wild type mice show an increase in vascular permeability upon local IgE-dependent MC activation (passive cutaneous anaphylaxis) ……. 49

3.3.3 Mast cell-deficient mice (W^{sh}) reconstituted with CnAα deficient MCs show diminished FcεRI-mediated vascular permeability after challenge … 50

3.4 The role of CnAα in the production and secretion of *de novo* synthesized cytokines and chemokines in the late phase FcεRI-mediated immune response ……... 52

3.4.1 CnAα deficient MCs release decreased amounts of cytokines TNF, IL-6, IL-13, and IL-4 upon FcεRI-mediated activation …… 52

3.4.2 CnAα deficient MCs selectively impair gene transcription of specific cytokines upon FcεRI-mediated activation …….. 53

3.4.3 CnAα deficient MCs demonstrate impaired release of specific chemokines upon IgE-dependent activation ……..... 55

3.4.4 Mast cell-deficient mice (W^{sh}) reconstituted with CnAα wild type and deficient MCs show similar FcεRI-mediated inflammatory responses (late phase cutaneous anaphylaxis reaction) ……... 56

3.5 CnAα and the effects on key signaling components within the FcεRI-mediated inflammatory pathway ……. 58

3.5.1 Activation of MAPK signaling pathway family members is not impaired in CnAα deficient MCs ……. 58

3.5.2 Phosphorylation of IκBα is impaired in CnAα deficient MCs in an IgE-dependent manner …… 60

3.5.3 NF-κB translocation into the nucleus is impaired in activated CnAα deficient MCs ……... 61

CHAPTER 4 – DISCUSSION …… 63

4.1 Results in the context of established literature ……... 63

4.1.1 CnAα and development of mast cells …… 64

4.1.2 Basal mediator production and FcεRI-mediated calcium signaling 65

4.1.3 Early phase response 67

4.1.4 Late phase response 68

4.1.5 CnAα and FcεRI-mediated signaling components 70

4.2 Limitations 72

4.3 Future directions 74

4.4 Concluding remarks 76

REFERENCES 79

LIST OF TABLES

Table 1: Mast cell mediators and functions 11

Table 2: Primer information for genotyping and RT-qPCR 26

Table 3: Flow cytometry antibody information 28

Table 4: Western blotting antibody information 34

Table 5: Compiled summary of flow cytometric data 43

Table 6: Cytokine protein and mRNA expression pattern 55

LIST OF FIGURES

Figure 1.1: Phases in the allergic immune response 5

Figure 1.2: Simplified categories of pharmaceutical targets 12

Figure 3.1: Genotyping results and primer alignment schema 39

Figure 3.2a: Mast cell toluidine blue staining 40

Figure 3.2b: Toluidine blue staining of CnAα wild type and deficient mast cells 41

Figure 3.3a: Flow cytometry plots for surface expression of c-Kit 42

Figure 3.3b: Flow cytometry plots for surface expression of FcεRI 43

Figure 3.4: Intracellular calcium flux of wild type and CnAα deficient mast cells 44

Figure 3.5: Basal level of pre-formed beta-hexosaminidase within granules 45

Figure 3.6: Basal gene expression of classical cytokines TNF, IL-6, IL-13, and IL-4 46

Figure 3.7a: CnAα deficient mast cells show significantly decreased degranulation of beta-hexosaminidase in the early phase 48

Figure 3.7b: Stained CnAα deficient mast cells show observable residual granulation after stimulation 48

Figure 3.8: CnAα wild type mice have increased vascular permeability in ear tissues challenged with allergen 49

Figure 3.9a: Mast cell-deficient mice ears reconstituted with CnAα deficient mast cells have significantly decreased vascular permeability compared to ears reconstituted with wild type mast cells 51

Figure 3.9b: Mast cell-deficient mice ears reconstituted with CnAα deficient mast cells have increased observable dye leakage compared to ears reconstituted with wild type mast cells 51

Figure 3.10: CnAα deficient mast cells have significantly decreased levels of cytokines in the supernatant after stimulation 53

Figure 3.11: CnAα deficient mast cells have significantly decreased mRNA levels of TNF and IL-4 after stimulation, but not IL-6 and IL-13 54

Figure 3.12: CnAα deficient mast cells have decreased levels of CCL1, CCL2, CCL3, CXCL5, and CCL9 in the supernatant after stimulation 56

Figure 3.13a: Mast cell-deficient mice ears reconstituted with CnAα wild type and deficient mast cells displayed similar late phase inflammation .. 57

Figure 3.13b: Mast cell-deficient mice hind paws reconstituted with CnAα wild type and deficient mast cells displayed similar late phase inflammation 57

Figure 3.14: CnAα wild type and deficient mast cells display similar MAPK protein activation following stimulation 59

Figure 3.15: CnAα deficient mast cells showed significantly decreased IκBα phosphorylation and degradation 60

Figure 3.16: CnAα deficient mast cells showed significantly decreased levels of NF-κB transcription factor in the nucleus 62

Figure 4.1: Condensed summary of findings at the level of the mast cell associated with CnAα deficiency 78

Abstract

Allergies are a health challenge resulting from excessive and inappropriate responses to innocuous antigens (allergens), and a key cellular player is the mast cell. Mast cell activation by allergen initiates a response through the FcεRI culminating in the release of multiple inflammatory mediators. Calcineurin inhibitors have been used to treat allergies; however, there is a lack of understanding of the contributions of calcineurin isoforms. Knowledge of calcineurin isoforms may reveal specific targets to treat allergy. Therefore FcεRI-mediated events were examined in mast cells from mice lacking calcineurin Aα. While appearing morphologically normal, the cells show decreased degranulation and release of cytokines and chemokines in vitro. Upon adoptive transfer into mice, the reduction in mediator release was detected in the early but not late phase response. The reduction in mediator release was associated with a reduction in the NF-κB signaling pathway. Thus, specifically blocking calcineurin Aα may reduce FcεRI-mediated allergy.

List of Abbreviations and Symbols Used

^{32}P	Phosphorus-32
AAAAI	The American Academy of Allergy, Asthma and Immunology
AAIA	Allergy/Asthma Information Association
Ag	Antigen
AP-1	Activator protein 1
APS	Ammonium persulfate
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
Bcl10	B cell lymphoma 10
bp	Base pair
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
CaM	Calmodulin
Carma1	Caspase recruitment domain-containing membrane-associated guanylate kinase protein -1
CBM	Carma1-Bcl10-Malt1
CCL	Chemokine ligand
CD	Cluster of differentiation
CDC	Centers for Disease Control and Prevention
cDNA	Complementary DNA
CnA	Calcineurin catalytic A subunit
CnAα	Calcineurin catalytic A, alpha isoform
CnAβ	Calcineurin catalytic A, beta isoform
CnAγ	Calcineurin catalytic A, gamma isoform
CnB	Calcineurin regulatory B subunit
CO_2	Carbon dioxide
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
C_t	Cycle threshold
CXCL	Chemokine (C-X-C motif) ligand
DCs	Dendritic cells
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DNFB	1-fluoro-2,4-dinitrobenzene
DNP	2,4-dinitrophenol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N,N'-tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMSA	Electromobility shift assay
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
FcεRI	High affinity IgE receptor

FITC	Fluorescein
FK506	Tacrolimus
F_{max}	Maximum fluorescence
F_{min}	Minimum fluorescence
H_2O	Water
H_2SO_4	Sulphuric acid
HBSS	Hank's balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HRP	Horseradish peroxidase
HZ	Heterozygous
IgE	Immunoglobulin E
IKK-β	Inhibitor of nuclear factor kappa-B kinase
IL	Interleukin
ITAMs	Immunoreceptor tyrosine-based activation motifs
IWK	Izaak Walton Killam
IκBα	NF-kappa-B inhibitor, alpha
JNK	c-Jun N-terminal kinase
kb	Kilo-base pair
KO	Knock-out
LMC	Liver-derived mast cell
LTB_4	Leukotriene B4
Malt1	Mucosa-associated lymphoid tissue lymphoma translocation protein 1
MAPK	Mitogen-activated protein kinase
MC	Mast cell
MC_C	Mast cell, containing chymase only
Mcpt5	Mast cell protease 5
MC_T	Mast cell, containing tryptase only
MC_{TC}	Mast cell, containing tryptase and chymase
MEM	Minimal Essential Media
MFI	Mean fluorescence intensity
mg	Milligram
mL	Millilitre
mM	Micromolar
mm	Millimetre
mRNA	Messenger RNA
mSCF	Murine stem cell factor
NaOH	Sodium hydroxide
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NKs	Natural killer cells
nm	Nanometre
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

p-NAG	Poly-N-acetyl glucosamine
Rcan1	Regulator of calcineurin 1
RIPA	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
RNase	Ribonuclease
RPMI	Roswell Park Memorial Institute
RT-qPCR	Real time quantitative polymerase chain reaction
SCF	Stem cell factor
SDS	Sodium dodecyl sulphate
SNAP-23	Synaptosomal-associated protein 23
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein
SPF	Specific pathogen free
TBE	Tris/Borate/EDTA
TE	Tris/EDTA
TEMED	Tetramethylethylenediamine
T_h	Helper T cells
TMB	3,3′,5,5′-Tetramethylbenzidine
T_{mem}	Memory T cells
TNF	Tumour necrosis factor
TNP	2, 4, 6-trinitrophenol
UV	Ultraviolet
WT	Wild type
xg	Multiple of standard acceleration due to gravity at Earth's surface
α	Alpha
β	Beta
γ	Gamma
µg	Microgram
µL	Microlitre
µm	Micrometre
µM	Micromolar

Acknowledgements

I would like to take this opportunity to thank all those that have helped me throughout the course of my journey towards achieving an MSc degree – your support and guidance were and continue to be invaluable.

I would like to thank my committee members Dr. Karen Bedard and Dr. Jean-François Légaré for their continuous support and input into my work throughout the course of my MSc degree. I would also like to give a big thank you to the administrative staff in the department of Pathology for their hard work and reminders to me of things that I would have forgotten to do had it not been for them.

Coming into the 8E Immunology Wing of the IWK Health Centre, I had only been in Halifax for about 2 weeks – you all welcomed me and showed me the way. I will not forget the contribution and support each of you made to help me get to where I am today. There were many individuals that have come and gone from the floor and from the Lin Lab, but you all have made an impact on my life; there were countless long-lasting memories in and out of the work environment that we have made. I would like to especially thank two individuals that I have had the pleasure to work with – Zheng Pang and Fang Liu. You have been my mentors, my support, and my friends. Without your patience and guidance, I would not have been able to succeed in my experiments and gain the knowledge I will keep with me moving forward.

To my family and friends back home and here in Halifax, words cannot express the gratitude I have for your constant support and motivational words to keep me running. As the first member of my family to move away from home and pursue a

graduate degree, there were so many uncertainties and worries but we made it pretty far, eh? I must give a sincere thank you to Chloe Wong for being a constant despite all the variables that I have encountered. Your presence and support – academically, socially, and emotionally – were monumental in my successes during the progress of my degree. Thank you, thank you, thank you.

Finally, I would like to give thanks to two very important people: Dr. Tong-Jun Lin and Dr. Andrew Stadnyk. Tong-Jun allowed me this opportunity to pursue a MSc degree in his lab and I will not forget the lessons, guidance, patience, and motivation you have given me. Coming into the lab, I did not know very much and it was not an easy start, but he always gave me an opportunity to learn and to succeed. I wish you the best during your recovery and hopefully we can meet again some day in the future. Andy, I could not have been more fortunate in having you as my mentor and supervisor during recent times of uncertainty. You took on the responsibility of me when you did not have to, and you have given me an opportunity to achieve my MSc degree. You have taught me to be critical, to look at things from different perspectives, and you always had my back. I cannot thank the both of you enough for giving me the opportunity to learn despite my shortcomings, and to continue chasing my goals.

CHAPTER 1: INTRODUCTION

1.1 – Allergies

1.1.1: Allergic inflammation

Allergic inflammation, or allergy, is a result of the immune response mounted against "harmless" antigens. To be more specific, it is an excessive and inappropriate response against specific though otherwise innocuous allergens that the immune system had been previously sensitized to[1]. The ensuing inflammatory response is a spectrum of events, typically characterized by increased vascular permeability to allow for the chemotaxis of immune cells, and their subsequent activation and release of inflammatory mediators[2]. At the same time, there is an anti-inflammatory response that is also stimulated to try to maintain a homeostatic balance between both processes[3]. It is important for this anti-inflammatory response to occur, as an uncontrolled strong inflammatory response to an allergen can be fatal, what we know as anaphylactic shock[2]. Examples of allergic inflammation in which the response is pro-inflammatory include asthma, allergic rhinitis, atopic dermatitis, and food allergy, amongst others[4].

1.1.2: Clinical epidemiology of allergy

During the past many years, statistics on the prevalence and incidence have been recorded that paint a vivid and chilling picture of the burden of allergy. Reports from the American Academy of Allergy, Asthma, and Immunology (AAAAI), the Centers for Disease Control and Prevention (CDC), and the Allergy Asthma Information Association (AAIA) show that about 20-25% of Canadians have allergic rhinitis, about 10-30% of the

world's population suffers from allergic rhinitis, and 40% of the population has been sensitized to foreign particles in the environment. In the case of food allergy, a study looking at about 38,000 children up to 18 years of age showed a prevalence of 8%, with 30.4% allergic to multiple foods[5].

It is important to consider that these statistics do not include other forms of allergy such as reactions to insect bites or drugs. Nevertheless, the prevalence has risen in the industrialized world over the last half-century and sensitization of children in classroom environments to common allergens have risen to about 50% according to the World Allergy Organization in 2011. In the United States, it is reported that roughly 1 in 5 individuals have an allergy[6]. To further complicate the condition, there are situations where one existing allergy can increase susceptibility to others - patients with asthma or allergic rhinitis were found likely to be sensitized for proteins in food or exhibit an allergic reaction to foods[7]. In other studies, it was shown that there is a gender disparity in the prevalence and severity of allergic diseases, that may be explained by sex hormones and others during growth[8].

Considering the magnitude of this health challenge, it becomes imperative to understand that allergies are a complex disease and there are variables yet to be unraveled that are involved in the spectrum of allergic responses – these need to be understood in order to develop preventative or appropriate therapeutics measures.

1.1.3: Important cellular players in allergy

Despite the complexity of allergies, researchers have made considerable progress on delineating the processes in the immune response that occur upon exposure to an allergen. This has led to a better understanding of the sequence of events occurring from allergen encounter to resolution of the allergic response. The inflammation in the allergic response is a result of a coordination of signaling cascades of various immune cell types and secretions[9]. One immune cell type heavily implicated and identified as the primary culprit in the allergic response and ensuing pathophysiology is the mast cell. Upon activation of mast cells by allergens there is release of multiple inflammatory products[10, 11] that have effects within the microenvironment manifesting as the overall allergic inflammatory response[12].

1.1.4: Phases of allergic inflammation

The mediators released by the mast cell in the allergic response can be organized into two categories – pre-formed mediators stored within granules and *de novo* synthesized mediators[11]. These two categories function in two phases of the allergic immune response, with the former in the acute phase and the latter in the late phase. Furthermore, the products released in the acute phase help in the transition to the late phase allergic immune response[1, 2].

Mast cell pre-formed mediators are found stored within cytoplasmic granules and have immediate and direct effects due to their rapid release in relatively high concentrations, and targets following mast cell activation with allergen[10]. Some examples

of mediators released from granules during degranulation include histamine, tryptase, and heparin[13]. Moreover, some inflammatory cytokines such as TNF and chemokines such as CCL2 (MCP-1) have been discovered stored within granules and also released during mast cell degranulation[13, 14]. The rapid release of these mediators promotes changes in the surrounding tissues including increasing vascular permeability and the recruitment of leukocytes that in turn, play a role in the inflammation associated with the late phase response[2, 11].

De novo synthesized mediators include lipid mediators and a plethora of cytokines and chemokines. These mediators are mainly produced in the late phase of the allergic immune response and further aid in promoting local chemotaxis of other cell types, as well as promoting inflammation. An example of a lipid mediator is leukotriene B4 (LTB_4), which has profound effects in allergy. LTB_4 is a potent mediator that promotes the chemotaxis of neutrophils, eosinophils, and lymphocytes[15, 16]. LTB_4 also acts on endothelial cells to increase permeability, airway reactivity, and goblet cell secretion[17].

De novo synthesized cytokines and chemokines also play an important role in the late phase reaction of the allergic immune response. They further promote the existing inflammation and may be associated with pathological pain that is present in some instances of allergic inflammation[18]. Cytokines and chemokines are abundant in the late phase reaction, released from mast cells and recruited leukocytes, as well as the pleiotropic effects they exhibit. Even pre-formed and released TNF and CCL2 that are released from granules are also newly synthesized in the late phase allergic immune response.

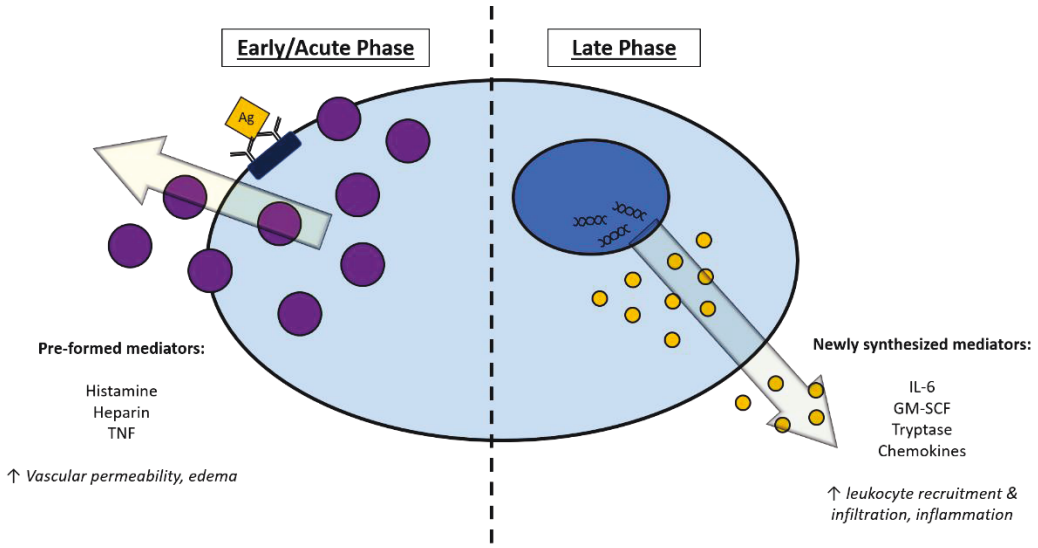

Figure 1.1: The phases in the allergic immune response and the effects of some of the mediators listed in each. Persistent production of these mediators can result in chronic allergic conditions and promote unceasing pro-inflammatory conditions leading to alterations of tissue structure function.

1.2 – Mast cells and allergic inflammation

1.2.1: Mast cell characteristics

Human mast cells arise from $CD34^+$, $CD117^+$, and $CD13^+$ pluripotent progenitor cells and are of hematopoietic origin[19]. In mice, mast cell progenitors differentiate from granulocyte/macrophage progenitors, which originate from common myeloid progenitors that develop from multipotent progenitors[11]. Progenitors circulate in the blood and migrate to tissue sites where they mature into mast cells, expressing phenotypes influenced by surrounding cytokines at those tissue sites[20]. The main tissue distribution of mast cells are at host-external environment interface such as in the lungs, intestines and

skin, and this property allows them to be early responders to foreign antigens from the environment.

Due to resident mast cells populating different sites in multiple microenvironments, it is not surprising that these populations display heterogeneity. In humans, there are at least three categories of mast cells, based on serine protease content (MC_T – tryptase only, MC_C – chymase only, and MC_{TC} – tryptase and chymase)[21]. Mast cells in mice and rats have been characterized into two categories – connective tissue-type and mucosal-type mast cells[22]. Variations in homing and recruitment mechanisms regulate the influx of mast cell progenitors to tissue sites where maturation takes place[22]. Furthermore, rather than distinct categories of mast cell phenotypes, it is more favourable that there exists a dynamic spectrum of phenotypes and function depending on the environmental interactions taking place during maturation.

Despite the heterogeneous populations of mast cells, what remains the same is the sentinel capability they have in the innate and acquired immune response[23, 24]. This is largely due to the multiple types of receptors expressed at the cell surface, capable of recognizing a multitude of antigens and coordinating a subsequent appropriate immune response[25, 26, 27]. These receptors allow the mast cell to be involved in recognition and participation in the immune response to foreign viral[28], bacterial[29], helminthic[30], fungal[31], and allergenic[32] sources, as well as hormones and other mediators and peptides[33, 34] found within the host[27]. One of these receptors is the high affinity IgE receptor (FcεRI), of which activation initiates the FcεRI-mediated signaling pathway and allergic immune response – this is also the signaling pathway of interest studied in this thesis.

1.2.2: IgE – high affinity IgE receptor – (FcεRI) mediated signaling pathway

The general signaling pathway and key players activated following crosslinking the FcεRI have been established from extensive research although, there is evidence of mast cell activation through IgE receptors differing on an individual basis[35, 36].

FcεRI is a tetrameric receptor consisting of an α chain that binds IgE, β chain, and two γ chains containing immunoreceptor tyrosine-based activation motifs (ITAMs) responsible for signaling. Dimerization of FcεRI by antigen (Ag)-crosslinked IgE results in internalization of the complex and the initiation of the FcεRI-mediated signaling pathway leading to the allergic immune response. Phosphorylation of ITAMs recruit proteins in the signaling cascade from the cytoplasm. Subsequent series of phosphorylations and recruitment of multiple adaptor proteins and lipids eventually leads to the mobilization of calcium and ultimately activation of transcription factors such as nuclear factor of activated T-cells (NFAT) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)[37]. The FcεRI-mediated signaling pathway is more complex than just described and has been extensively reviewed in literature in detail[38, 39].

The FcεRI-mediated signaling cascade essentially results in two main events: 1) degranulation of pre-formed mediators, and 2) *de novo* production and secretion of lipid mediators and cytokines/chemokines. Upon release, these mediators play indispensable roles in the inflammation associated with the allergic immune response[2, 24].

1.2.3: Mast cell-secreted products

Mast cells can produce and release different mediator profiles depending on the type of stimulus[10], but activation through the FcεRI will result in release of pre-formed mediators from granules, and the production and secretion of lipid mediators, cytokines, chemokines, and growth factors. These mediators have specific roles in the early and late phase mast cell FcεRI-mediated immune response and have been well-studied by researchers. It is important to understand that most of the secreted products are studied under *in vitro* mast cell-specific settings, and it is more difficult to assess the role played by each mediator in an *in vivo* setting[24]. Furthermore, in addition to mast cells, there are multiple cell types responding to these mediators, and direct specific actions on each cell type are difficult to measure *in vivo*.

Pre-formed mediators

The release of pre-formed mediators from mast cells dictate the early phases of the immune response as degranulation of these products is rapid and robust. Furthermore, some of the mediators released upon degranulation act to recruit other leukocytes and initiate an inflammatory cascade of events. The granules found within mast cells have been classified into categories based on the contents; however, this is not firm and it is likely there is granular heterogeneity depending on multiple factors including species and tissue site[10, 40]. What is consistent are the different classes of mediators pre-formed and stored within the granules, including but not limited to lysosomal enzymes, biogenic amines, mast cell-specific proteases, cytokines, and chemokines[41].

Lysosomal enzymes stored within granules include β-hexosaminidase and β-glucuronidase[42, 43], both of which have degradative functions; they have been found to digest carbohydrates such as peptidoglycan from bacterial cell walls[44]. Because of the abundance of β-hexosaminidase within the rapidly released granules[42], it has been widely used as a measure of degranulation[45] despite a lack of evidence showing any role for β-hexosaminidase in the FcεRI-mediated immune response to allergen.

Biogenic amines such as serotonin and histamine are found within mast cell granules and are released upon FcεRI-mediated mast cell activation[46, 47]. Serotonin may play a role in behavioural aspects associated with allergic rhinitis[48] through transmission of mast cell-mediated signals to local nerve endings[41]. It may also further promote inflammatory responses by enhancing mast cell migration to the site of inflammation and challenge[49]. Histamine has been well characterized in allergic inflammation, especially in smooth muscle contraction and increasing vascular permeability in local areas of release[50]. These have profound implications in allergic diseases such as asthma and histamine has been one target for pharmaceutical approaches to therapy[51].

Mast cell granules also contain serine proteases such as tryptase and chymase. Found in high concentrations within granules, they are readily degranulated with other products such as histamine[52, 53]. Blood tryptase levels have been used clinically as a measure of anaphylaxis, as there are increased levels of tryptase in allergic instances[54]. Furthermore, tryptase can also be used as a measure of total mast cell numbers[55]. In allergy, chymase potentially plays a protective role, as there was increased airway hyperresponsiveness and inflammation in the upper respiratory tracts in a chymase-deficient mice model of allergy[56].

Select cytokines have also been found stored within granules and released upon degranulation. TNF is the first cytokine found to be stored in granules[57], and has pro-inflammatory functions in allergy. It indirectly facilitates the recruitment of leukocytes such as neutrophils to the environment[58]. Another cytokine that has been found stored in granules is IL-4[59]. The role of IL-4 is likely to be mediating T_h2 responses such as promoting IgE isotype switching and secretion[60].

Newly synthesized cytokines/chemokines

Apart from degranulation of pre-formed mediators, mast cells also synthesize and release a plethora of cytokines and chemokines upon FcεRI-mediated mast cell activation. Cytokines and chemokines have been shown to be released by FcεRI-mediated mast cell activation although many of these were identified from transformed cell lines and may not be an accurate physiological representation[24]. Some of the typical cytokines found to be released from human mast cells or primary mast cells in mice include TNF[61, 62, 63], IL-4[64, 65], IL-6[66, 67], and IL-13[68], and chemokines CCL1[69, 70], CCL2[71], CCL3[70, 72], CCL9[70], and CXCL5[73, 74]. These cytokines and chemokines are synthesized upon FcεRI-mediated mast cell activation and have important functions (briefly outlined in Table 1) in the early and late phase allergic immune response.

Table 1: Brief list of the newly synthesized cytokines and chemokines released from the mast cell upon FcεRI-mediated activation, and corresponding simplified functions.

Mediator	Function
TNF	Leukocyte recruitment, inflammation
IL-4	IgE isotype switching, T_H2 cell differentiation
IL-6	Inflammation, induction of acute phase protein synthesis
IL-13	IgE isotype switching, regulation of immune responses
CCL1	Lymphocyte, monocyte recruitment
CCL2	T_{mem}, dendritic cell, monocyte recruitment
CCL3	Polymorphonuclear leukocyte recruitment
CCL9	Dendritic cell recruitment
CXCL5	Neutrophil recruitment

1.2.4: Drugs and pharmaceuticals

Identifying the active mediators derived from mast cells has led to a better understanding on how to treat diseases impacted by mast cells. Currently there are a wide variety of therapeutic interventions available against allergic inflammation. These approaches target different components of the allergic immune response, including released products or components within the FcεRI-mediated signaling pathway. There are advantages and disadvantages to each type of approach and most are only partially effective, which encourages the need for further research to develop improved therapeutics. In a simplified categorization, the pharmaceuticals can be arranged into four groups based on the components they target – released mediators, receptors, mast cell signaling components, and mast cell development and survival[11, 75]. In an FcεRI-mediated context (i.e. presuming the mast cells are already present in the tissues), the drug targets

can be explained as mast cell-derived products, mast cell secretion, and mast cell signaling (Figure 1.2).

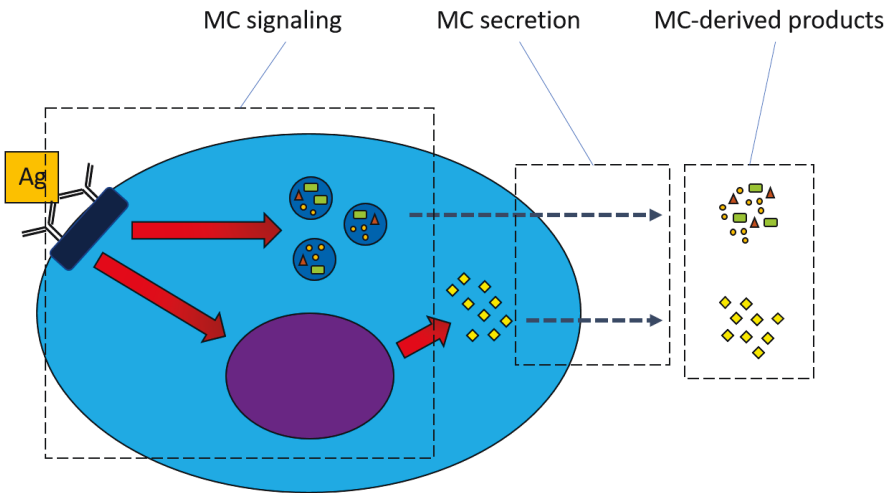

Figure 1.2: Drug targets simplified into three main categories based on mast cell FcεRI-mediated responses – targeting signaling components, secretory mechanisms, and released products. Signaling components are represented by red arrows, granules by blue circles, and secretory mechanisms by the dotted arrows.

Blocking mast cell-derived products

Many released pre-formed mediators, cytokines, and chemokines produced by mast cells during FcεRI-mediated activation have been targeted with pharmaceuticals. Some of these block mediator binding sites such as histamine receptors (histamine receptor antagonists; anti-histamines) and others bind to cytokines such as TNF and IL-1 to prevent interaction with target cells using blocking monoclonal antibodies[75, 76, 77]. These pharmaceuticals function to prevent the binding of these mediators after release to their targets and the conditions that would ensue, mainly regulating the recruitment and

activation of leukocytes in the pro-inflammatory environment[78, 79].

Blocking secretion

Another possibility of pharmaceutical intervention would be the signaling mechanisms involved in the secretion of mediators. Ideally, this would prevent the mediators from being released in the first place and would not necessitate the use of receptor antagonists or blocking monoclonal antibodies to prevent inflammatory conditions because of the released products. These types of pharmaceuticals can range from mast cell stabilizers[80, 81] to drugs that target proteins involved in secretory trafficking[82]. However, progress has been slowed on pharmaceuticals specifically targeting secretion pathways due to conflicting results[83] and the need for further testing of promising safer types of pharmaceutical intervention[84]. Furthermore, specificity of these drugs to secretory events during FcεRI-mediated mast cell activation must be accounted for since there are homeostatic processes that require functional secretory mechanisms common to both[82, 85, 86]. Regardless, regulation of secretion remains an attractive target since preventing mediators from being released can have a profound benefit in reducing disease[81].

Blocking FcεRI-mediated signaling pathway components

With research improving our knowledge on the various components within the FcεRI-mediated signaling pathway and important proteins that regulate specific functions, we can develop a great deal of potential therapeutic targets against allergy. Furthermore, we

can circumvent some of the complexities inherent in signaling pathways by either blocking signaling upstream of mediator synthesis or mediator release – inhibiting key signaling elements will allow us to do both. This is the basis for many current drugs being tested or used to treat mast cell-mediated diseases. It is also the foundation for current research where the contributions of specific proteins in the FcεRI-mediated immune response are studied in induced gene-deficient animal models or cell-based models, with a goal to develop therapeutic approaches. One class of pharmaceutical with demonstrated anti-inflammatory properties in other clinical settings are calcineurin inhibitors. The mechanism(s) and contribution(s) of calcineurin to the FcεRI-mediated immune response are the focus of this research.

1.3 – Mast cell models

There is considerable challenge in studying such a complex cell type and delineating the different responses to stimuli in different disease settings. The reason is because it is not possible to completely reconstruct the same environmental settings found within the host that the mast cells are exposed to during antigen challenge. Furthermore, mast cells may populate specific areas within the host, but they are not found in high numbers; to achieve a monoculture of these mature mast cells from peripheral tissues is challenging as purification processes result in a very low yield of mast cells, not to mention the potential of undesirable effects such as inadvertent activation[87, 88]. However, different mast cell models have been developed and techniques refined to attempt to better understand the processes and functions that take place physiologically[89].

Groups of researchers have successfully developed several mast cell lines of rodent and human origin for *in vitro* experiments. In rodents, the RBL-2H3 (rat) and MC-9 (mouse) transformed cell lines have been established. In humans, there are two cell lines that encompass mast cells in different stages of maturity. The HMC-1 cell line consists of immature mast cells[90], while the LAD2 cell line are more differentiated and matured mast cells[91]. It is important to understand that these mast cells are genetically altered and thus may not reflect the phenotypic responses seen in physiological settings.

Another method of deriving mast cells for experiments *in vitro* is to harvest progenitors and use conditioned media to mature and maintain mast cell monocultures. A popular source of these progenitors is from the bone marrow, and this has been widely used in mast cell research. Other tissue sites where progenitors can be harvested include umbilical cord blood, fetal liver, and peripheral blood[88, 92], but the source is important as cord blood and peripheral blood-derived progenitors have been shown to give rise to different mast cell phenotypes[93]. Culture conditions are important for maturation of progenitors as it influences the phenotypic properties of mature mast cells. Typical conditioned media for primary mast cell cultures contains IL-3 and stem cell factor (SCF)[94, 95]. Once again, it is important to highlight that these mast cells are derived from conditions that do not mimic those seen *in vivo* and cannot be conclusive representations of the physiological effects taking place within the host organism. This drives the need for research of mast cells using *in vivo* models and the development of techniques that allow the integration of *in vitro* results with *in vivo* mast cell models.

The development of mice deficient in mast cells was critical to the field of mast cell research, specifically W/W^{v} and W^{sh}/W^{sh} mice strains[96, 97]. These strains have genetic

mutations that affect CD117 (c-Kit), which is the receptor for SCF. Of the two mouse strains, the W^{sh}/W^{sh} is a better mouse model to use as there is greater mast cell deficiency compared to W/W^{v} mice, mice are fertile, and are not predisposed to other conditions that have the potential to confound mast cell-related research[97, 98]. Mast cell-deficient mice allows researchers to study the impact that mast cells (and lack thereof) have in different disease settings. Taking it a step further, the adoptive transfer of cultured primary mast cells back into these mice allows for results that are mast cell-specific and a comparison between *in vitro* and *in vivo* systems, and has been widely used in research[99, 100].

In the pursuit for the optimal animal model to represent *in vivo* conditions, the development of a mast cell-specific mast cell protease (*Mcpt*)5-*Cre* mouse system was achieved, with the *Cre* under the control of the promoter of *Mcpt-5* in connective tissue mast cells[101]. Using a Cre/loxP system to edit genes of interest mitigates the potential confounders that a systemic genetic deficiency will have on the development of the mice and allows for conditional control of gene deficiencies. This is a powerful tool for analyzing the effects a gene may have *in vivo* in mast cell-mediated disease settings in mice[102], and many strains are commercially available with floxed genes of interest.

The refinement and impact of CRISPR/Cas9 system has also allowed for genetic engineering and holds vast clinical promise. Through the ability to manipulate gene expression, this system has been used to edit genes of organisms ranging from plants to vertebrates[103]. In mast cells and allergy, CRISPR/Cas9 has been used to study roles of specific genes in the immune response. Through editing of the mouse genome, researchers have been successful in creating mouse models of disease for *in vivo* experimentation[104].

1.4 – Calcineurin

1.4.1: Characteristics of calcineurin

Calcineurin is a serine threonine protein phosphatase conserved in all eukaryotes[105] with critical functions in multiple cell types and signaling pathways. It is composed of two subunits – catalytic subunit A (CnA) and regulatory subunit B (CnB). Each subunit contains specific domains that play roles in the calcium-dependent activation of calcineurin. The catalytic subunit A consists of three isoforms, α (CnAα), β (CnAβ), and γ (CnAγ). CnAα and CnAβ are ubiquitously expressed and distributed, while CnAγ is found in limited tissues[106].

The activation of calcineurin from its inactive state has been widely studied and well characterized[107]. Briefly, elevated levels of calcium, upon FcεRI-mediated activation, will bind to CnB and binding partner calmodulin (CaM). Conformational changes to calcineurin and the binding of CaM to the calmodulin-binding domain will remove an autoinhibitory domain on CnA[108].[109] This results in the activation of calcineurin and allow it to interact with its substrates.

Calcineurin binds and interacts with a variety of substrates to regulate many physiological functions, ranging from cell cycle and apoptosis to cytoskeletal functions[110]. Examples of important interactions of calcineurin are with transcription factors such as NFAT and NF-κB to regulate immune responses[111]. Calcineurin interacts with NFAT directly by dephosphorylating NFAT at specific sites to unmask a nuclear localization signal, allowing NFAT to translocate into the nucleus and initiate the transcription of various genes[111]. Activation of NF-κB is indirect and likely through

dephosphorylation of Bcl-10 and formation of the Carma1-Bcl10-Malt1 complex in the signaling pathway, leading to phosphorylation and degradation of IκBα to release inhibition of NF-κB[112, 113, 114]. The activation of these transcription factors and subsequent gene expression results in many physiological functions, and dysregulation has been the culprit of various disease settings[110]. This provided the impetus for research on calcineurin inhibitors as a form of therapeutic towards its associated pathologies.

1.4.2: Calcineurin inhibitors as therapeutics

Due to the importance of calcineurin in mediating many physiological functions including immune responses, the development of calcineurin inhibitors such as cyclosporine A and tacrolimus (FK506) were instrumental in organ transplant successes[115]. The action of calcineurin inhibitors work to suppress the T lymphocytes in the immune response through the inhibition of interactions with transcription factors involved in production of undesirable mediators[116]. However, the successful use of calcineurin inhibitors has been found to also result in adverse pathologies including nephrotoxicity and hypertension, warranting further research on the mechanisms involved[117, 118].

1.4.3: Calcineurin in FcεRI-mediated immune responses

Calcineurin inhibitors have been used for anti-inflammatory effects in mast cell-related FcεRI-mediated incidences of allergy such as atopic dermatitis[119, 120]. The safety of using calcineurin inhibitors in allergies has been acknowledged, and long-term studies

are in progress[121]. Activation of mast cells in an IgE-dependent manner results in calcineurin-dependent activation of NFAT as well as NF-κB through Malt1 and Bcl10, leading to mediator synthesis that promotes allergic inflammation[114, 122, 123]. Remarkably, since most of our knowledge on calcineurin and allergies come from the use of calcineurin inhibitors, we are still lacking genetic and definitive evidence of the roles calcineurin plays in the FcεRI-mediated immune response – specifically the contributions of the different isoforms.

1.4.4: Calcineurin A – alpha isoform

The focus of my research is specifically on the α isoform of calcineurin. As one of the isoforms ubiquitously expressed in various tissues, there has been research done on CnAα in other physiological settings. Unfortunately, research on this isoform has been limited due to early lethality of homozygous CnAα gene knockout mice[124]. Most of the literature describes the phenotypes associated with CnAα deficiency in non-immune settings including submandibular gland function and secretion[124], renal development and function[125], and keratinocyte differentiation and survival[126]. Specifically, maturation was affected including the CnAα deficient mice being smaller, with smaller livers and kidneys[127], and decreased size and maturation of glomeruli in the kidneys[125] compared to wild type counterparts. CnAα deficiency also resulted in abnormal cytoskeletal components in brain cells[128] and decreased differentiation of epidermal cells[126] in mice. In contrast, T and B cell maturation was found to be normal in CnAα deficient mice[129]. In the immune system, CnAα deficiency has been shown to result in an impaired *in vivo* T cell response to antigen[129]. Otherwise, there is a paucity of literature or research on

calcineurin isoforms in mast cells and its role in the FcεRI-mediated immune response, despite the use of calcineurin inhibitors to treat allergy. Thus, it is imperative to delineate and understand the mechanisms in which CnAα contributes to the FcεRI-mediated signaling pathway in mast cells so that improvements can be made in the therapeutic approaches concerning calcineurin.

1.5 – Rationale, Hypothesis, Objectives

1.5.1: Rationale

While calcineurin inhibitors have been applied in incidences of allergic inflammation, there is a lack of genetic and definitive evidence of the mechanistic role of calcineurin and the contributions of the isoforms in the FcεRI-mediated immune response. Previous work from our laboratory has shown that an endogenous regulator of calcineurin (Rcan1) plays an important role in suppression of the FcεRI-mediated immune response through regulation of calcineurin – Rcan1 deficiency resulted in enhanced inflammatory phenotypes seen *in vitro* and *in vivo* FcεRI-mediated models of allergy[123]. Thus, while calcineurin is an attractive therapeutic target in allergy, it is important to characterize calcineurin and the isoforms regarding the role they play in mast cell FcεRI-mediated activation. Through studying the mechanisms and contributions of the different isoforms of calcineurin, a fundamental understanding can be formed and serve as the basis for future therapeutics against allergy.

1.5.2: Objectives

The objectives of this research project are to determine the following:

1. The role of CnAα on the development of mast cells
2. The contributions of CnAα on FcεRI-mediated degranulation (early phase response)
3. The contributions of CnAα on FcεRI-mediated cytokine and chemokine production (late phase response)
4. The effects of CnAα on the FcεRI-mediated signaling pathway

1.5.3: Hypotheses

Based on existing knowledge on calcineurin in the FcεRI-mediated signaling pathway and immune response, we hypothesize that CnAα deficiency will not result in any impairments on mast cell development. We also hypothesize that CnAα is necessary for both early and late phase responses to FcεRI-mediated challenge, and deficiency will result in impaired levels of released pre-formed mediators, *de novo* mediators, and observable inflammation. Finally, we hypothesize that CnAα deficiency will result in impaired activation of key FcεRI-mediated signaling pathway components, relative to wild type.

CHAPTER 2: MATERIALS AND METHODS

2.1: Animals

All experiments followed protocols approved by the University Committee on Laboratory Animals, Dalhousie University, in accordance with the guidelines of the Canadian Council of Animal Care. CnA$\alpha^{+/-}$ mice were generously donated by Dr. Jennifer Gooch (Emory University, Georgia, ATL, USA), and mast cell-deficient W^{sh}/W^{sh} mice were obtained from The Jackson Laboratory (B6Cg-kit W-sh/HNiHJacBsmJ NistltF4, Bar Harbor, Maine). Mice colonies were bred and housed in the Izaak Walton Killam (IWK) Health Centre Animal Care Facility, and food and water were provided *ad libitum* prior to and during experimentation.

2.2: Cell isolation, cell culture, and activation

2.2.1: Extraction of cells from liver tissue

Entire livers were isolated in aseptic conditions from neonatal mice bred from heterozygous CnA$\alpha^{+/-}$ breeding pairs. The livers from individual neonates were ground and pressed through a 40µm cell strainer to produce a cell suspension in Roswell Park Memorial Institute (RPMI) 1640 medium, collected into 50mL conical tubes. Contents were centrifuged at 500xg for 5 minutes at 4˚C and an aliquot of resuspended cells was stained and counted using trypan blue exclusion to determine the number of live cells. An extra clipping of neonatal tissue from each newborn pup was taken for genotyping purposes. Flasks containing CnAα knock-out cells and corresponding littermate wild-type cells were maintained and used for experiments, and heterozygous cells were discarded.

2.2.2: Mast cell monoculture

Isolated liver-derived cells were resuspended at a density of 0.5 x 10^6 cells per millilitre of complete mast cell growth media in T-25 cell culture flasks (Sarstedt, Montreal, Quebec). Complete mast cell media consists of 500mL of RPMI 1640 with L-glutamine, 10% heat-inactivated fetal bovine serum (Gibco, Thermo Fisher Scientific, Waltham, Massachusetts), 10% WEHI-3B conditioned medium, 1% of penicillin/streptomycin (Gibco, Thermo Fisher Scientific), 50µM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, Missouri), and 200nM prostaglandin E2 (Sigma-Aldrich). Mouse stem cell factor (mSCF) (Peprotech, Rocky Hill, New Jersey) was supplemented into each flask at a concentration of 30ng/mL. Non-adherent cells were resuspended twice a week in fresh complete mast cell media supplemented with fresh mSCF and transferred to a new flask once per week. After 4-6 weeks, the purity of liver-derived cultured mast cells was greater than 95%.

2.2.3: WEHI-3B cell line monoculture for IL-3

WEHI-3 (American Type Culture Collection, Manassas, Virginia; ATCC® TIB-68™) cells were cultured in complete medium containing RPMI 1640 with L-glutamine, 10% FBS, 1% Minimum Essential Medium (MEM) non-essential amino acid (Gibco, Thermo Fisher Scientific), 1% Penicillin/Streptomycin, 1% 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) (Gibco, Thermo Fisher Scientific), 50µM 2-mercaptoethanol, and 1-2mL 1M NaOH. Complete medium was filtered using a 0.22µm pore size prior to use for cell culture. Culture flasks were kept at 70-80% confluency and media was gently transferred into 50mL conical tubes when the colour turned yellow in

the flasks, without disturbing the adherent cells. Fresh media was added to each flask to replace harvested media each time it was taken. The conical tubes were centrifuged at 480xg for 5 minutes to pellet any cell debris, and the supernatant was transferred to a sterile bottle. All batches of supernatant were mixed together to ensure an even concentration of IL-3 throughout, stored frozen at -20˚C, and filtered before use in mast cell culture.

2.2.4: TIB-141 cell line monoculture for anti-DNP IgE

IGEL b4 (ATCC® TIB-141™) cells were cultured in filtered complete medium (0.22μm pore size) consisting of Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Thermo Fisher Scientific), 10% FBS, and 1% Penicillin/Streptomycin at 37˚C and 10% CO_2 culture conditions. Cells were cultured at a density of 0.1 x 10^6 cells/mL. Cell media was changed every two days, and starting from the second passage, supernatant was collected after centrifugation of contents from culture flasks into baked glassware then stored at -20˚C. The supernatant contains the anti-DNP IgE used for mast cell sensitization. Supernatants collected over a week were mixed together to ensure an even IgE concentration.

2.2.5: Activation of mast cells

Cultured mast cells were sensitized with IgE supernatant cultured from the IGEL b4 (ATCC® TIB-141™) cell line overnight for about 18 hours prior to stimulation with 10ng/mL 2,4,6-Trinitrophenyl Bovine Serum Albumin, TNP-BSA (LGC Biosearch

Technologies, Petaluma, California) for various durations depending on the time points of interest. Flasks containing stimulated cells were centrifuged at 480xg for 5 minutes at 4˚C after stimulation and supernatant and cell pellets were processed accordingly for different assays. All sensitization and activation of mast cells used *in vitro* followed this procedure.

2.3: Genotyping of cell cultures

2.3.1: DNA extraction from tissue

An extra clipping of tissue from each neonate was used to genotype their respective flask of cultured cells. DNA was isolated using a REDExtract-N-Amp™ PCR ReadyMix™ kit (Sigma-Aldrich) containing extraction solution, tissue preparation solution, neutralization solution, and REDTaq® ReadyMix™ PCR Reaction Mix. Tissues were minced with sterile scissors and immersed in 80µL extraction solution and 8µL tissue preparation solution for 20 minutes at room temperature in a 1.5mL Eppendorf tube after a brief vortex mixing. Each tube was then immersed for 10 minutes in a heating block at 100˚C, then 100µL of neutralization buffer added to each followed by another brief vortex prior to a final centrifugation at 2400xg for 10 minutes. The supernatant contained the DNA isolate and was used for PCR while the pellet containing tissue debris was discarded.

2.3.2: PCR amplification

The PCR mixture for each sample totaled 20µL and contained 10µL REDTaq® ReadyMix™ PCR Reaction Mix (Sigma-Aldrich), 4µL molecular grade H_2O, 2µL common primer, 1µL CnAα wild-type primer, 1µL CnAα knock-out primer, and 2µL isolated DNA (primer sequences are in Table 2). The PCR protocol was 95˚C denaturation for 5 minutes followed by 30 cycles of 90 second amplification steps consisting of 95˚C denaturation (30 seconds), 58˚C primer annealing (30 seconds), and 72˚C extension (30 seconds), and then a final extension phase at 72˚C for 5 minutes.

Table 2: Primer information for genotyping of CnAα wild type and knockouts, as well as primers used for real time quantitative polymerase chain reaction experiments.

	Primer	Predicted Size	Primer Sequence 5' – 3'
Genotyping	CnAα wild type (reverse)	~ 247 bp	CAG GGA ATG GGT AGA CAT GG
	CnAα mutant (reverse)	~ 360 bp	GCT ACT TCC ATT TGT CAC GTC C
	CnAα common (forward)		TGT CAA TGA GAT GGC CCT AGT
	Primer	**Predicted Size**	**Primer Sequence 5' – 3'**
RT-qPCR	TNF forward	~174 bp	CAT CTT CTC AAA ATT CGA GTG ACA A
	TNF reverse		TGG GAG TAG ACA AGG TAC AAC CC
	IL-6 forward	~77 bp	TAG TCC TTC CTA CCC CAA TTT CC
	IL-6 reverse		TTG GTC CTT AGC CAC TCC TTC
	IL-13 forward	~108 bp	CTG TGT CTC TCC CTC TGA CCC
	IL-13 reverse		GCC AGG TCC ACA CTC CAT ACC
	IL-4 forward	~77 bp	CAT GCA CGG AGA TGG ATG TGC
	IL-4 reverse		AAG CCC TAC AGA CGA GCT CAC
	HPRT forward	~229 bp	CAC AGG ACT AGA ACA CCT GC
	HPRT reverse		GCT GGT GAA AAG GAC CTC T

2.3.3: Agarose gel electrophoresis and visualization

PCR products were run at 150 volts for 30 minutes on a fresh 1.5% agarose gel (UltraPure, Invitrogen) stained using SafeView™ Classic (Applied Biological Materials Inc., Vancouver, Canada), and visualized on a Gel-Doc 2000 apparatus (Bio-Rad

Laboratories, Hercules, California) using the corresponding QuantityOne software. A 100kb TrackIt DNA Ladder (Invitrogen, Thermo Fisher Scientific) was used to determine PCR product sizes. Predicted PCR product sizes are listed in Table 2.

2.4: Toluidine Blue Staining

An aliquot containing 5 x10^5 mast cells from 6-week old CnAα knock-out and littermate wild-type cell culture flasks were taken and resuspended at a density of 1 x 10^6 cells/mL in RPMI 1640. Cells were subjected to a cytocentrifugation at 5xg for 5 minutes onto microscope slides (FisherBrand, Fisher Scientific, Hampton, New Hampshire) and allowed to air dry overnight. The following day, the microscope slides were fixed in Carnoy's fixative solution (30ml 100% ethanol, 15mL chloroform, and 5mL glacial acetic acid) for 10 minutes, then 66% ethanol for 10 minutes, 0.5% acetic acid for 1 minute, and finally toluidine blue solution for 2 hours. Slides were rinsed in distilled H_2O and air dried prior to the addition of a drop of DPX mounting medium (Sigma-Aldrich) and a coverslip. Specimens were viewed on a Nikon Eclipse E600 microscope with a DXM 200 camera attachment using corresponding Nikon ACT-1 software version 2.20, and images processed using Adobe Photoshop 5.0.

2.5: Flow cytometry

1 x 10^6 wild type and CnAα deficient mast cells were sensitized overnight and labeled with FITC-conjugated rat anti-mouse IgE (clone: R35-72) or FITC Rat IgG1 κ isotype control (clone: R3-34) antibodies and prepared for flow cytometry. Similarly,

non-sensitized wild type and CnAα mast cells were labeled with FITC-conjugated anti-mouse CD117 (clone: 2B8) or FITC-conjugated rat IgG2b κ isotype control (clone: eB149/10H5) (Table 3). Flow cytometry was done using a BD Biosciences FACSCalibur (BD Biosciences, Franklin Lakes, New Jersey). Unstained cells were used to establish a gate for live mast cells within the samples and then gates for $CD117^+$ and IgE^+ cells were established. Data analysis was done using FlowJo V10 software (BD Biosciences). Percentages of number of gated live mast cells expressing CD117, IgE, as well as mean fluorescence index (MFI) were taken.

Table 3: Antibodies used for flow cytometry (FACSCalibur) to assess surface receptor expression, and their corresponding isotype controls.

Antibody	Fluorophore	Company	Clone Name
Rat anti-mouse IgE	FITC	BD Biosciences	R35-72
Rat IgG1 κ isotype control	FITC	BD Biosciences	R3-34
Rat anti-CD117 (c-Kit)	FITC	eBioscience	2B8
Rat IgG2b κ isotype control	FITC	eBioscience	eB149/10H5

2.6: Intracellular calcium mobilization assay

2×10^6 mast cells from wild type and CnAα deficient cultures were sensitized overnight and the next day resuspended in Hank's Balanced Salt Solution (HBSS; Gibco, Thermo Fisher Scientific) and incubated with 4μg/mL Fura-2-acetoxymethyl ester (Fura-

2 AM; Invitrogen, Thermo Fisher Scientific), 2.5mM probenecid (Invitrogen, Thermo Fisher Scientific), and 0.02% pluronic F-127 (Invitrogen, Thermo Fisher Scientific). Samples were incubated in the dark for 30 minutes and then washed twice. Cells were resuspended in 1mL 1% FBS-HBSS and counted using a haemocytometer before being adjusted to a density of 0. 5 x 10^6 cells/mL, into two tubes. All samples were then transferred to a UV-glass cuvette with a magnetic stir bar, and fluorescence read every 0.1 seconds (with continuous stirring) using a Shimadzu RF-5301PC fluorophotometer and corresponding manufacturer's software. The UV-glass cuvette was washed and dried thoroughly each time prior to another aliquot of cells being added for measuring intracellular calcium levels. To determine maximum fluorescence (F_{max}), TNP-BSA was added at 30 seconds and 20µL of 10% Triton X-100 added at 300 seconds. To determine minimum fluorescence (F_{min}), TNP-BSA was added at 30 seconds, 20µL of 0.2M $CaCl_2$ at 300 seconds, and 20µL of 0.5M EGTA at 400 seconds. F_{max} was determined after the addition of Triton X-100, and F_{min} determined after addition of EGTA under saturating calcium conditions. To calculate the fluorescence ratio (F), the equation below was used and [Ca^{2+}] values plotted over time.

$$\mathbf{[Ca^{2+}] = K_d\,(F - F_{min})\,/\,(F_{max} - F)}$$; [Ca^{2+}]: Intracellular calcium, Fura-2 K_d = 145nM

2.7: Degranulation Assay – Beta-hexosaminidase release

Sensitized wild type and CnAα deficient mast cells were resuspended in HBSS and plated in triplicates at a density of 1 x 10^6 cells/mL into a 96-well plate, then stimulated for 20 minutes. Supernatant was collected and transferred into another 96-well

plate, while the pellet was gently resuspended with a 1% NP-40-HBSS solution to lyse the cells. The plate was centrifuged again, the supernatant collected, and transferred to another plate. This plate contained the residual β-hexosaminidase remaining in the cells. 50µL of supernatant from degranulation and supernatant from cell pellets were taken and incubated with 50µL of 1mM p-Nitrophenyl-N-Acetyl-β-D-Glucosaminide (p-NAG) (Sigma-Aldrich) for 90 minutes at 37˚C. 200µL of 0.1M carbonate buffer was added to stop the reaction, and bubbles were removed with a needle tip. Plates were immediately read on a spectrophotometer at a wavelength of 405nm, and optical densities copied to and analyzed using Microsoft Excel. Total relative β-hexosaminidase was determined through the comparison of the summed supernatants between wild type and CnAα deficient mast cells. Percentage of β-hexosaminidase released from mast cells through degranulation was determined using the formula below.

$$\textbf{\% Release} = \frac{\textbf{O.D.supernatant} - \textbf{O.D.background}}{\textbf{O.D.supernatant} - \textbf{O.D.background} + \textbf{O.D.pellet} - \textbf{O.D.background}}$$

2.8: Real-time quantitative polymerase chain reaction (RT-qPCR)

2.8.1 – RNA isolation from mast cells and cDNA synthesis

1.2 x 10^7 sensitized mast cells from each genotype were either untreated or stimulated with TNP-BSA for various durations (15, 30, 60, 180 and 360 minutes; 2 x 10^6 cells per condition), and then centrifuged at 480xg for 5 minutes. The cell pellet was resuspended in 1mL of Trizol (Life Technologies) and total RNA isolation was done

following the manufacturer's protocol. RNA concentrations were measured using a NanoDrop 2000c UV-Vis Spectrophotometer (Thermo Fisher Scientific) and purity assessed using A260/A280 ratios. cDNA synthesis was done using a RNA to cDNA EcoDry Premix (Takara Bio USA, Mountain View, California) following the manufacturer's protocol and thermocycler settings. Briefly, 1µg of RNA was resuspended with RNase free H_2O to a final volume of 20µL and then added into the provided tube to dissolve the reaction mixture for reverse transcription. The tubes were briefly vortexed, incubated at 42˚C for 60 minutes, and the reaction stopped by heating to 70˚C for 10 minutes using a thermocycler (Applied Biosystems).

2.8.2 – mRNA measurement using RT-qPCR

Samples were prepared for RT-qPCR using the following master mix components: 10µL molecular grade H_2O (Invitrogen, Thermo Fisher Scientific), 4µL SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories), 2µL 10mM forward primer, 2µL reverse primer, and 2µL cDNA (total volume is 20µL per well). Gene expression was measured using a CFX Connect Real-time system (Bio-Rad Laboratories), and data processed on Biorad CFX Manager 3.1 software. Melt curve analyses were run to determine target specificity. Gene expression levels were normalized to hypoxanthine guanine phosphoribosyltransferase (HPRT) housekeeping gene and analyzed using the double delta Ct (ΔΔCt) method. Primer sequences used for the genes analyzed are in Table 2.

2.9: Enzyme-Linked ImmunoSorbent Assay (ELISA)

Sensitized cells from both genotypes were stimulated with 10ng/mL TNP-BSA for various durations at a cell density of 1 x 10^6 cells/mL in 1.5mL microfuge tubes. After stimulation, tubes were centrifuged and supernatant collected for ELISA. ELISA kits for cytokines TNF, IL-6, IL-13, and IL-4 were used following the manufacturer's protocol (R&D Biosystems) and plated on Nunc Maxisorp flat-bottom 96-well plates (Thermo Fisher Scientific). Briefly, plates were coated on the first day with capture antibody, standards and samples added on the second, and finished with secondary antibody and streptavidin-horseradish peroxidase (HRP) on the third day. The substrate for HRP used in the enzymatic reaction was a 1X TMB solution (eBioscience, Thermo Fisher Scientific), and the reaction was stopped with 0.3M H_2SO_4. Plates were immediately read using a SpectraMax 190 plate reader (Molecular Devices) and processed using corresponding SOFTmax PRO 4.3 LS computer software.

2.10: Inflammatory protein array

Sensitized cells from both genotypes were either untreated or stimulated for 3 hours with TNP-BSA for collection of supernatant. The 3-hour secretory profile between wild type and CnAα deficient mast cells was compared using the RayBiotech Mouse Inflammation Array C1 (RayBiotech, Norcross, Georgia) following the manufacturer's instructions. Membranes were scanned using the ChemiDoc Imaging System (Bio-Rad) and processed with Image Lab software (Bio-Rad).

2.11: Gel electrophoresis and western blotting

2.11.1 – Collection of cell lysate and sample loading preparation

6 X 10^6 cells from both genotypes and per condition (untreated, 5, 20, 60, 180, and 360 minutes) were stimulated with TNP-BSA and then centrifuged to collect the cell pellet. Pellets were lysed using a lysis buffer consisting of radioimmunoprecipitation assay (RIPA) buffer and HALT Protease and Phosphatase Inhibitor Cocktail (diluted to 1X final concentration; Thermo Fisher Scientific). The final protein concentration was assessed using a bicinchoninic acid assay (Thermo Fisher Scientific) per manufacturer's instructions and read on a SpectraMax 190 plate reader. Lysates were denatured using a SDS sample buffer (GenScript, Cedarlane Labs) and boiled at 95˚C for 5-7 minutes, with a final volume of 15µL. Denatured samples were loaded onto a 12% SDS-PAGE gel with a Precision Plus Protein Standard (10-250kD) protein marker (Bio-Rad Laboratories) for identification of band sizes.

2.11.2 – Western blotting

Using a Mini PROTEAN 3 Cell system (Bio-Rad), samples were run at 120V until the leading edge of the dye approached the bottom of the gel. The gel was then transferred to a 0.2µm Immun-Blot polyvinylidene fluoride membrane for one hour at 75V at 4˚C, with constant stirring of the transfer buffer containing 25mM Tris, 190mM glycine, 20% methanol, and 0.1% SDS. Membranes were blocked with 5% skim milk in Tris-buffer saline and 0.1% Tween-20 (TBST) (Sigma-Aldrich) for one hour, to prevent non-specific binding. Primary antibodies were used to detect proteins of interest on the

membranes overnight at 4˚C on a shaker. Corresponding HRP-conjugated secondary antibodies were added the next day and Western Lightning Plus – Enhanced Chemiluminescence (ECL) substrate (Perkin Elmer) was used for the reaction with HRP. Membranes with ECL were exposed onto Carestream Kodak BioMax light film (Sigma-Aldrich) and developed using a SRX-101A Medical Film Processor (Konica Minolta). Films were digitally saved by scanning using a CanoScan LiDE 120 (Canon, USA), and quantitative analysis done using ImageJ software. All antibody information can be found in Table 4.

Table 4: Antibody information for proteins of interest looked at using western blotting.

Antibody (Ab)	Clone/Species	Company/Cat.#	Dilution
Phospho-p38 MAPK (Thr180/Tyr182)	D3F9/Rabbit	Cell Signaling Technology #4511	1:1000
Total p38 (C-20)	Rabbit	Santa Cruz Biotechnology sc-535	1:1000
Phospho-SAPK/JNK (Thr183/Tyr185)	Rabbit	Cell Signaling Technology #9251	1:1000
Total SAPK/JNK	Rabbit	Cell Signaling Technology #9252	1:1000
Phospho-MAPK ERK1/2 (Thr202/Tyr204)	Rabbit	Cell Signaling Technology #9101	1:1000
Total MAPK ERK1/2	Rabbit	Cell Signaling Technology #9102	1:1000
Phospho-IκBα (Ser32)	14D4/Rabbit	Cell Signaling Technology #2859	1:1000
Total-IκBα	Rabbit	Cell Signaling Technology #9242	1:1000
PP2B-Aα	D-9/Mouse	Santa Cruz Biotechnology sc-17808	1:1000
SNAP-23	A-5/Mouse	Santa Cruz Biotechnology sc-166244	1:1000
Actin (I-19)	Goat	Santa Cruz Biotechnology sc-1616	1:1000
Goat anti-rabbit IgG-HRP		Santa Cruz Biotechnology sc-2004	1:2000
Goat anti-mouse IgG-HRP		Santa Cruz Biotechnology sc-2005	1:2000
Mouse anti-goat IgG-HRP		Santa Cruz Biotechnology sc-2354	1:2000

2.12: Electrophoretic mobility shift assay (EMSA)

2.12.1 – Collection of nuclear fraction, DNA probe labelling, and sample preparation

1 x 10^7 sensitized cells were stimulated for each condition (untreated, 5, 20, 60, 180, and 360 minutes) for wild type and CnAα deficient mast cells. Samples were centrifuged to collect the cell pellets and then washed once with phosphate buffered saline (PBS) (Gibco, Thermo Fisher Scientific). Cell pellets were then processed using a nuclear protein extraction kit following manufacturer's protocols (Active Motif, Carlsbad, California).

DNA probing for NF-κB transcription factor was done through a reaction consisting of 5µL DNase/RNase free water (Sigma-Aldrich), 2µL 1.75pmol/µL NF-κB oligonucleotide (Promega, Madison, Wisconsin), 1µL 10X T4 Kinase buffer (Promega), 1µL T4 Kinase (Promega), and 1µL ^{32}P ATP (Perkin Elmer, Waltham, Massachusetts) in a 37˚C water bath for 30 minutes. The phosphorylation reaction was halted by the addition of 1µL 0.5M EDTA and 89µL TE buffer (Qiagen, Toronto, Ontario). The solution was passed through a Sephadex G-25M column (GE Healthcare, Pittsburgh, Pennsylvania) and the eluate (labelled probe) was used to prepare samples. The double-stranded oligonucleotide used was a NF-κB binding consensus sequence on the mouse IL-6 promoter 5' - TTA TCA AAT GTG GGA TTT TCC CAT - 3'.

Nuclear protein concentrations were read using a BCA Assay on a SpectraMax 190 plate reader and analyzed using SOFTmax PRO 4.3 LS software. 10µg of nuclear protein was assayed from each sample and reacted for 30 minutes with 2µL binding buffer (Promega), 1µL poly(deoxyinosinic-deoxycytidylic) (Sigma-Aldrich), 1µL of

labelled DNA probe, and DNase/RNase free water to a sample volume of 10µL. Lastly, 1.5mL of loading buffer was added at the end and samples pulsed prior to loading. A blank sample without nuclear protein was also prepared in the same procedure.

2.12.2 – EMSA

6% native polyacrylamide gels were prepared as follows: 3mL 10X TBE buffer, 42.5mL distilled water, 1.88mL 80% glycerol, 0.6mL 10% ammonium persulfate (APS; Bio-Rad), 12mL 30% acrylamide/bis-acrylamide (Bio-Rad), and 50µL TEMED (Bio-Rad). Solidified gels were placed in a PROTEAN II xi Cell (Bio-Rad) device and separated through electrophoresis in 0.5X Tris-Boric acid-EDTA (TBE) buffer. Gels were pre-run for 30 minutes at 100V before samples were loaded, and then ran for 90 minutes at 100V. Gels were removed from the apparatus, arranged on top of filter paper (Whatman, Sigma-Aldrich), and vacuum dried using a gel drier for 2 hours. The dried gel-filter paper assembly was then placed in an autoradiography cassette (Fisher Scientific) with a sheet of Carestream Kodak BioMax light film (Sigma-Aldrich) for autoradiography exposure. Films were processed using a SRX-101A Medical Film Processor (Konica Minolta). Films were digitally saved by scanning using a CanoScan LiDE 120 (Canon, USA), and quantitative analysis done using ImageJ software.

2.13: Mast cell reconstitution into W^{sh} mice

Mast cell-deficient W^{sh} mice were anaesthetized and intradermally reconstituted with 5 x 10^5 mast cells using 31-gauge insulin needles on 1mL syringes (BD Biosciences)

loaded with either wild type or CnAα deficient mast cells at a density of 25 x 10^6 cells/mL. Intradermal injections of mast cells were done in the ear pinna or hind foot pads of W^{sh} mice, depending on the *in vivo* model of anaphylaxis being assessed, and left to reconstitute for 6 weeks. Wild type mast cells were reconstituted into the right ears and foot pads of mice, whereas CnAα deficient mast cells were injected into the left ears and foot pads. Non-reconstituted W^{sh} littermates were used as controls. All mice used were gender and age-matched.

2.14: IgE-dependent passive cutaneous anaphylaxis (PCA)

W^{sh} mice reconstituted for 6 weeks with wild type mast cells in right ears and CnAα deficient mast cells in left ears were anaesthetized and sensitized with 20µL of 1ng/µL anti-DNP IgE mAbs (Sigma-Aldrich) using an insulin syringe into the dorsal side of the ear pinnae overnight. 10µL of 10mg/mL DNP-BSA (Biosearch Technologies, Novato, CA) was mixed with 190µL 1% Evan's Blue dye (Sigma-Aldrich) and left overnight at 4˚C. The following day, DNP-BSA in Evan's Blue dye (working concentration of 500µg/mL) was intravenously injected into each sensitized mouse with a 30-gauge needle through the tail vein. The reaction was allowed to occur for 30 minutes before mice were sacrificed and ear tissues harvested into 2mL tubes. Ear tissues were cut into pieces and immersed in 2mL *N, N*-dimethyl formamide (Sigma-Aldrich) and for 2 hours in an 80˚C water bath to extract the blue dye. Tubes were centrifuged and 200µL of supernatant was collected from each to measure absorbance at 620nm.

2.15: IgE-dependent late phase cutaneous anaphylaxis (LPA)

Reconstituted W^{sh} mice were anaesthetized and passively sensitized by intravenous injection of 2μg anti-DNP IgE mAbs through the tail vein using a 30-gauge needle. After 24 hours, a cutaneous reaction was elicited by the topical application of 20μL dinitrofluorobenzene (DNFB, 0.3% wt./v.; Sigma-Aldrich) in acetone/olive oil (4:1) to both sides of the ears and hind foot paws and allowed to react for 24 hours. Mice were then sacrificed, and the thickness of the ears and hind foot pad were measured using a digital micrometer. Ear punches (8mm) were taken and hind paws removed pre-weighed 1.5mL microfuge tubes. The weights of the hind paws and extracted ear tissues were determined using a balance and subtracting the weight of their respective pre-weighed 1.5mL microfuge tubes.

2.16: Statistical analyses

Statistical analyses of results were done using analysis of variance and *t* tests as appropriate, and results were significant when $p<0.05$. Data displayed in figures are represented as mean ± standard error of the mean.

CHAPTER 3: RESULTS

Section 3.1: Genotyping of CnAα neonatal tissue and mast cell monocultures

CnAα heterozygous mice were bred and neonatal mice were housed in a specific-pathogen-free (SPF) environment. Using littermate wild type and knockouts diminished potential confounders such as the microbiome and maternal stress that otherwise may alter the phenotype in both parent and newborns used for experiments. Three wild type and three CnAα knockout cell culture flasks were established from liver harvested for mast cell monocultures. To ensure there was no cross-contamination during cell culture, throughout the course of the experiments periodic genotyping was performed on aliquots from each flask (see Figure 3.1).

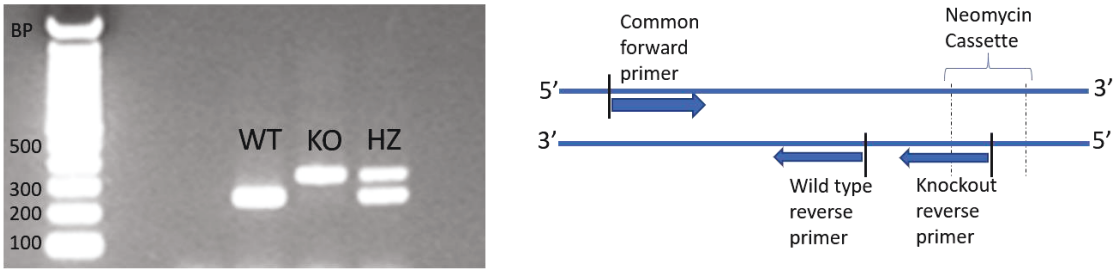

Figure 3.1: Representation of genotyping results of wild type (WT), CnAα deficient (KO), and CnAα heterozygous (HZ) positive control samples with DNA ladder, and corresponding gene sequence schema. A neomycin cassette was inserted and used to disrupt the sequence coding for a portion of the catalytic domain resulting in CnAα deficiency. Approximate base pair (bp) size information can be found in table 2.

Section 3.2: Morphological properties of CnAα wild type and deficient mast cells

The first set of experiments were to determine whether CnAα deficiency led to alterations in mast cell development that would potentially lead to modifications in activation in an IgE-dependent manner. It is important to identify any structural differences such as morphology, granularity, and expression of key surface receptors to establish a baseline standard for comparison between wild type and CnAα deficient mast cells.

3.2.1: CnAα wild type and deficient MCs show similar morphology

Morphological characteristics of the cultured mast cells were assessed to determine if CnAα had an impact on the development of mast cell shape and granularity. This was done using toluidine blue staining (Figure 3.2a). It was determined that there were no significant differences in shape and granularity between cells of the two genotypes. A representative photomicrograph of each genotype is shown in Figure 3.2b (original magnification x 100).

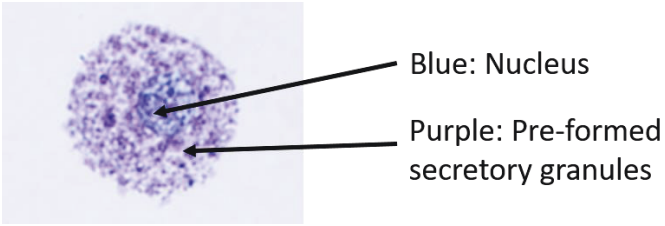

Figure 3.2a: MCs were stained with toluidine blue to determine mast cell morphology. The blue colour represents the nucleus, and the purple represents the granules.

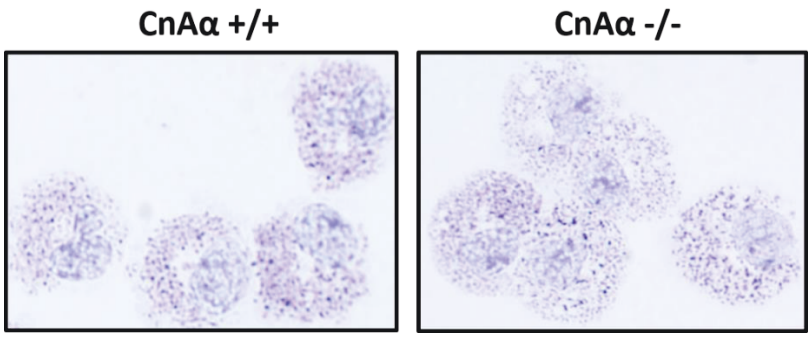

Figure 3.2b: Mast cell morphology is similar between CnAα wild type and deficient MCs. A representative photomicrograph of each genotype following toluidine blue staining is shown (original magnification = 100X) of several CnAα wild type and deficient mast cells. Specimens were viewed using Nikon ACT-1 software version 2.20, and images processed using Adobe Photoshop 5.0.

3.2.2: CnAα wild type and deficient MCs display similar surface expression of CD117 and FcϵRI

Mast cell monocultures were analyzed using flow cytometry to determine the expression levels of specific mast cell surface markers. Analysis by flow cytometry allows for an objective comparison of the mast cell morphology based on forward and side scatter properties. The forward and side scatter properties of mast cells of the two strains were similar, affirming our toluidine blue staining results (Figure 3.3a, left-most graphs). We then measured the expression of two surface markers, CD117 and FcϵRI – the receptor for SCF, and the receptor for IgE immunoglobulins, respectively. Using FITC anti-CD117 antibodies, it was determined that there was no significant difference between the expression levels in wild type (96.2% of gated MCs; mean fluorescence intensity (MFI) - 117) and CnAα deficient (93.6% of gated MCs; MFI - 133) mast cells (Figure 3.3a). Similarly, using a FITC anti-IgE antibody to indirectly determine expression of FcϵRI on IgE-sensitized MCs, there was comparable expression between

wild type (95.7% of gated MCs; MFI - 313) and CnAα deficient (93.1% of gated MCs; MFI - 297) mast cells (Figure 3.3b). The staining results are summarized in Table 5. These results indicate that CnAα deficient mast cells are not lacking in the development of the important growth factor receptor (SCF), and there is no deficiency in the level of FcεRI expression. Understandably, using an anti-IgE antibody is an indirect method of measuring FcεRI, but it also allows us to form a secondary interpretation - that IgE saturation of the receptors between both genotypes is normal and are similar. Antibodies against FcεRI alpha subunits are available but would only give a comparison of surface receptor expression and exclude measurement of IgE binding.

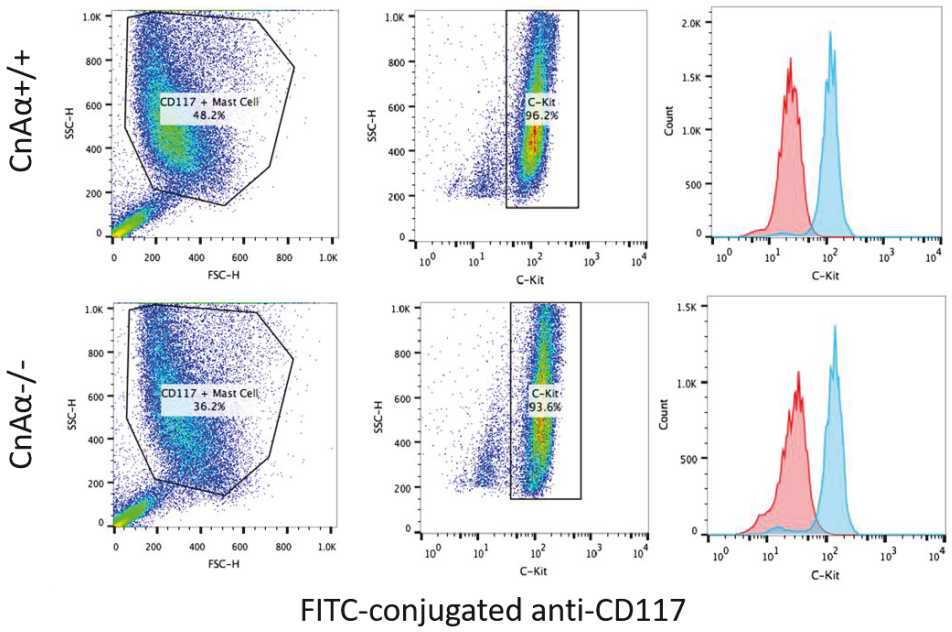

Figure 3.3a: Mature MCs were examined using flow cytometry for surface expression of c-Kit (FITC-conjugated anti-CD117) on gated live CnAα wild type and deficient mast cells. On the histograms on the right, blue peaks are the antibody of interest and red peaks are the corresponding isotype control. Wild type and CnAα deficient mast cells showed similar surface expression of c-Kit at 96.2% and 93.6% of gated live cells (MFI – 117 vs. 133), respectively.

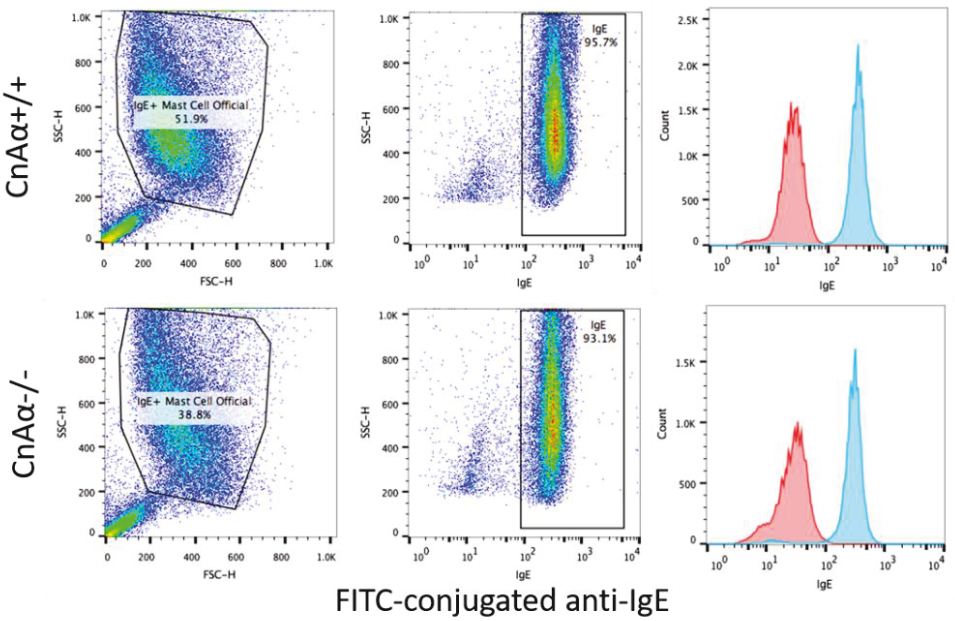

Figure 3.3b: MC maturation was examined using flow cytometry (FACSCalibur) for surface expression of FcεRI using FITC-conjugated anti-IgE antibodies on sensitized gated live CnAα wild type and deficient mast cells. On the histograms on the right, blue peaks are the antibody of interest and red peaks are the corresponding isotype control. Wild type and CnAα mast cells showed similar surface expression of FcεRI at 95.7% and 93.1% of sensitized gated live cells (MFI – 313 vs. 297), respectively.

Table 5: Compiled table of percent of gate mast cells expressing each receptor, comparing between CnAα wild type and deficient mast cells. Values represent percentage of gated live mast cells expressing each receptor and respective median fluorescent intensity (MFI).

	Percent of "mast cell" gated cells	
	C-Kit (CD117)	**FcεRI**
WT/MFI	96.2% / 117	95.7% / 313
KO/MFI	93.6% / 133	93.1% / 297

3.2.3: CnAα wild type and deficient MCs display similar calcium mobilization

Calcium signaling is an important event that precedes and promotes the activation of calcineurin from its inactive to active form and is critical to IgE-dependent signaling cascades. Thus, it is important to assess intracellular calcium fluxes within activated mast cells. Sensitized wild type and CnAα deficient MCs were incubated with an intracellular calcium indicator, Fura 2-AM, prior to stimulation. The stimulated intracellular calcium flux was found to be unaffected in CnAα deficient mast cells compared to wild type controls (Figure 3.4). These results indicate that calcium mobilization is not different between wild type and CnAα deficient mast cells, and signaling leading up to activation of calcineurin is not impaired.

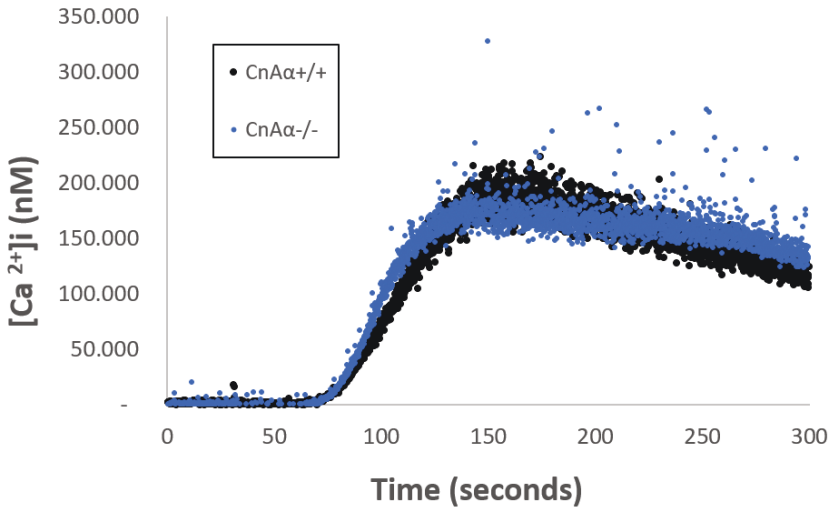

Figure 3.4: CnAα wild type and deficient MCs display similar intracellular calcium flux. MCs were sensitized overnight with IgE and loaded wth Fura-2 AM, a high affinity intracellular calcium indicator. MCs were then stimulated with TNP at 30 seconds and intracellular calcium levels were measured up to 5 minutes (at intervals of every 0.1 seconds), using a Shimadzu RF-5301PC spectrophotometer and associated computer software. Figure is one representative of *n=2* blots from wild type versus CnAα deficient MC experiments.

3.2.4: Basal granular beta-hexosaminidase concentrations are similar in CnAα wild type and deficient MCs

Mast cells respond to FcεRI-mediated activation with the release of many potent mediators, some of which are found pre-stored in granules. To establish whether there is a deficiency in these products within granules comparing wild type to CnAα deficient MCs, basal levels of β-hexosaminidase were measured. Levels of pre-formed and stored β-hexosaminidase was measured by totalling the amount released upon activation with TNP-BSA and the amount left within the mast cell following cell and granule lysis. The total amount of β-hexosaminidase was found to be similar between both wild type and CnAα deficient MCs (Figure 3.5). These results indicate that CnAα deficiency does not affect the mediator levels synthesized and stored in granules.

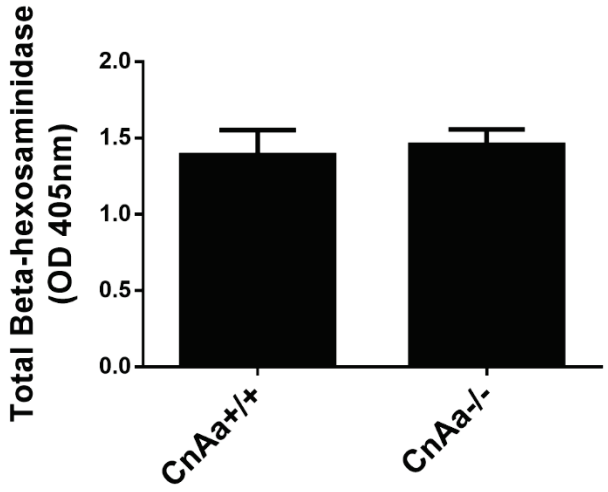

Figure 3.5: Total basal levels of beta-hexosaminidase were similar between wild type and CnAα deficient mast cells. Values were determined from the concentrations (spectrometer wavelength at 405nm) found released in the supernatant and remaining within granules in both genotypes of mast cells. Data shown is a comparison of all CnAα mast cell monocultures. (*n=3* wild type versus CnAα deficient MC experiments)

3.2.5: Basal gene expression of mediators are similar in CnAα wild type and deficient MCs

Another class of mediators produced by mast cells upon FcεRI-mediated activation are *de novo* synthesized cytokines, and we sought to determine if CnAα deficiency impacted basal levels of these cytokines. To assess this, transcripts of several typical cytokines were analyzed in untreated cultured mast cells using RT-qPCR. The results showed transcript levels of all the classical cytokines at baseline were similar between the two genotypes (Figure 3.6), as portrayed by Ct values and on an agarose gel. These results indicate that the deficiency in CnAα does not affect the constitutive level of classical *de novo* synthesized mediator mRNAs.

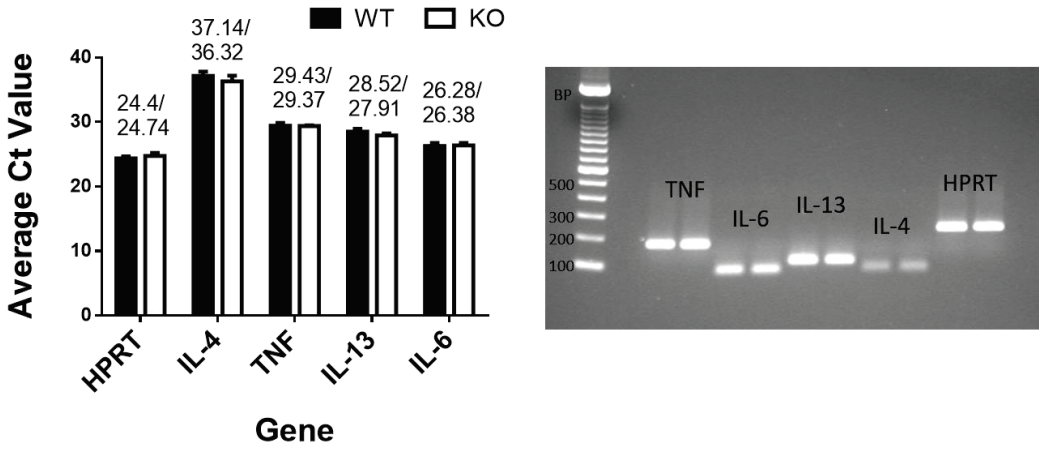

Figure 3.6: Total basal gene transcript levels of classical cytokines TNF, IL-6, IL-13, and IL-4 are similar between both genotypes (HPRT is the housekeeping gene). Gene expression at baseline is shown as Ct values, with insignificant differences between both wild type and CnAα deficient mast cells for all classical cytokines assessed (*n=3* wild type versus CnAα deficient MC experiments). Right image is an agarose gel separation of the RT-qPCR products.

Section 3.3: The role of CnAα in the early phase FcεRI-mediated degranulation in mast cells

One of the major responses of mast cells to IgE-dependent activation is the degranulation of pre-formed mediators stored within their abundant granules. These mediators have different functions such as changing the microenvironment by altering epithelial permeability and initiating an inflammatory cascade. As a critical component of the early phase allergic inflammatory response it is imperative to assess if CnAα deficiency plays a role in the degranulation of mast cells in an IgE-dependent manner.

3.3.1: CnAα deficient MCs display an impaired ability to release pre-formed mediators stored within granules

To determine if CnAα deficiency can affect degranulation, a β-hexosaminidase assay was used to look at the amount released from sensitized mast cells upon activation with TNP-BSA. Following 20 minutes of stimulation with TNP-BSA it was found that CnAα deficient mast cells released approximately 50% less β-hexosaminidase relative to wild type counterparts (Figure 3.7a). Using toluidine blue staining of mast cells before and after stimulation with TNP-BSA, it was observed that CnAα deficient mast cells retained more granules compared to wild type, which had relatively lower or lacked any remaining granules after 20 minutes of stimulation (Figure 3.7b). These findings indicate that CnAα is important in facilitating the emptying of granules containing pre-formed mediators such as β-hexosaminidase following IgE-dependent activation of mast cells.

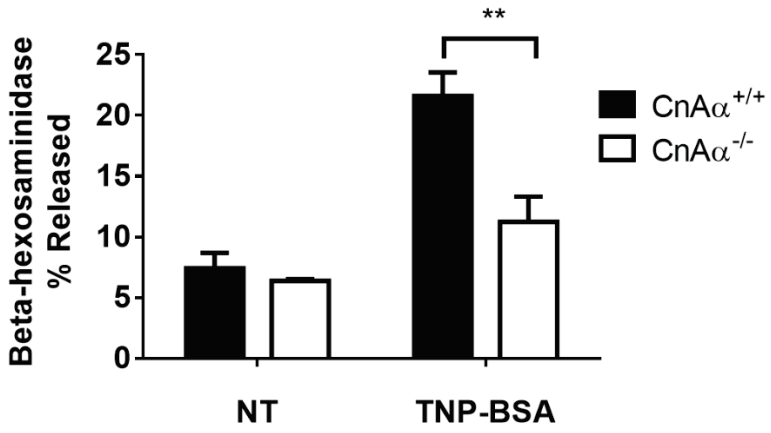

Figure 3.6a: CnAα deficient mast cells have impaired degranulation of β-hexosaminidase. Sensitized CnAα wild type and deficient mast cells were activated with 10ng/mL TNP-BSA and assessed for their degranulation activity of pre-formed mediators using a β-hexosaminidase release assay. Data is representative of mean values +/- SEM. *n=3* wild type versus CnAα deficient MC experiments; ***p<0.01.*

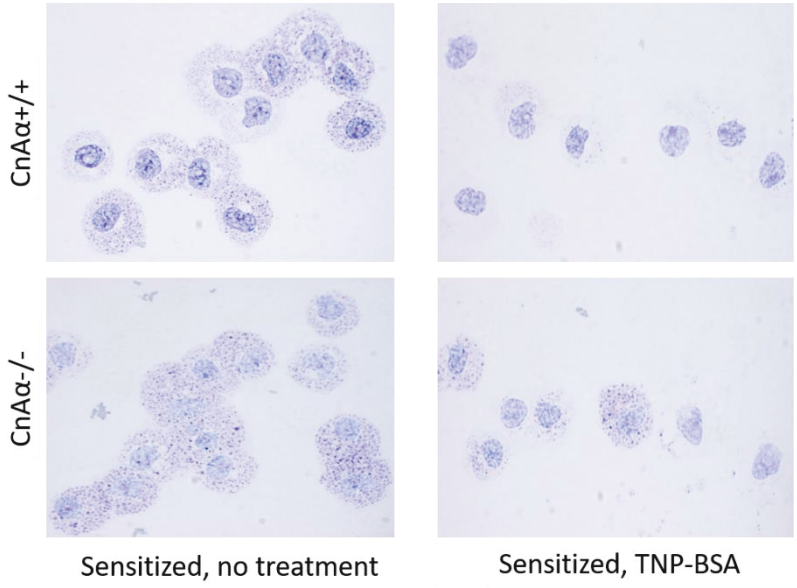

Figure 3.6b: Visual representative of sensitized wild type and CnAα MCs before and after 10ng/mL TNP-BSA stimulation. Mast cells were stained using toluidine blue to observe granulation before and after stimulation of both genotypes of mast cells. Mast cell monocultures with the same genotype were combined to obtain these representative images after stimulation.

3.3.2: CnAα wild type mice show an increase in vascular permeability upon local IgE-dependent MC activation (passive cutaneous anaphylaxis)

To understand if the differences in the rapid degranulation seen *in vitro* were mirrored *in vivo*, changes to local vascular permeability (passive cutaneous anaphylaxis) were measured on CnAα wild type mice in an IgE-dependent fashion. The Evan's Blue dye leakage assay was applied to CnAα wild type mice in two groups, both sensitized intradermally in both ears with anti-DNP IgE. The next day, one group was injected with a saline-Evan's blue dye mixture and the second group with TNP-BSA mixed in Evan's Blue dye, both for 20 minutes. The group challenged with TNP-BSA experienced significantly greater increases in vascular permeability as indicated by amount of Evan's blue dye leakage (Figure 3.8). Thus, FcεRI-mediated activation of mast cells within ear tissues of wild type mice resulted in degranulation of pre-formed mediators that promoted an increase in vascular permeability.

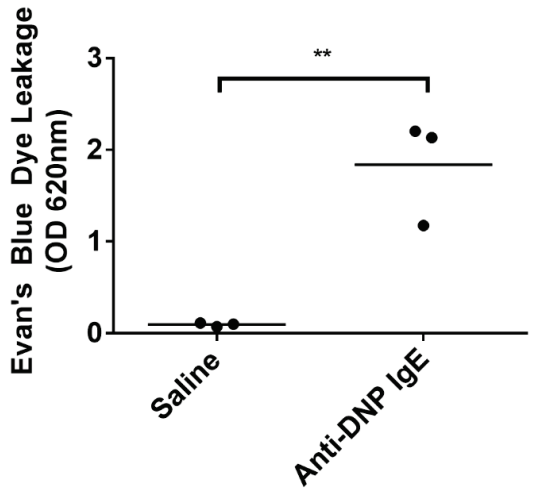

Figure 3.8: Evan's Blue Dye Leakage was used as a measure of vascular permeability *in vivo* to determine mast cell degranulation in a model of passive cutaneous anaphylaxis. Mice stimulated with anti-DNP IgE displayed changes in vascular permeability while

mice with the saline treatment did not, observed as increased sample absorbance. Dye was extracted from isolated ear tissues and absorbance read in a spectrophotometer. *n = 3; **p<0.01.*

3.3.3: Mast cell-deficient mice (W^{sh}) reconstituted with CnAα deficient MCs show diminished FcεRI-mediated vascular permeability after challenge

Considering that CnAα knock out mice experience early lethality, *in vivo* findings relied on experiments performed on mast cell-deficient (Wsh) mice reconstituted with cultured mast cells from CnAα knock out or wild type control monocultures at localized ear tissue sites. Passive cutaneous anaphylaxis was assessed by measuring vascular permeability using the Evan's Blue dye leakage as above. It was determined that, in the ears reconstituted with CnAα knock out mast cells, there was significantly less vascular permeability when challenged with TNP-BSA antigen compared to ears reconstituted with wild type mast cells (Figure 3.9a). Qualitatively, greater blue dye leaked after stimulation with antigen in the right ear of the mouse (Figure 3.9b) reconstituted with wild type mast cells compared to knock out mast cells in the left ear. These findings indicate that, like the *in vitro* findings, the reduction in vascular permeability signifies impaired degranulation associated with CnAα deficiency.

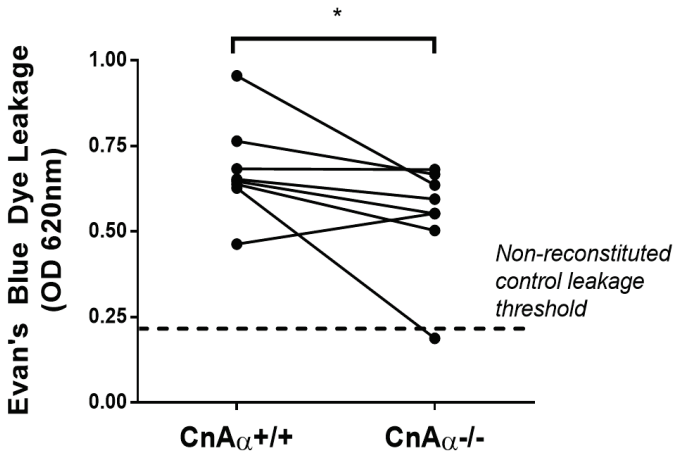

Figure 3.9a: Mast cell-deficient (Wsh) mice ear tissues reconstituted with wild type MCs displayed higher vascular permeability than ear tissues with CnAα deficient MCs. Mice ears containing reconstituted mast cells were sensitized intradermally with anti-DNP IgE and activated 24 hours later with DNP mixed with Evan's Blue Dye via tail vein injection for 30 minutes. Ear tissues were collected and Evan's Blue Dye extracted, and then absorbance read on a spectrophotometer. *n = 8; *p<0.05*

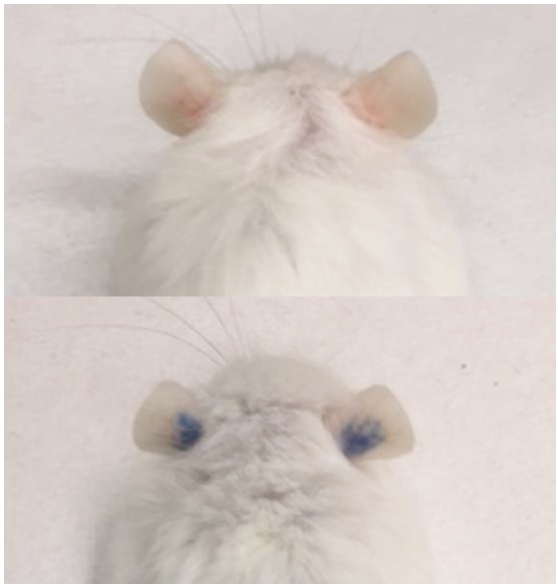

Figure 3.9b: Mice ears reconstituted with wild type MCs (right ear) displayed higher vascular permeability than ears reconstituted with CnAα deficient MCs (left ear), observed as less Evan's Blue Dye leakage. The top image is a sensitized unstimulated animal, while the bottom image is a representative image 30 minutes after DNP stimulation.

Section 3.4: The role of CnAα in the production and secretion of *de novo* synthesized cytokines and chemokines in the late phase FcεRI-mediated immune response

It was determined earlier that constitutive mRNA levels of a number of cytokines are similar in the two genotypes of mast cells. An important outcome of mast cell activation is new synthesis and release of cytokines, including chemokines, following activation in an IgE-dependent manner. We next addressed the impact CnAα deficiency has on typical cytokines TNF, IL-6, IL-13, and IL-4 *in vitro*, and analyze the inflammatory effects in an *in vivo* model of late phase cutaneous anaphylaxis in a reconstituted mast cell-deficient (Wsh) mice.

3.4.1: CnAα deficient MCs release decreased amounts of cytokines TNF, IL-6, IL-13, and IL-4 upon FcεRI-mediated activation

We assessed levels of typical cytokines released by wild type and CnAα deficient mast cells to determine if this gene deficiency resulted in any discrepancies. Our ELISA results showed that CnAα deficient mast cells responded to FcεRI-mediated activation in an impaired manner evident from significantly lower levels in comparison to their wild type counterparts at 3, 6, and 24 hours after stimulation. (Figure 3.10). Taken together, all the classical cytokines released by mast cells upon activation (TNF, IL-6, IL-13, and IL-4) showed pronounced reductions in levels in the supernatants of CnAα deficient mast cells.

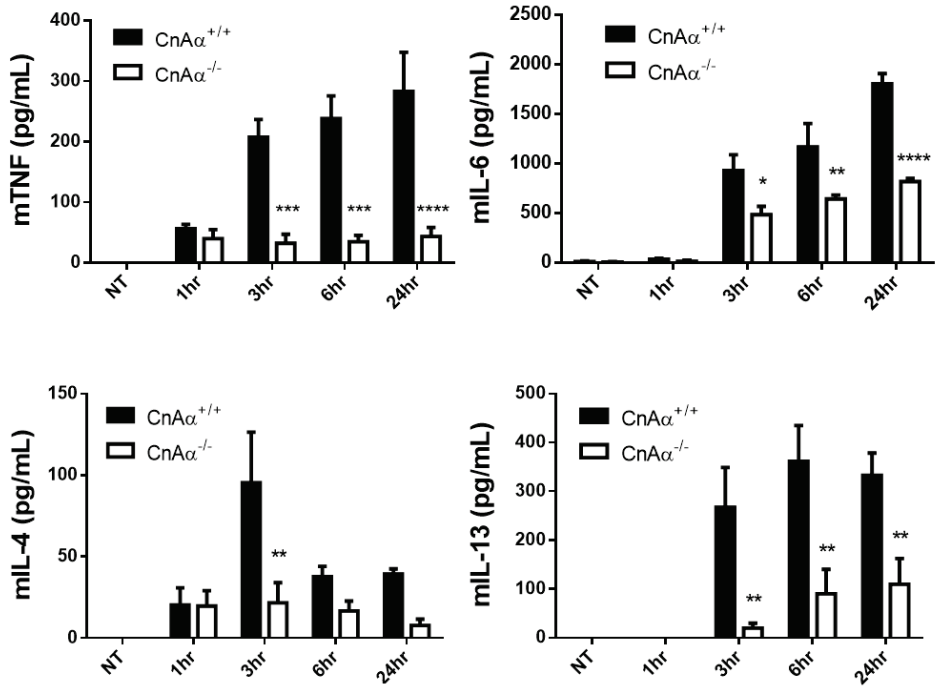

Figure 3.10: CnAα deficient MCs have significantly reduced cytokine levels released into the supernatant upon FcεRI-mediated stimulation with TNP-BSA compared to wild type over various durations. Sensitized mast cells were activated with 10ng/mL TNP-BSA and supernatants used for ELISA experiments. Data is representative of mean values +/- SEM; *n=6, *p,0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

3.4.2: CnAα deficient MCs selectively impair gene transcription of specific cytokines upon FcεRI-mediated activation

Considering that all the secreted classical cytokines were reduced from IgE-dependent activation of CnAα deficient mast cells compared to wild type, we next assessed whether this is possibly due to reduced gene transcript levels associated with the deficiency or if there were any other mechanisms. mRNA levels of TNF, IL-6, IL-13, and IL-4 were analyzed using RT-qPCR, and the results showed two separate trends – TNF

and IL-4 gene transcript levels are significantly diminished in CnAα deficient mast cells compared to wild type, but IL-6 and IL-13 were similar between both genotypes (Figure 3.11). Essentially, the two trends can be seen as "blunted protein and transcript levels" and "blunted protein but no difference in transcript levels." These two patterns in protein and corresponding gene transcript levels are summarized in Table 6, with only IL-6 and IL-13 RNA not being significantly impacted by CnAα deficiency. This indicates that CnAα may differentially regulate cytokine gene expression on a cytokine-based manner.

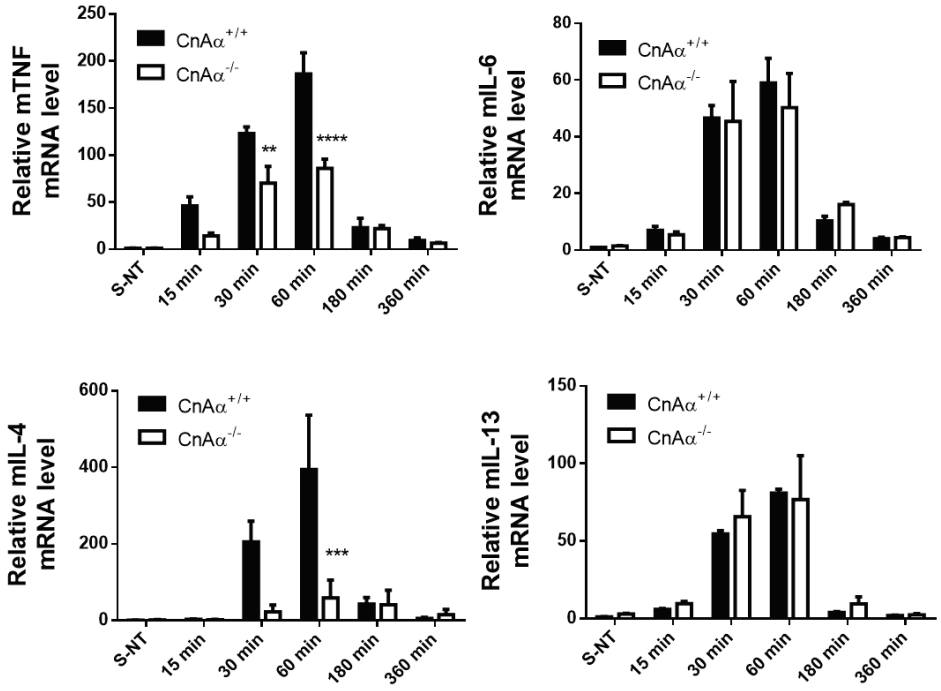

Figure 3.11: CnAα deficient MCs have significantly decreased gene expression of TNF and IL-4, but not IL-13 and IL-6 relative to wild type upon FcεRI-mediated stimulation. Sensitized cells were stimulated with 10ng/mL TNP-BSA for various durations and cDNA synthesized from isolated RNA for RT-qPCR. Data is representative of the mean +/- SEM; *n=3, *p<0.05, **p<0.01, ***p<0.001.*

Table 6: Two patterns (left column) seen in *de novo* synthesized classical mediators after IgE-dependent activation of mast cell. Patterns are in terms of CnAα deficient mast cells relative to wild type counterparts.

KO relative to WT	Protein	RNA
Blunted	TNF, IL-6, IL-13, IL-4	TNF, IL-4
No Change	--	IL-6, IL-13

3.4.3: CnAα deficient MCs demonstrate impaired release of specific chemokines upon IgE-dependent activation

Chemokines are also *de novo* synthesized mediators released by mast cells contributing to IgE-dependent inflammation, so several chemokines were analyzed using an inflammatory panel pre-coated with primary antibodies. Of the different mediators tested using the array, there was no difference in constitutive (i.e untreated) released levels from wild type and CnAα deficient mast cells; however, after FcεRI-mediated activation, in the one protein array used, there was less of several chemokines released by the CnAα deficient mast cells compared to their wild type counterparts (Figure 3.12). Specifically, CCL1 (A), CXCL5/LIX (B), CCL2 (C), CCL3 (D), and CCL9/MIP-1γ (E) were observed to be lower in the supernatants of knock out mast cells, indicated by less dense spots on the antibody-coated membranes. Thus, components of inflammation resulting from *de novo* synthesized cytokines and chemokines are reduced due to CnAα deficiency and promote a decreased inflammatory response *in vitro*.

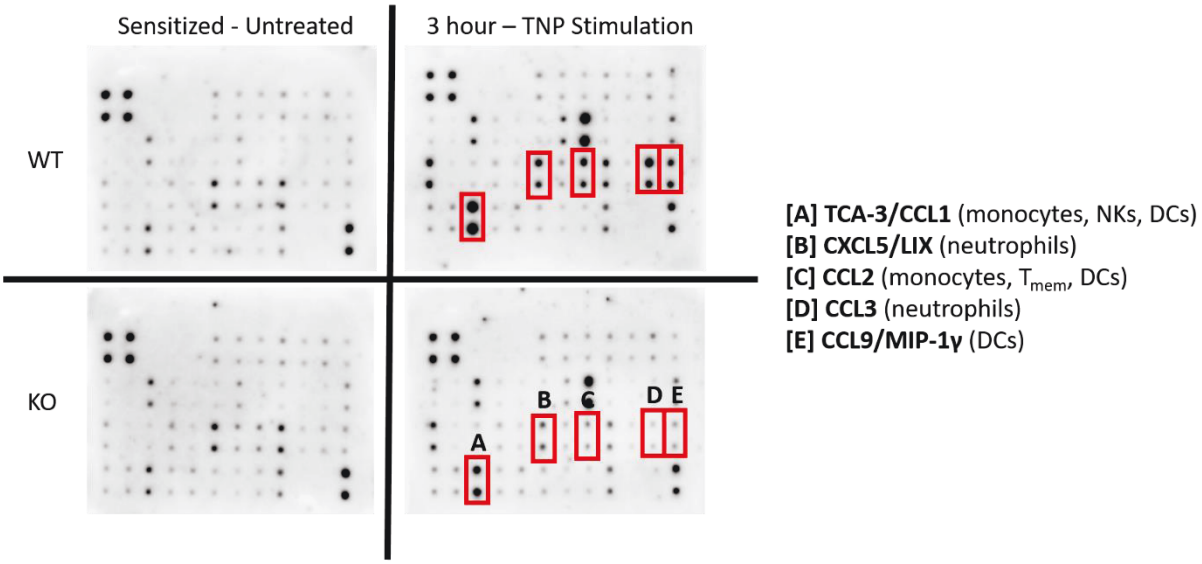

Figure 3.12: CnAα deficient MCs have reduced chemokine secretion compared to wild type counterparts upon FcεRI-mediated stimulation. The representation above is from a RayBiotech inflammatory array with pre-coated antibodies for various inflammatory mediators. Specific chemokines with reduced levels are boxed in red.

3.4.4: Mast cell-deficient mice (W^{sh}) reconstituted with CnAα wild type and deficient MCs show similar FcεRI-mediated inflammatory responses (late phase cutaneous anaphylaxis reaction)

Observing differences in the magnitude of secreted cytokines and chemokines between the two mast cell genotypes led us to assess the *in vivo* effects of inflammation associated with *de novo* synthesized cytokines and chemokines. W^{sh} mice were reconstituted with each genotype of mast cells in localized ear and hind paw sites then challenged with TNP-BSA antigen. Thickness (mm) and mass (mg) of isolated ear and hind paw tissues were used as a measure of inflammation. Regarding ear tissues, those reconstituted with wild type mast cells displayed similar thickness as those reconstituted with CnAα deficient mast cells. Similarly, the mass of the extracts did not significantly

differ from each other, indicative of similar IgE-dependent late phase cutaneous reactions and inflammatory responses (Figure 3.13a). For the hind paws, a similar outcome was also observed, where the thickness and mass of hind paws reconstituted with wild type versus CnAα deficient mast cells did not differ significantly (Figure 3.13b). This outcome indicates that any deficiency in secretion by CnAα deficient mast cells observed *in vitro* cannot be discerned from the *in vivo* FcεRI-mediated late phase inflammatory responses.

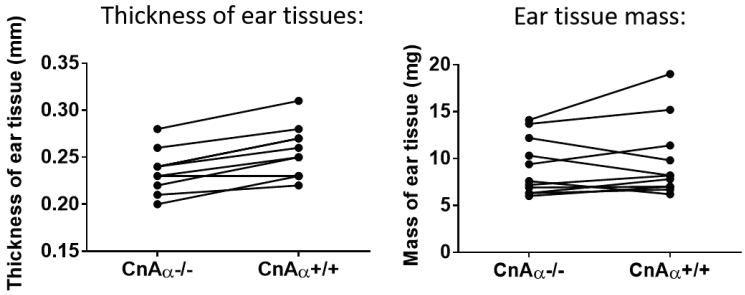

Figure 3.12a: Mast cell-deficient (W^{sh}) mice reconstituted with wild type and CnAα deficient MCs into ear tissues show similar late phase inflammatory responses *in vivo* after 24 hours of IgE-mediate antigen stimulation in a model of late phase cutaneous anaphylaxis reaction. Ear thickness was measured using a digital caliper and isolated ear tissues were weighed to determine inflammatory responses from localized stimulated mast cells in an IgE-dependent manner. *n=11*

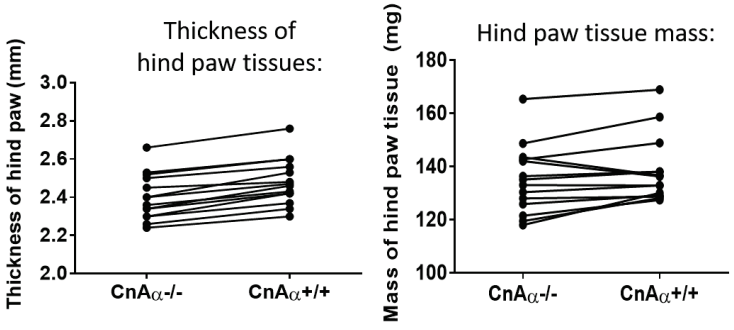

Figure 3.12b: Mast cell-deficient (Wsh) mice reconstituted with wild type and CnAα deficient MCs into hind paw tissues show similar late phase inflammatory responses *in vivo* after 24 hours of IgE-mediate antigen stimulation in a model of late phase cutaneous anaphylaxis reaction. Hind paw thickness was measured using a digital caliper and

isolated hind paw tissues were weighed to determine inflammatory responses from localized stimulated mast cells in an IgE-dependent manner. *n=11*

Section 3.5: CnAα and the effects on key signaling components within the FcεRI-mediated inflammatory pathway

The next course of action was to elucidate the factors influenced by CnAα deficiency that underlie the phenotypic response seen in some of the major outcomes of IgE-dependent mast cell activation measured earlier. To do this, we looked at various signaling elements downstream of the IgE receptor.

3.5.1: Activation of MAPK signaling pathway family members is not impaired in CnAα deficient MCs

The MAPK signaling pathway family members p38, JNK, and ERK are key downstream elements that play a significant role in the transcription of various genes including those seen in inflammatory responses. Activation of these three pathways by phosphorylation, detected by Western blotting, appears to be similar from observations of the corresponding blots and when quantified using densitometric analysis (Figure 3.14). These results indicate that the MAPK signaling pathway family members function similarly despite a deficiency in CnAα. It also promotes the notion that they may not play a significant role in regulating the responses seen in granule degranulation, cytokine synthesis, and release in a CnAα dependent manner.

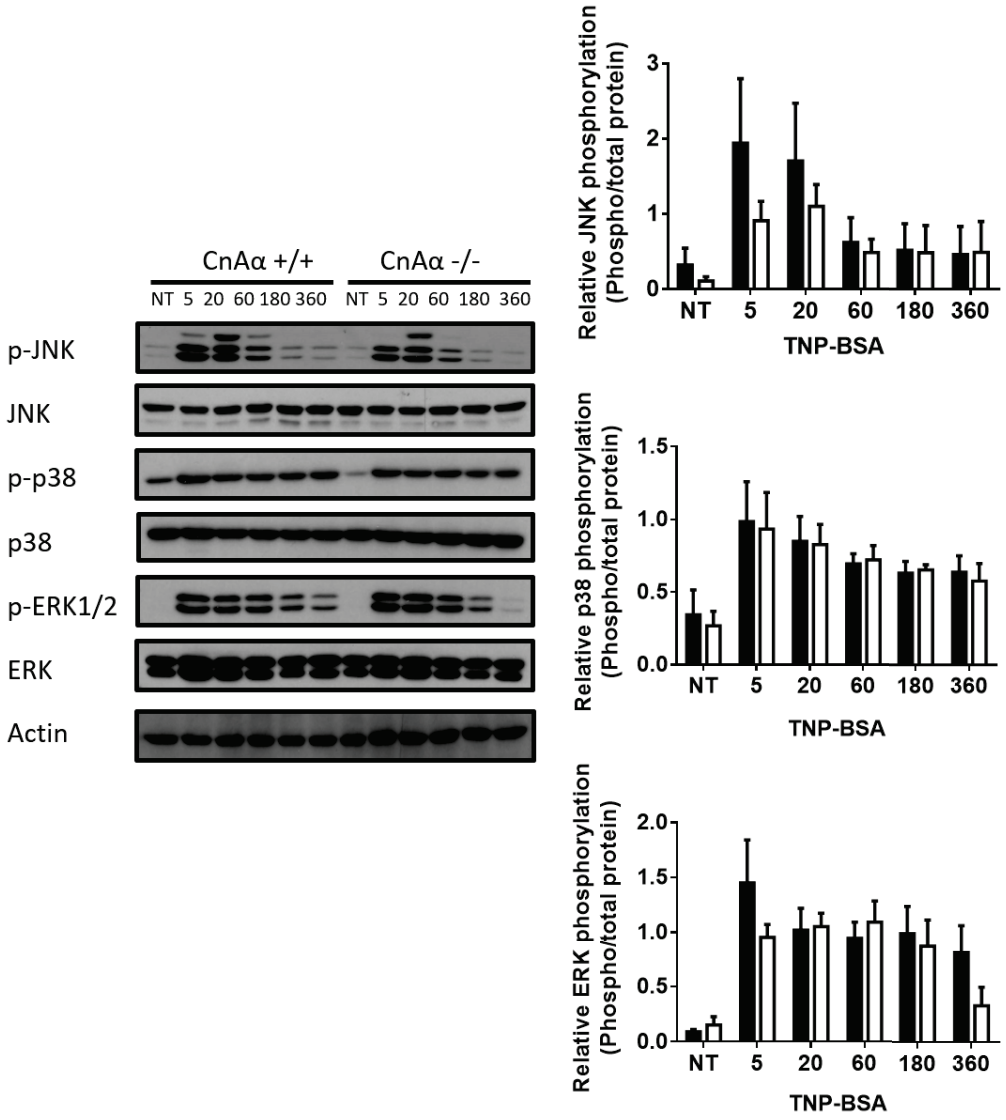

Figure 3.14: Wild type and CnAα deficient MCs display similar MAPK signaling following IgE-dependent activation. Sensitized mast cells were activated with 10ng/mL TNP-BSA and protein isolated for probing with important members of the MAPK signaling family p38, JNK, and ERK using SDS-PAGE gels. Phosphorylation and total protein was measured over various durations of stimulation and quantified using ImageJ software. Wild type protein levels are shown in black bars, and CnAα deficient mast cell protein levels in white bars. *n=3*

3.5.2: Phosphorylaton of IκBα is impaired in CnAα deficient MCs in an IgE-dependent manner

The NF-κB signaling is an important pathway that can mediate inflammatory responses. To determine the activation of NF-κB we measured IκBα, which prevents NF-κB from translocating into the nucleus. Phosphorylation of IκBα leads to its ubiquitination and subsequent degradation, releasing NF-κB of its inhibition, permitting translocation into the nucleus to promote the transcription of various inflammatory genes. Probing blots for phosphorylated IκBα, we found there was less phosphorylation in CnAα deficient mast cells upon activation. Specifically, there was significantly impaired phosphorylation of IκBα at 5 and 20 minutes after TNP-BSA stimulation in CnAα deficient mast cells compared to wild type cells (Figure 3.15). These findings indicate that this signaling path is impaired by CnAα deficiency.

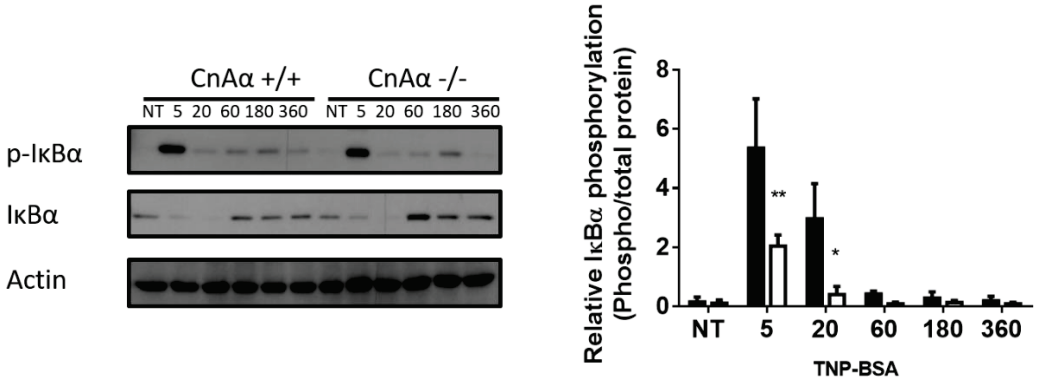

Figure 3.15: Wild type and CnAα deficient MCs display reduced IκBα phosphorylation and degradation at various durations of FcεRI-mediated activation. Sensitized mast cells were activated with 10ng/mL TNP-BSA and protein isolated for probing phosphorylated and total IκBα using SDS-PAGE gels. Phosphorylation and total protein was measured over various durations of stimulation and quantified using ImageJ software. Wild type protein levels are shown in black bars, and CnAα deficient mast cell protein levels in white bars. The left blot is one of three used to calculate the relative changes in the graph (right). *n=3, *p<0.05, **p<0.01.*

3.5.3: NF-κB translocation into the nucleus is impaired in activated CnAα deficient MCs

Given that IκBα phosphorylation and degradation is impaired in CnAα deficient mast cells, the next step was to look at NF-κB levels in the nucleus after stimulation. This was done using an electromobility shift assay. The banding in an EMSA is due to protein binding to radiolabelled oligonucleotides containing a NF-κB binding consensus sequence. The band common to all lanes in the blot on the left (Figure 3.16) were used in densitometric analysis. Looking at the quantified densitometric analysis results of the blots, CnAα deficient mast cells exhibited significantly lower NF-κB levels in the nucleus 20 minutes after FcϵRI-mediated stimulation compared to wild type (Figure 3.16). This finding indicates that there is less NF-κB protein in the nucleus of stimulated CnAα deficient mast cells. This result corresponds with the impaired IκBα phosphorylation and degradation levels associated with CnAα deficiency. The decreased NF-κB levels may also contribute to the decreased inflammatory phenotype observed, specifically protein and mRNA levels of specific cytokines and chemokines.

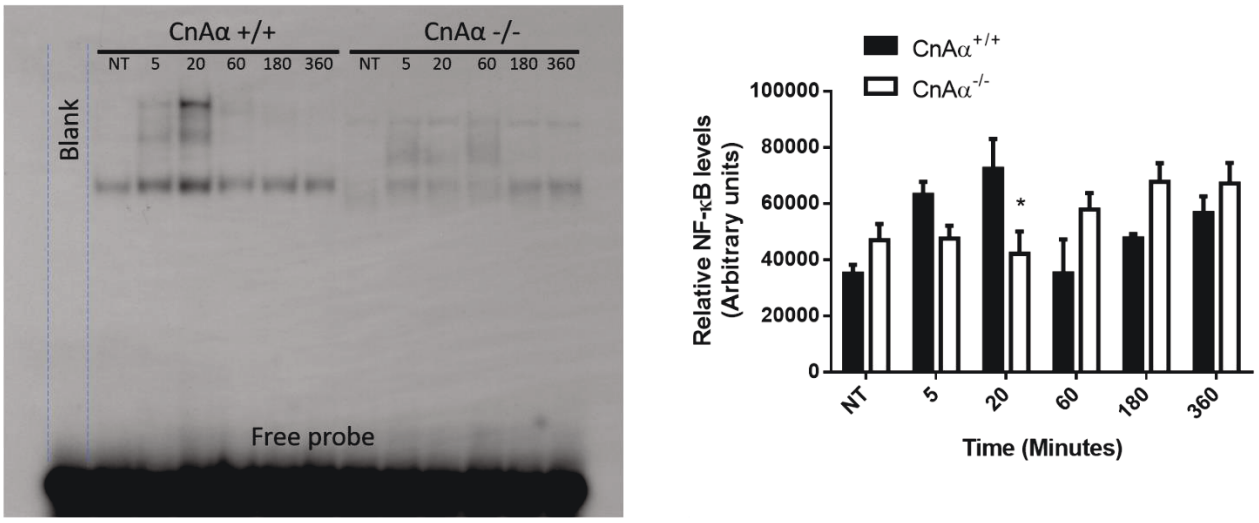

Figure 3.16: Wild type and CnAα deficient MCs display reduced NF-κB levels in the nucleus at various durations of FcεRI-mediated activation. Sensitized mast cells were activated with 10ng/mL TNP-BSA and nuclear protein isolated. A representative blot of three experiments is on the left. Relative NF-κB levels are plotted (arbitrary units) after densitometric analysis and quantification using ImageJ software (right). Wild type protein levels are shown in black bars, and CnAα deficient mast cell protein levels in white bars. *n=3, *p<0.05.*

CHAPTER 4: DISCUSSION

Various leukocytes are charged with defending our bodies, requiring these cells to read and interpret signals from the environment and react in a pre-programmed manner to launch a response. Calcineurin is integral to the flow from interpreting to responding, though we have much to learn about the response. When responses are undesirable, even harmful, calcineurin has been targeted to try to prevent the response from occurring. Various calcineurin inhibitors have been used based on the anti-inflammatory effects they elicit in many clinical settings, including allergies. Found distributed in most mammalian tissues and with an evolutionarily conserved catalytic domain, calcineurin and the different isoforms are likely to have specific contributions towards cells/tissues the isoforms are found distributed in [105, 127]. Certain isoforms may further regulate functions in specific tissues including development and homeostasis. In this thesis, I sought to understand and elucidate the contributions that CnAα makes in the FcεRI-mediated immune response by mast cells. By characterizing the involvement of each isoform in allergy, we can provide the foundation to improve current calcineurin inhibitor therapeutics through increasing specificity to key isoforms and mast cell-specific actions.

Section 4.1: Results in the context of established literature

Most of the current literature on calcineurin in allergy are from results on applications of calcineurin inhibitors – there is a scarcity of fundamental knowledge on the contributions each calcineurin isoform has on the FcεRI-mediated immune response.

This section will seek to place the results for each objective in the context of current established literature.

4.1.1: CnAα and development of mast cells

When determining the development of mast cells, our first assessment was of the structural and granular properties. As mentioned before, a role for CnAα in maturation was established in non-immune cells/tissues such as the kidney and epidermal cells, but CnAα did not influence immune T and B cell growth and development. Despite the spectrum of effects, this promotes the notion of isoform specific contributions in certain cell/tissue types. Armed with this knowledge, we hypothesized that CnAα deficiency would not affect mast cell maturation and contents because there was no recorded impact on the maturation of other immune cell types such as T and B lymphocytes, so far examined. Staining of cultured mast cells displayed similar morphology (size and structure) between both genotypes and this was confirmed by similar forward scatter profiles from flow cytometric analysis. Development of surface receptors c-Kit and FcεRI were also similar between both genotypes, justifying that CnAα does not play a role in the development of mature mast cells.

Existing literature on CnAα deficient mice showed that the gene product played a very important role in submandibular glands, as deficiency resulted in decreased vesicle number and protein content[124]. Considering any impact of CnAα on mast cell granular properties, we hypothesized that there may be a defect in granularity in CnAα deficient mast cells. When assessing our results from staining and side scatter profiles, we did not

observe any significant differences between both genotypes in contrast to the effects seen in salivary glands. Furthermore, when looking at pre-formed granular content within mast cells, there was no significant difference in basal β-hexosaminidase levels found within granules of both mast cell genotypes. This may be a result of tissue-specific effects associated with CnAα, with a dispensable role in the development of mast cell granulation.

Given the lack of structural and granular differences in CnAα deficient and wild type mast cells, there is reason to believe that CnAα does not play a role in the development of mast cells. These results are similar to those seen in T and B cell maturation and importantly indicate that there are cell/tissue-specific contributions of CnAα.

4.1.2: Basal mediator production and FcϵRI-mediated calcium signaling

Prior to determining the effects of CnAα deficiency on the FcϵRI-mediated immune response, it was important to measure the basal levels of all mediators to establish if there were any deficits resulting from the gene deficiency. From the RT-qPCR results, there were no significant differences in C_t values of both genotypes for TNF, IL-4, IL-6, IL-13, and housekeeping gene HPRT mRNA levels. Gene expression of certain mediators such as TNF are important during development of mast cells as the proteins are found to be stored in pre-formed granules[14]. Furthermore, the addition of mast cell growth factor SCF can induce the production of TNF, IL-6, and IL-13 through binding and internalization of c-Kit without activation of FcϵRI-dependent signaling

components[130]. The action of this gene expression is through the transcription factor AP-1 and is likely to be calcineurin-independent, as previous reports showed that most AP-1 regulated transcription was not sensitive to calcineurin inhibitors[131, 132]. Thus, CnAα deficiency does not affect the basal gene expression of mediators in the cultured mast cells.

Calcium signaling is an important event in the FcεRI-mediated signaling pathway preceding calcineurin activation and triggers other signaling events. Calcium-independent activation of mast cells through toll-like receptor ligands did not result in mast cell degranulation or have an influence on release of pre-formed β-hexosaminidase when combined with FcεRI-dependent mast cell activation[133]. These findings confirmed previous work showing a correlation between intracellular calcium concentrations and histamine release – histamine release was maximal at peak intracellular calcium concentrations and decreased parallel to calcium in RBL-2H3 cells[134]. When increased intracellular calcium in the cytosol are not maintained or calcium-dependent calcineurin activity prevented with calcineurin inhibitors, gene transcription is halted and there is a rapid export of transcription factor NFAT back into the cytoplasm to an inactive state[135]. As calcineurin is activated by calcium, it was not surprising that CnAα deficiency did not result in any alterations in intracellular calcium flux upon FcεRI-mediated activation of both genotypes of cultured mast cells. Furthermore, this ruled out signaling events upstream of CnAα activation that may affect the observed CnAα-dependent results in the FcεRI-mediated immune response.

4.1.3: Early phase response

CnAα deficient mast cells showed impaired degranulation compared to wild type as indicated by reduced β-hexosaminidase released from granules *in vitro*. The *in vivo* model of the early phase response – passive cutaneous anaphylaxis – also showed similar findings as ear tissues of mast cell-deficient mice reconstituted with CnAα deficient mast cells displayed reduced vascular permeability upon FcεRI-mediated activation. The reduction is likely due to decreased release of mediators such as histamine from mast cell granules, as histamine is a potent inducer of vascular permeability in allergic inflammation[136].

Calcineurin inhibitors have been previously shown to inhibit the release of preformed mediator histamine from granules upon IgE-dependent mast cell activation[137]. Further research identified inhibitor of nuclear factor kappa-B kinase 2 (IKK-β) as a necessary component of the signaling pathway leading to degranulation, as a deficiency resulted in impaired FcεRI-mediated degranulation of mast cells[138]. The importance of IKK-β in this instance was to phosphorylate synaptosomal-associated protein 23 (SNAP-23)[138], which is involved in the mast cell exocytotic mechanisms[139]. Importantly, this mechanism was found to be independent of NF-κB, which is corroborated by findings that toll-like receptor signaling did not induce mast cell degranulation[133]. Activation of the CBM complex by CnAα may be responsible for downstream signaling through IKK-β and subsequent activation of exocytotic machinery regulating mast cell degranulation. Although the exact mechanism has not been established, the *in vitro* and *in vivo* results demonstrate the significant contribution of CnAα in the release of preformed mediators during the early phase response from FcεRI-mediated mast cell activation.

4.1.4: Late phase response

Analysis of the results from the late phase response showed different functions by CnAα in the FcεRI-mediated immune response. Despite significantly impaired levels of all mediators tested in the supernatants of CnAα deficient mast cells, there were two different patterns in mRNA level changes of the cytokines *in vitro*. Unlike TNF and IL-4, IL-6 and IL-13 mRNA levels were not different between CnAα deficient and wild type mast cells, indicating that the decrease in protein found in the supernatant was not due to deficient gene expression. CnAα may regulate specific gene expression through interactions with transcription factors and possibly regulate mechanisms associated with secretory pathways. In human skeletal muscle cells, it was reported that calcineurin regulated the gene expression of TNF and IL-6 differently, where calcineurin inhibitors decreased ionomycin-induced IL-6 but not TNF mRNA levels[140]. Possible mechanisms that could be playing a role in the differential regulation of these cytokines include selective post-transcriptional regulation such as mRNA stabilization and translation. Considering the lower levels of all cytokines examined so far, we chose to look at multiple chemokines produced *in vitro* using an inflammatory array. There were significantly reduced levels of multiple chemokines in the supernatants of CnAα deficient mast cells compared to wild type. This finding is similar to a study showing FK506 inhibited CCL-1 and CCL-3 gene expression and significantly decreased levels found in the supernatants of IgE-dependent activated cultured human peripheral blood-derived mast cells[141]. Although these results were compared to a different species subset of mast cells, it suggests that calcineurin inhibition results in an impaired chemokine response *in vitro*.

The findings of decreased protein levels of all cytokines tested indicated CnAα may play a role in the regulation of secretory mechanisms involved in release of *de novo* synthesized mediators. The mechanisms behind secretion upon mast cell activation have not been established and may be complex considering the evidence for distinct subsets of proteins in different types of stimuli involving different cytokines[10, 142]. It is known that SNARE proteins are involved with secretory mechanisms in mast cell activation. Different members of the SNARE protein family have roles in packaging, intracellular trafficking, and extracellular secretion of cytokines and chemokines[143, 144]. One member of the SNARE protein family was previously mentioned – SNAP-23. This protein was required for the release of many chemokines including CCL2 and CCL3 from IgE-dependent activation of mature human mast cells[143]. SNAP-23 may also be involved in the early phase response as previously mentioned, due to activation by IKKβ and subsequently leading to mast cell degranulation. Considering the findings that all cytokines are reduced in CnAα deficient mice, CnAα is likely involved in regulation of SNAP-23 activation.

In the *in vivo* model of late phase cutaneous anaphylaxis, there was no significant difference detected between tissues reconstituted with wild type versus CnAα deficient mast cells. In these adoptive transfer experiments, mast cell-specific responses alone are impacted by the CnAα deficiency and there is no systemic gene deficiency. The impaired release of late phase mediators *in vitro* did not have enough impact *in vivo* as other local and recruited immune cell types at the sites of challenge may be contributing to the overall observed inflammation. In a report from our lab on Rcan1 deficiency (an endogenous inhibitor of calcineurin), the late phase cutaneous anaphylaxis was

significantly increased in Rcan1 deficient mice, suggesting that calcineurin is important in promoting late phase responses *in vivo*[123]. However, it is important to note that this experiment was performed in mice with systemic deficiency in the endogenous calcineurin inhibitor, and it remains possible that the loss of Rcan1 in multiple cell types contributed to the heightened response. Either the impact of CnAα in the mast cells is masked by the added effect of infiltrating leukocytes or it does not play a significant role in the late phase response of FcεRI-mediated mast cell allergic inflammation.

4.1.5: CnAα and FcεRI-mediated signaling components

We sought to delineate the mechanisms involved in the phenotypic responses associated with CnAα deficiency in FcεRI-mediated mast cell activation, so we examined key components in the known signaling pathway. We first screened members of the mitogen-activated protein kinase (MAPK) family, p38 kinases (p38), c-Jun N-terminal kinases (JNK), and extracellular signal-regulated kinase-1 and -2 (ERK1/2). Activation of these MAPK family members results in activation of various transcription factors that contribute to production of mediators released in FcεRI-mediated mast cell activation[64, 145].

Our findings demonstrated CnAα does not play a significant role in activation of MAPK family members in FcεRI-mediated mast cell signaling. These results were comparable to a previous report using Rcan1 deficient mast cells, which did not exhibit significant differences in phosphorylation of p38, JNK, or ERK1/2 compared to wild type mice[123].

The NF-κB signaling pathway is crucial in the transcription of many pro-inflammatory mediators in many types of diseases[146], and allergic inflammation is no exception. Our results showed significant reduction in phosphorylation and degradation of IκBα protein, thus impairing the release and translocation of NF-κB into the nucleus to perform gene transcription functions. In a previous report from our lab, deficiency in Rcan1 led to significantly greater NF-κB activity, which corroborates with our findings[123]. Use of pharmaceutical calcineurin inhibitors on a different cell type in another study showed reduced NF-κB activity preceded by reduced IκBα degradation in Jurkat T cells[147].

The NF-κB signaling pathway is activated by calcineurin likely through the upstream CBM complex formation, which is also important in T cell receptor activation[113]. Other investigations uncovered the role of calcineurin in transient dephosphorylation of Bcl-10 necessary for NF-κB activation in T_h cells[112]. The interaction between Bcl-10 and Malt1 in mast cells was analyzed in another report and concluded that this interaction separates FcεRI-mediated degranulation from NF-κB-associated signaling and cytokine production. Responses from the early phase were not diminished in Bcl-10 and Malt1 deficient models but late phase responses such as cytokine production and NF-κB activity were significantly reduced compared to wild type[148].

Impaired NF-κB is the likely explanation behind CnAα leading to the early and late phase phenotypic responses observed. Deficiency in this gene would result in decreased dephosphorylation of Bcl-10 in the CBM complex and thus impair IκBα degradation and subsequent NF-κB activation. It is important to understand that this is

not the only signaling axis that may be impaired by a CnAα deficiency – there are others yet to be assessed that could important roles in regulation of the phenotypic responses.

Section 4.2: Limitations

The work and results from this study are not without limitations – there are several that may be influential in regulating the phenotypic responses we found associated with CnAα gene deficiency or limit the confidence in applying the findings in a physiological setting.

One main limitation is that the mast cells were cultured using cell culture-specific conditions, which means that the results from *in vitro* findings may not be entirely representative of the response of mast cells which developed *in vivo*. It is well-established that mast cell phenotype is heterogeneous and depends on different factors in the microenvironment[11]. The culture conditions may allow for the survival and expansion of cultured mast cells to be used for *in vitro* analysis but may not recapitulate the conditions of the mast cells found within tissues. This confounder can be extended to encompass the idea that mediator expression within the mast cells can differ depending on the microenvironment as well[149, 150]. This may limit the conclusions that can be made on the observed *in vitro* responses and the application to *in vivo* observations. Our *in vivo* models used mast cell-deficient mice reconstituted with mast cells grown in culture-specific conditions.

Another limitation that may have an impact in our findings is the source of mast cells. Traditional murine models of mast cell studies use bone marrow-derived mast cells

as mast cell progenitors circulate from this important hematopoietic source and mature at tissue sites, but our culture was from liver-derived mast cells from neonates. Although the liver is a site of hematopoiesis during fetal development, there is evidence that bone marrow-derived mast cell differentiation processes may be different from those derived from the fetal liver. Furthermore, analysis using next generation sequencing did not show complete matching gene expression between bone marrow-derived and fetal liver-derived mast cells. Analysis of cytokine profile also showed minor differences, notably a significantly higher IL-6 level in supernatants of FcεRI-mediated activation of liver-derived mast cells. However, despite possibly having differences in differentiation and 1.4% difference in gene expression, it was concluded that using mast cells derived from the liver in settings where bone marrow-derivation was not feasible remains a valuable and appropriate alternative to study mast cell-associated allergic inflammation[151]. Previously, our lab has successfully used this method of cell culture to conduct studies regarding mast cell signaling[70, 152] and although it was concluded there are no significant impairments associated with using liver-derived mast cells, there is a minor difference in mast cell source that must be acknowledged.

Finally, there is another limitation to our study that needs to be addressed associated with the experimental procedure for analyzing late phase responses *in vivo*. When reconstituting mice ears and hind paws, wild type mast cells were always injected into the right-sided tissues and CnAα deficient mast cells in left-sided tissues of the mice. When determining thickness using a digital micrometer, there may be unconscious biases that affect the final measurements of wild type and knockout reconstituted tissues. This

could be bypassed if the reconstitutions were done blinded, and thus remains a limitation that must be addressed in the experimental procedure.

Section 4.3: Future directions

The characterization of calcineurin isoforms and the contributions towards FcεRI-mediated immune responses are not complete. There are still mechanisms associated with the alpha isoform that are unknown and promote the phenotypic responses from our work.

Of importance are further analyses into the different transcription factors that may be affected by CnAα deficiency in FcεRI-mediated signaling of mast cells, which may lead to explanations regarding differential cytokine mRNA regulation. Due to some technical issues, we were unable to analyze NFAT activation and levels in the nucleus. As NFAT is a direct substrate of calcineurin and an important transcription factor activated in an FcεRI-mediated manner[153], this is a critical component of the signaling pathway that is necessary to analyze.

As the function of transcription factors are to transcribe a multitude of genes, it will be important to determine how the transcription factors interact with specific promoters as well as mRNA levels of those genes. This would allow a for the delineation to the contribution of specific transcription factors to transcription of specific genes. By analyzing the mRNA levels of other mediators such as chemokines we had found to be impaired in released amounts from CnAα deficient mast cells, we will be able to establish a bigger profile on regulation of potent mast cell-derived products by CnAα.

Furthermore, secretory mechanisms are important to further study as levels of all tested mediators were reduced in CnAα deficient mast cells compared to wild type. This overall reduction of mediators released in combination with current incomplete knowledge of molecular mechanisms regarding the release of cytokines and chemokines from mast cells remain an anomaly but could potentially be a contribution of CnAα to the FcεRI-mediated immune response. Moreover, measuring the intracellular concentrations of the cytokines would establish if protein levels were consistent or not between both genotypes, thus leading to the phenotypic response seen *in vitro*.

Finally, determining the role of the beta isoform is of importance, as CnAβ has been stated to be the predominant isoform in lymphocytes and play a predominant role in immune settings[106, 154, 155]. Furthermore, given that there is residual calcineurin activity likely to be mediated by the beta isoform, it would be of importance to identify the contribution of CnAβ as well as possible calcineurin-independent mechanisms to establish the contribution of each towards this immune response. Nevertheless, CnAα definitively and CnAβ likely contribute to the FcεRI-mediated immune response and this is a crucial area that needs to be explored.

With the development of innovative technology in molecular biology and development of novel *in vivo* models of mast cell deficiency, there is a positive outlook in the capacity to perform the necessary future experiments and at the same time, consider associated limitations. Addressing these future experiments will ideally complete our understanding on specific calcineurin isoform contributions and is necessary before developing potential therapeutics targeting calcineurin.

Section 4.4: Concluding remarks

Allergies remain an increasing health concern and there is a drive to develop improved therapeutics. The mast cell FcεRI-mediated signaling pathway is a complex network of proteins that all participate in processes leading to allergic inflammation. These signaling components are ideal candidates for therapeutic intervention, such as the protein phosphatase calcineurin. However, there remains more research that needs to be done to improve current calcineurin inhibitors, especially when it functions in a systemic inhibitory manner and thus has only been used in specific cases of allergy. In stark contrast to knowledge of calcineurin in organ transplantation, our understanding of calcineurin in allergy is limited to the anti-inflammatory effect of calcineurin inhibitors – we currently lack definitive and genetic evidence of calcineurin isoforms and contributions to the FcεRI-mediated immune response.

Our research work in this thesis has been to delve into the contributions that CnAα makes in the FcεRI-mediated immune response to characterize the role of specific isoforms in allergy. We looked at the role of CnAα in the development of mast cells, the contributions to the early phase allergic response, the late phase response, and touched the surface of possible involved signaling mechanisms. In a summary of our findings (Figure 4.4), we have established that CnAα does not play a role in the development of mast cells and activation. However, CnAα gene deficiency was associated with a significantly reduced ability to release pre-formed β-hexosaminidase *in vitro* in the early phase allergic response and reduced vascular permeability *in vivo*. There was an overall decrease in released *de novo* synthesized mediators TNF, IL-6, IL-4, IL-13, CCL-1, CCL-2, CCL-3, CXCL5, and CCL-9 in the late phase response associated with CnAα

deficiency, but differential regulation of mRNA levels *in vitro*. However, there did not seem to be any impairments in inflammation observed *in vivo* using a model to assess late phase reactions. Looking at important components involved in the signaling cascade, we did not find significant differences in activation of MAPK proteins p38, JNK, and ERK1/2, but impaired phosphorylation of IκBα in CnAα deficient mast cells. Consequently, there was a significantly impaired reduction in NF-κB levels found in the nucleus of CnAα deficient mast cells, which may partially explain the phenotypic responses seen in the late phase allergic response.

These results display a general overview of the contribution of CnAα on the FcεRI-mediated immune response. Through further analyses of possible mechanisms and characterization of other isoforms in allergy, especially with the advancement of techniques and technology, we can establish a foundation of knowledge upon which future improved therapeutics can be developed for allergy.

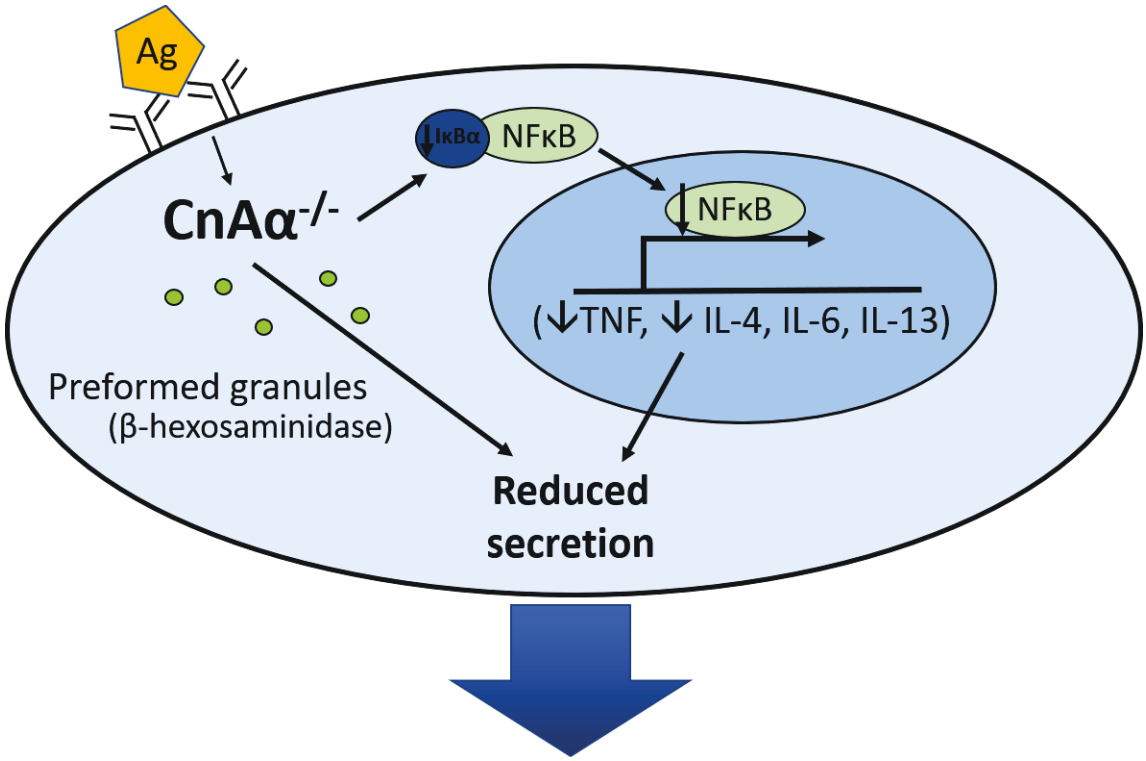

Figure 4.1: Condensed model at the mast cell level to represent our findings in this thesis. Activation of CnAα deficient mast cells through FcεRI resulted in decreased release of pre-formed granules, *de novo synthesized* cytokines and chemokines, decreased TNF and IL-4 mRNA gene expression, and impaired NF-κB levels in the nucleus.

REFERENCES

1. da Silva, E.Z., Jamur, M.C. & Oliver, C. Mast cell function: a new vision of an old cell. *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society* **62**, 698-738 (2014).

2. Galli, S.J., Tsai, M. & Piliponsky, A.M. The development of allergic inflammation. *Nature* **454**, 445-454 (2008).

3. Barnes, P.J. Pathophysiology of allergic inflammation. *Immunol Rev* **242**, 31-50 (2011).

4. Williams, C.M. & Galli, S.J. The diverse potential effector and immunoregulatory roles of mast cells in allergic disease. *The Journal of allergy and clinical immunology* **105**, 847-859 (2000).

5. Gupta, R.S. *et al.* The prevalence, severity, and distribution of childhood food allergy in the United States. *Pediatrics* **128**, e9-17 (2011).

6. Ebert, C.S., Jr. & Pillsbury, H.C., 3rd. Epidemiology of allergy. *Otolaryngologic clinics of North America* **44**, 537-548, vii (2011).

7. Liu, A.H. *et al.* National prevalence and risk factors for food allergy and relationship to asthma: results from the National Health and Nutrition Examination Survey 2005-2006. *The Journal of allergy and clinical immunology* **126**, 798-806 e713 (2010).

8. Jensen-Jarolim, E. & Untersmayr, E. Gender-medicine aspects in allergology. *Allergy* **63**, 610-615 (2008).

9. Broide, D.H. Molecular and cellular mechanisms of allergic disease. *The Journal of allergy and clinical immunology* **108**, S65-71 (2001).

10. Moon, T.C., Befus, A.D. & Kulka, M. Mast cell mediators: their differential release and the secretory pathways involved. *Front Immunol* **5**, 569 (2014).

11. Moon, T.C. *et al.* Advances in mast cell biology: new understanding of heterogeneity and function. *Mucosal immunology* **3**, 111-128 (2010).

12. Bischoff, S.C. Role of mast cells in allergic and non-allergic immune responses: comparison of human and murine data. *Nature reviews. Immunology* **7**, 93-104 (2007).

13. Theoharides, T.C., Kempuraj, D., Tagen, M., Conti, P. & Kalogeromitros, D. Differential release of mast cell mediators and the pathogenesis of inflammation. *Immunol Rev* **217**, 65-78 (2007).

14. Gordon, J.R. & Galli, S.J. Mast cells as a source of both preformed and immunologically inducible TNF-alpha/cachectin. *Nature* **346**, 274-276 (1990).

15. Liu, M. & Yokomizo, T. The role of leukotrienes in allergic diseases. *Allergol Int* **64**, 17-26 (2015).

16. Ohnishi, H., Miyahara, N. & Gelfand, E.W. The role of leukotriene B(4) in allergic diseases. *Allergol Int* **57**, 291-298 (2008).

17. Miyahara, N. *et al.* Leukotriene B4 release from mast cells in IgE-mediated airway hyperresponsiveness and inflammation. *Am J Respir Cell Mol Biol* **40**, 672-682 (2009).

18. Zhang, J.M. & An, J. Cytokines, inflammation, and pain. *Int Anesthesiol Clin* **45**, 27-37 (2007).

19. Kirshenbaum, A.S. *et al.* Demonstration that human mast cells arise from a progenitor cell population that is CD34(+), c-kit(+), and expresses aminopeptidase N (CD13). *Blood* **94**, 2333-2342 (1999).

20. Metcalfe, D.D., Baram, D. & Mekori, Y.A. Mast cells. *Physiol Rev* **77**, 1033-1079 (1997).

21. Welle, M. Development, significance, and heterogeneity of mast cells with particular regard to the mast cell-specific proteases chymase and tryptase. *J Leukoc Biol* **61**, 233-245 (1997).

22. Dahlin, J.S. & Hallgren, J. Mast cell progenitors: origin, development and migration to tissues. *Molecular immunology* **63**, 9-17 (2015).

23. Galli, S.J., Nakae, S. & Tsai, M. Mast cells in the development of adaptive immune responses. *Nat Immunol* **6**, 135-142 (2005).

24. Mukai, K., Tsai, M., Saito, H. & Galli, S.J. Mast cells as sources of cytokines, chemokines, and growth factors. *Immunol Rev* **282**, 121-150 (2018).

25. Galli, S.J., Grimbaldeston, M. & Tsai, M. Immunomodulatory mast cells: negative, as well as positive, regulators of immunity. *Nature reviews. Immunology* **8**, 478-486 (2008).

26. Marshall, J.S. Mast-cell responses to pathogens. *Nature reviews. Immunology* **4**, 787-799 (2004).

27. Yu, Y., Blokhuis, B.R., Garssen, J. & Redegeld, F.A. Non-IgE mediated mast cell activation. *Eur J Pharmacol* **778**, 33-43 (2016).

28. King, C.A., Anderson, R. & Marshall, J.S. Dengue virus selectively induces human mast cell chemokine production. *J Virol* **76**, 8408-8419 (2002).

29. Siebenhaar, F. *et al.* Control of Pseudomonas aeruginosa skin infections in mice is mast cell-dependent. *Am J Pathol* **170**, 1910-1916 (2007).

30. Ierna, M.X., Scales, H.E., Saunders, K.L. & Lawrence, C.E. Mast cell production of IL-4 and TNF may be required for protective and pathological responses in gastrointestinal helminth infection. *Mucosal immunology* **1**, 147-155 (2008).

31. Urb, M., Pouliot, P., Gravelat, F.N., Olivier, M. & Sheppard, D.C. Aspergillus fumigatus induces immunoglobulin E-independent mast cell degranulation. *J Infect Dis* **200**, 464-472 (2009).

32. Brown, J.M., Wilson, T.M. & Metcalfe, D.D. The mast cell and allergic diseases: role in pathogenesis and implications for therapy. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* **38**, 4-18 (2008).

33. Alysandratos, K.D. *et al.* Neurotensin and CRH interactions augment human mast cell activation. *PLoS One* **7**, e48934 (2012).

34. Aung, G. *et al.* Catestatin, a neuroendocrine antimicrobial peptide, induces human mast cell migration, degranulation and production of cytokines and chemokines. *Immunology* **132**, 527-539 (2011).

35. Christensen, L.H., Holm, J., Lund, G., Riise, E. & Lund, K. Several distinct properties of the IgE repertoire determine effector cell degranulation in response to allergen challenge. *The Journal of allergy and clinical immunology* **122**, 298-304 (2008).

36. Gadermaier, E., Levin, M., Flicker, S. & Ohlin, M. The human IgE repertoire. *Int Arch Allergy Immunol* **163**, 77-91 (2014).

37. Roth, K., Chen, W.M. & Lin, T.J. Positive and negative regulatory mechanisms in high-affinity IgE receptor-mediated mast cell activation. *Arch Immunol Ther Exp (Warsz)* **56**, 385-399 (2008).

38. Gilfillan, A.M. & Tkaczyk, C. Integrated signalling pathways for mast-cell activation. *Nature reviews. Immunology* **6**, 218-230 (2006).

39. Turner, H. & Kinet, J.P. Signalling through the high-affinity IgE receptor Fc epsilonRI. *Nature* **402**, B24-30 (1999).

40. Puri, N. & Roche, P.A. Mast cells possess distinct secretory granule subsets whose exocytosis is regulated by different SNARE isoforms. *Proc Natl Acad Sci U S A* **105**, 2580-2585 (2008).

41. Wernersson, S. & Pejler, G. Mast cell secretory granules: armed for battle. *Nature reviews. Immunology* **14**, 478-494 (2014).

42. Schwartz, L.B. & Austen, K.F. Enzymes of the mast cell granule. *J Invest Dermatol* **74**, 349-353 (1980).

43. Schwartz, L.B., Austen, K.F. & Wasserman, S.I. Immunologic release of beta-hexosaminidase and beta-glucuronidase from purified rat serosal mast cells. *J Immunol* **123**, 1445-1450 (1979).

44. Fukuishi, N. *et al.* Does beta-hexosaminidase function only as a degranulation indicator in mast cells? The primary role of beta-hexosaminidase in mast cell granules. *J Immunol* **193**, 1886-1894 (2014).

45. Kuehn, H.S., Radinger, M. & Gilfillan, A.M. Measuring mast cell mediator release. *Curr Protoc Immunol* **Chapter 7**, Unit7 38 (2010).

46. Kushnir-Sukhov, N.M., Brown, J.M., Wu, Y., Kirshenbaum, A. & Metcalfe, D.D. Human mast cells are capable of serotonin synthesis and release. *The Journal of allergy and clinical immunology* **119**, 498-499 (2007).

47. Riley, J.F. Histamine in tissue mast cells. *Science* **118**, 332 (1953).

48. Ciprandi, G. *et al.* Serotonin in allergic rhinitis: a possible role for behavioural symptoms. *Iran J Allergy Asthma Immunol* **10**, 183-188 (2011).

49. Kushnir-Sukhov, N.M. *et al.* 5-hydroxytryptamine induces mast cell adhesion and migration. *J Immunol* **177**, 6422-6432 (2006).

50. White, M.V. The role of histamine in allergic diseases. *The Journal of allergy and clinical immunology* **86**, 599-605 (1990).

51. Simons, F.E. & Simons, K.J. Histamine and H1-antihistamines: celebrating a century of progress. *The Journal of allergy and clinical immunology* **128**, 1139-1150 e1134 (2011).

52. Pejler, G., Ronnberg, E., Waern, I. & Wernersson, S. Mast cell proteases: multifaceted regulators of inflammatory disease. *Blood* **115**, 4981-4990 (2010).

53. Caughey, G.H. Mast cell tryptases and chymases in inflammation and host defense. *Immunol Rev* **217**, 141-154 (2007).

54. Payne, V. & Kam, P.C. Mast cell tryptase: a review of its physiology and clinical significance. *Anaesthesia* **59**, 695-703 (2004).

55. Caughey, G.H. Tryptase genetics and anaphylaxis. *The Journal of allergy and clinical immunology* **117**, 1411-1414 (2006).

56. Waern, I. *et al.* Mouse mast cell protease 4 is the major chymase in murine airways and has a protective role in allergic airway inflammation. *J Immunol* **183**, 6369-6376 (2009).

57. Olszewski, M.B., Groot, A.J., Dastych, J. & Knol, E.F. TNF trafficking to human mast cell granules: mature chain-dependent endocytosis. *J Immunol* **178**, 5701-5709 (2007).

58. Thomas, P.S. Tumour necrosis factor-alpha: the role of this multifunctional cytokine in asthma. *Immunol Cell Biol* **79**, 132-140 (2001).

59. Wilson, S.J., Shute, J.K., Holgate, S.T., Howarth, P.H. & Bradding, P. Localization of interleukin (IL) -4 but not IL-5 to human mast cell secretory granules by immunoelectron microscopy. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* **30**, 493-500 (2000).

60. Steinke, J.W. & Borish, L. Th2 cytokines and asthma. Interleukin-4: its role in the pathogenesis of asthma, and targeting it for asthma treatment with interleukin-4 receptor antagonists. *Respir Res* **2**, 66-70 (2001).

61. Gibbs, B.F. *et al.* Human skin mast cells rapidly release preformed and newly generated TNF-alpha and IL-8 following stimulation with anti-IgE and other secretagogues. *Exp Dermatol* **10**, 312-320 (2001).

62. Gordon, J.R. & Galli, S.J. Release of both preformed and newly synthesized tumor necrosis factor alpha (TNF-alpha)/cachectin by mouse mast cells stimulated via the Fc epsilon RI. A mechanism for the sustained action of mast cell-derived TNF-alpha during IgE-dependent biological responses. *J Exp Med* **174**, 103-107 (1991).

63. Ohkawara, Y. *et al.* Human lung mast cells and pulmonary macrophages produce tumor necrosis factor-alpha in sensitized lung tissue after IgE receptor triggering. *Am J Respir Cell Mol Biol* **7**, 385-392 (1992).

64. MacNeil, A.J., Yang, Y.J. & Lin, T.J. MAPK kinase 3 specifically regulates Fc epsilonRI-mediated IL-4 production by mast cells. *J Immunol* **187**, 3374-3382 (2011).

65. Bradding, P. *et al.* Interleukin-4, -5, and -6 and tumor necrosis factor-alpha in normal and asthmatic airways: evidence for the human mast cell as a source of these cytokines. *Am J Respir Cell Mol Biol* **10**, 471-480 (1994).

66. Bradding, P. *et al.* Immunolocalization of cytokines in the nasal mucosa of normal and perennial rhinitic subjects. The mast cell as a source of IL-4, IL-5, and IL-6 in human allergic mucosal inflammation. *J Immunol* **151**, 3853-3865 (1993).

67. Burd, P.R. *et al.* Interleukin 3-dependent and -independent mast cells stimulated with IgE and antigen express multiple cytokines. *J Exp Med* **170**, 245-257 (1989).

68. Burd, P.R., Thompson, W.C., Max, E.E. & Mills, F.C. Activated mast cells produce interleukin 13. *J Exp Med* **181**, 1373-1380 (1995).

69. Gombert, M. *et al.* CCL1-CCR8 interactions: an axis mediating the recruitment of T cells and Langerhans-type dendritic cells to sites of atopic skin inflammation. *J Immunol* **174**, 5082-5091 (2005).

70. Wu, Z. *et al.* Mast cell FcepsilonRI-induced early growth response 2 regulates CC chemokine ligand 1-dependent CD4+ T cell migration. *J Immunol* **190**, 4500-4507 (2013).

71. Ndaw, V.S. *et al.* TGF-beta1 Suppresses IL-33-Induced Mast Cell Function. *J Immunol* **199**, 866-873 (2017).

72. Yano, K. *et al.* Production of macrophage inflammatory protein-1alpha by human mast cells: increased anti-IgE-dependent secretion after IgE-dependent enhancement of mast cell IgE-binding ability. *Lab Invest* **77**, 185-193 (1997).

73. Juremalm, M. & Nilsson, G. Chemokine receptor expression by mast cells. *Chem Immunol Allergy* **87**, 130-144 (2005).

74. Lukacs, N.W. *et al.* Mast cells produce ENA-78, which can function as a potent neutrophil chemoattractant during allergic airway inflammation. *J Leukoc Biol* **63**, 746-751 (1998).

75. Cildir, G., Pant, H., Lopez, A.F. & Tergaonkar, V. The transcriptional program, functional heterogeneity, and clinical targeting of mast cells. *J Exp Med* **214**, 2491-2506 (2017).

76. Akdis, C.A., Jutel, M. & Akdis, M. Regulatory effects of histamine and histamine receptor expression in human allergic immune responses. *Chem Immunol Allergy* **94**, 67-82 (2008).

77. Deveci, F. *et al.* Evaluation of the anti-inflammatory effect of infliximab in a mouse model of acute asthma. *Respirology* **13**, 488-497 (2008).

78. Hickey, M.J. *et al.* Tumor necrosis factor-alpha induces leukocyte recruitment by different mechanisms in vivo and in vitro. *J Immunol* **158**, 3391-3400 (1997).

79. Jutel, M., Blaser, K. & Akdis, C.A. The role of histamine in regulation of immune responses. *Chem Immunol Allergy* **91**, 174-187 (2006).

80. Patalano, F. & Ruggieri, F. Sodium cromoglycate: a review. *Eur Respir J Suppl* **6**, 556s-560s (1989).

81. Finn, D.F. & Walsh, J.J. Twenty-first century mast cell stabilizers. *British journal of pharmacology* **170**, 23-37 (2013).

82. Woska, J.R., Jr. & Gillespie, M.E. SNARE complex-mediated degranulation in mast cells. *J Cell Mol Med* **16**, 649-656 (2012).

83. Oka, T., Kalesnikoff, J., Starkl, P., Tsai, M. & Galli, S.J. Evidence questioning cromolyn's effectiveness and selectivity as a 'mast cell stabilizer' in mice. *Lab Invest* **92**, 1472-1482 (2012).

84. Yang, Y. *et al.* Polyphenols differentially inhibit degranulation of distinct subsets of vesicles in mast cells by specific interaction with granule-type-dependent SNARE complexes. *Biochem J* **450**, 537-546 (2013).

85. Jewell, J.L., Oh, E. & Thurmond, D.C. Exocytosis mechanisms underlying insulin release and glucose uptake: conserved roles for Munc18c and syntaxin 4. *Am J Physiol Regul Integr Comp Physiol* **298**, R517-531 (2010).

86. Moresco, E.M. & Brandl, K. Linking membrane trafficking and intestinal homeostasis. *Tissue Barriers* **1**, e23119 (2013).

87. Metcalfe, D.D. Isolation of tissue mast cells. *Curr Protoc Immunol* **Chapter 7**, Unit 7 25 (2001).

88. Radinger, M., Jensen, B.M., Kuehn, H.S., Kirshenbaum, A. & Gilfillan, A.M. Generation, isolation, and maintenance of human mast cells and mast cell lines derived from peripheral blood or cord blood. *Curr Protoc Immunol* **Chapter 7**, Unit 7 37 (2010).

89. Reber, L.L., Marichal, T. & Galli, S.J. New models for analyzing mast cell functions in vivo. *Trends in immunology* **33**, 613-625 (2012).

90. Butterfield, J.H., Weiler, D., Dewald, G. & Gleich, G.J. Establishment of an immature mast cell line from a patient with mast cell leukemia. *Leuk Res* **12**, 345-355 (1988).

91. Kirshenbaum, A.S. *et al.* Characterization of novel stem cell factor responsive human mast cell lines LAD 1 and 2 established from a patient with mast cell sarcoma/leukemia; activation following aggregation of FcepsilonRI or FcgammaRI. *Leuk Res* **27**, 677-682 (2003).

92. Metcalfe, D.D. Mast cells and mastocytosis. *Blood* **112**, 946-956 (2008).

93. Andersen, H.B. *et al.* Comparison of short term in vitro cultured human mast cells from different progenitors - Peripheral blood-derived progenitors generate highly mature and functional mast cells. *J Immunol Methods* **336**, 166-174 (2008).

94. Dvorak, A.M., Seder, R.A., Paul, W.E., Morgan, E.S. & Galli, S.J. Effects of interleukin-3 with or without the c-kit ligand, stem cell factor, on the survival and cytoplasmic granule formation of mouse basophils and mast cells in vitro. *Am J Pathol* **144**, 160-170 (1994).

95. Rottem, M., Goff, J.P., Albert, J.P. & Metcalfe, D.D. The effects of stem cell factor on the ultrastructure of Fc epsilon RI+ cells developing in IL-3-dependent murine bone marrow-derived cell cultures. *J Immunol* **151**, 4950-4963 (1993).

96. Grimbaldeston, M.A. *et al.* Mast cell-deficient W-sash c-kit mutant Kit W-sh/W-sh mice as a model for investigating mast cell biology in vivo. *Am J Pathol* **167**, 835-848 (2005).

97. Katz, H.R. & Austen, K.F. Mast cell deficiency, a game of kit and mouse. *Immunity* **35**, 668-670 (2011).

98. Wolters, P.J. *et al.* Tissue-selective mast cell reconstitution and differential lung gene expression in mast cell-deficient Kit(W-sh)/Kit(W-sh) sash mice. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* **35**, 82-88 (2005).

99. Gaudenzio, N. *et al.* Analyzing the Functions of Mast Cells In Vivo Using 'Mast Cell Knock-in' Mice. *J Vis Exp*, e52753 (2015).

100. Tsai, M., Grimbaldeston, M.A., Yu, M., Tam, S.Y. & Galli, S.J. Using mast cell knock-in mice to analyze the roles of mast cells in allergic responses in vivo. *Chem Immunol Allergy* **87**, 179-197 (2005).

101. Peschke, K., Dudeck, A., Rabenhorst, A., Hartmann, K. & Roers, A. Cre/loxP-based mouse models of mast cell deficiency and mast cell-specific gene inactivation. *Methods Mol Biol* **1220**, 403-421 (2015).

102. Scholten, J. *et al.* Mast cell-specific Cre/loxP-mediated recombination in vivo. *Transgenic Res* **17**, 307-315 (2008).

103. Hsu, P.D., Lander, E.S. & Zhang, F. Development and applications of CRISPR-Cas9 for genome engineering. *Cell* **157**, 1262-1278 (2014).

104. Goodman, M.A., Moradi Manesh, D., Malik, P. & Rothenberg, M.E. CRISPR/Cas9 in allergic and immunologic diseases. *Expert Rev Clin Immunol* **13**, 5-9 (2017).

105. Aramburu, J., Heitman, J. & Crabtree, G.R. Calcineurin: a central controller of signalling in eukaryotes. *EMBO Rep* **5**, 343-348 (2004).

106. Williams, C.R. & Gooch, J.L. Calcineurin inhibitors and immunosuppression - a tale of two isoforms. *Expert Rev Mol Med* **14**, e14 (2012).

107. Rusnak, F. & Mertz, P. Calcineurin: form and function. *Physiol Rev* **80**, 1483-1521 (2000).

108. Rumi-Masante, J. *et al.* Structural basis for activation of calcineurin by calmodulin. *J Mol Biol* **415**, 307-317 (2012).

109. Ye, Q. *et al.* Structural basis of calcineurin activation by calmodulin. *Cell Signal* **25**, 2661-2667 (2013).

110. Li, H., Rao, A. & Hogan, P.G. Interaction of calcineurin with substrates and targeting proteins. *Trends Cell Biol* **21**, 91-103 (2011).

111. Crabtree, G.R. Calcium, calcineurin, and the control of transcription. *J Biol Chem* **276**, 2313-2316 (2001).

112. Frischbutter, S., Gabriel, C., Bendfeldt, H., Radbruch, A. & Baumgrass, R. Dephosphorylation of Bcl-10 by calcineurin is essential for canonical NF-kappaB activation in Th cells. *Eur J Immunol* **41**, 2349-2357 (2011).

113. Palkowitsch, L. *et al.* The Ca2+-dependent phosphatase calcineurin controls the formation of the Carma1-Bcl10-Malt1 complex during T cell receptor-induced NF-kappaB activation. *J Biol Chem* **286**, 7522-7534 (2011).

114. Klemm, S. & Ruland, J. Inflammatory signal transduction from the Fc epsilon RI to NF-kappa B. *Immunobiology* **211**, 815-820 (2006).

115. Chaverri, C. Impact of cyclosporine in the development of immunosuppressive therapy. *Transplant Proc* **36**, 80S-82S (2004).

116. Ho, S. *et al.* The mechanism of action of cyclosporin A and FK506. *Clin Immunol Immunopathol* **80**, S40-45 (1996).

117. Hoorn, E.J. *et al.* Pathogenesis of calcineurin inhibitor-induced hypertension. *J Nephrol* **25**, 269-275 (2012).

118. Naesens, M., Kuypers, D.R. & Sarwal, M. Calcineurin inhibitor nephrotoxicity. *Clin J Am Soc Nephrol* **4**, 481-508 (2009).

119. Castro, A.P. Calcineurin inhibitors in the treatment of allergic dermatitis. *J Pediatr (Rio J)* **82**, S166-172 (2006).

120. de Paulis, A. *et al.* Characterization of the anti-inflammatory effect of FK-506 on human mast cells. *J Immunol* **147**, 4278-4285 (1991).

121. Segal, A.O., Ellis, A.K. & Kim, H.L. CSACI position statement: safety of topical calcineurin inhibitors in the management of atopic dermatitis in children and adults. *Allergy Asthma Clin Immunol* **9**, 24 (2013).

122. Hutchinson, L.E. & McCloskey, M.A. Fc epsilon RI-mediated induction of nuclear factor of activated T-cells. *J Biol Chem* **270**, 16333-16338 (1995).

123. Yang, Y.J. *et al.* Rcan1 negatively regulates Fc epsilonRI-mediated signaling and mast cell function. *J Exp Med* **206**, 195-207 (2009).

124. Reddy, R.N. *et al.* Rescue of calcineurin Aalpha(-/-) mice reveals a novel role for the alpha isoform in the salivary gland. *Am J Pathol* **178**, 1605-1613 (2011).

125. Gooch, J.L., Toro, J.J., Guler, R.L. & Barnes, J.L. Calcineurin A-alpha but not A-beta is required for normal kidney development and function. *Am J Pathol* **165**, 1755-1765 (2004).

126. Pena, J.A., Losi-Sasaki, J.L. & Gooch, J.L. Loss of calcineurin Aalpha alters keratinocyte survival and differentiation. *J Invest Dermatol* **130**, 135-140 (2010).

127. Gooch, J.L. An emerging role for calcineurin Aalpha in the development and function of the kidney. *Am J Physiol Renal Physiol* **290**, F769-776 (2006).

128. Kayyali, U.S., Zhang, W., Yee, A.G., Seidman, J.G. & Potter, H. Cytoskeletal changes in the brains of mice lacking calcineurin A alpha. *J Neurochem* **68**, 1668-1678 (1997).

129. Zhang, B.W. *et al.* T cell responses in calcineurin A alpha-deficient mice. *J Exp Med* **183**, 413-420 (1996).

130. MacNeil, A.J., Junkins, R.D., Wu, Z. & Lin, T.J. Stem cell factor induces AP-1-dependent mast cell IL-6 production via MAPK kinase 3 activity. *J Leukoc Biol* **95**, 903-915 (2014).

131. Emmel, E.A. *et al.* Cyclosporin A specifically inhibits function of nuclear proteins involved in T cell activation. *Science* **246**, 1617-1620 (1989).

132. Mattila, P.S. *et al.* The actions of cyclosporin A and FK506 suggest a novel step in the activation of T lymphocytes. *EMBO J* **9**, 4425-4433 (1990).

133. Qiao, H., Andrade, M.V., Lisboa, F.A., Morgan, K. & Beaven, M.A. FcepsilonR1 and toll-like receptors mediate synergistic signals to markedly augment production of inflammatory cytokines in murine mast cells. *Blood* **107**, 610-618 (2006).

134. Beaven, M.A. *et al.* The mechanism of the calcium signal and correlation with histamine release in 2H3 cells. *J Biol Chem* **259**, 7129-7136 (1984).

135. Timmerman, L.A., Clipstone, N.A., Ho, S.N., Northrop, J.P. & Crabtree, G.R. Rapid shuttling of NF-AT in discrimination of Ca2+ signals and immunosuppression. *Nature* **383**, 837-840 (1996).

136. Ashina, K. *et al.* Histamine Induces Vascular Hyperpermeability by Increasing Blood Flow and Endothelial Barrier Disruption In Vivo. *PLoS One* **10**, e0132367 (2015).

137. Harrison, C.A., Bastan, R., Peirce, M.J., Munday, M.R. & Peachell, P.T. Role of calcineurin in the regulation of human lung mast cell and basophil function by cyclosporine and FK506. *British journal of pharmacology* **150**, 509-518 (2007).

138. Suzuki, K. & Verma, I.M. Phosphorylation of SNAP-23 by IkappaB kinase 2 regulates mast cell degranulation. *Cell* **134**, 485-495 (2008).

139. Guo, Z., Turner, C. & Castle, D. Relocation of the t-SNARE SNAP-23 from lamellipodia-like cell surface projections regulates compound exocytosis in mast cells. *Cell* **94**, 537-548 (1998).

140. Keller, C., Hellsten, Y., Steensberg, A. & Pedersen, B.K. Differential regulation of IL-6 and TNF-alpha via calcineurin in human skeletal muscle cells. *Cytokine* **36**, 141-147 (2006).

141. Kato, A. *et al.* Dexamethasone and FK506 inhibit expression of distinct subsets of chemokines in human mast cells. *J Immunol* **182**, 7233-7243 (2009).

142. Lacy, P. & Stow, J.L. Cytokine release from innate immune cells: association with diverse membrane trafficking pathways. *Blood* **118**, 9-18 (2011).

143. Frank, S.P., Thon, K.P., Bischoff, S.C. & Lorentz, A. SNAP-23 and syntaxin-3 are required for chemokine release by mature human mast cells. *Molecular immunology* **49**, 353-358 (2011).

144. Lorentz, A., Baumann, A., Vitte, J. & Blank, U. The SNARE Machinery in Mast Cell Secretion. *Front Immunol* **3**, 143 (2012).

145. Tsai, M., Chen, R.H., Tam, S.Y., Blenis, J. & Galli, S.J. Activation of MAP kinases, pp90rsk and pp70-S6 kinases in mouse mast cells by signaling through the c-kit receptor tyrosine kinase or Fc epsilon RI: rapamycin inhibits activation of pp70-S6 kinase and proliferation in mouse mast cells. *Eur J Immunol* **23**, 3286-3291 (1993).

146. Christian, F., Smith, E.L. & Carmody, R.J. The Regulation of NF-kappaB Subunits by Phosphorylation. *Cells* **5** (2016).

147. Frantz, B. *et al.* Calcineurin acts in synergy with PMA to inactivate I kappa B/MAD3, an inhibitor of NF-kappa B. *EMBO J* **13**, 861-870 (1994).

148. Klemm, S. *et al.* The Bcl10-Malt1 complex segregates Fc epsilon RI-mediated nuclear factor kappa B activation and cytokine production from mast cell degranulation. *J Exp Med* **203**, 337-347 (2006).

149. Lee, Y.M. *et al.* Alteration of protease expression phenotype of mouse peritoneal mast cells by changing the microenvironment as demonstrated by in situ hybridization histochemistry. *Am J Pathol* **153**, 931-936 (1998).

150. Xing, W., Austen, K.F., Gurish, M.F. & Jones, T.G. Protease phenotype of constitutive connective tissue and of induced mucosal mast cells in mice is regulated by the tissue. *Proc Natl Acad Sci U S A* **108**, 14210-14215 (2011).

151. Fukuishi, N. *et al.* Generation of mast cells from mouse fetus: analysis of differentiation and functionality, and transcriptome profiling using next generation sequencer. *PLoS One* **8**, e60837 (2013).

152. Yang, Y.J. *et al.* TRAF6 specifically contributes to FcepsilonRI-mediated cytokine production but not mast cell degranulation. *J Biol Chem* **283**, 32110-32118 (2008).

153. Fric, J. *et al.* NFAT control of innate immunity. *Blood* **120**, 1380-1389 (2012).

154. Bommireddy, R. *et al.* Calcineurin deficiency decreases inflammatory lesions in transforming growth factor beta1-deficient mice. *Clin Exp Immunol* **158**, 317-324 (2009).

155. Bueno, O.F., Brandt, E.B., Rothenberg, M.E. & Molkentin, J.D. Defective T cell development and function in calcineurin A beta -deficient mice. *Proc Natl Acad Sci U S A* **99**, 9398-9403 (2002).