University of Alberta

The Role of VAMP-7-Mediated Events in Eosinophil Degranulation: Implications from Animal Models of Allergic Airway Inflammation

by

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Abstract

Background: Eosinophil degranulation has been implicated in the development of inflammation in allergic asthma. Expressed by human and mouse eosinophils, VAMP-7 is a SNARE complex protein that has been shown to participate in receptor-mediated degranulation in many cell types including human mast cells, natural killer cells, and eosinophils. In this project, it was hypothesized that VAMP-7 is critical in eosinophil degranulation, and that deletion of the VAMP-7 gene in eosinophils will result in changes in one or more eosinophil effector functions in the development of allergic airway inflammation.

Objective: The role of VAMP-7 in eosinophil degranulation was studied using *in vitro* and *ex vivo* assays. The implication of VAMP-7-mediated events in the development of pathology and airway hyperresponsiveness was evaluated in the mouse model of allergic airway inflammation.

Method: Eosinophil-specific VAMP-7 deficiency was generated using the *Cre-Lox P* binary system. Eosinophils were isolated from the peripheral blood of progeny carrying a Cre-recombined null VAMP-7 allele. VAMP-7-deficient eosinophils were tested for degranulation using *in vitro* stimulation with platelet-activating factor and ionomycin. VAMP-7-deficient eosinophils were also stimulated by adoptive transfer into airways of interleukin-5 and human eotaxin-2 double transgenic mice lacking EPX. Lung function and disease pathology were evaluated in eosinophil-specific VAMP-7-deficient mice treated with an ovalbumin sensitization and challenge protocol that induced disease-like phenotype in mouse airways. Levels of eosinophil crystalloid granule proteins (i.e., EPX, MBP, and Ears) and immunoregulatory cytokines (IL-14 and IL13) were tested in supernatants and BAL samples.

Results: Using the *eCre-LoxP* binary system, a highly efficient *Cre*-mediated activity was achieved that resulted in VAMP-7 gene deletion in eosinophils. VAMP-7-deficient eosinophils demonstrated a significant reduction in granule protein release *in vitro.* This reduction in

secretion was reflected in three different types of eosinophil crystalloid granule proteins evaluated. Significant reduction in secreted EPX from VAMP-7-deficient eosinophils was confirmed following adoptive airway transfer into airways of IL-5/hE2/EPX $^{-/-}$ mice. Using mouse models of allergic airway inflammation, the contribution of VAMP-7-mediated degranulation from mouse eosinophils was demonstrated *in vivo.* Preliminary findings suggest that VAMP-7-mediated degranulation from airway eosinophils is important in establishing the pathology and airway hyperresponsiveness in mouse models of allergic asthma.

I would like to dedicate this thesis to my mother, Jane Wang, for her love and wisdom

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I would like to begin by first acknowledging my mentors Drs. James J. Lee, Redwan Moqbel, and Paige Lacy. Working with them on this project has provided me with invaluable experience and I truly appreciate their guidance over the past four and half years. I would like to thank the other members of the Lee Labs that have helped me along the way. Especially, Ms. Katie Zellner for her assistance in transgenic animal husbandry; Dr. Elisabeth Jacobsen for providing guidance and experience to all the questions I had about animal-based experiments; Dr. Rachel Condjella for her assistance with developing real-time PCR methods; Dr. Hui Lou for providing answers to all the questions I had about PCR and Cre-LoX binary system; Dr. Sergei Ochkur for his guidance in optimizing EPX ELISA; and Alfred Doyle, Will Lesure, John Kim, Cheryl Protheroe, Dana Colbert, Linda Mardel and Charlie Kern for their technical and logistical support.

I would like to thank Dr. Paige Lacy for providing guidance, mentorship, and support throughout this project. I would like to thank other members of my committee, Drs. Gary Eitzen, Harissios Vliagoftis, and Manfred Lindau for their support and guidance. I also would like to acknowledge the services of the Mayo Clinic Flow Cytometry and Cell Sorting Shared Resource, Histopathology and the Sequencing Core Facility. I would like to extend my gratitude to Dr. Hiroshito Harada for generating the VAMP-7 floxed mouse strain, and generously providing the VAMP-7 antisera for which I used in my experiments.

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Abbreviations

AF	*Aspergillus fumigatus*
AHR	Airway hyperresponsiveness
APC	Antigen presenting cell
ASM	Airway smooth muscle
BAL	Bronchoalveolar lavage
CCR3	C-C chemokine receptor type 3
CG	Crystalloid granule
CLC	Charcot-Leyden crystal
CMP	Common myeloid progenitors
DC	Dendritic cells
Ears	Eosinophil-associated ribonucleases
ECP	Eosinophil cationic protein
EDN	Eosinophil derived neurotoxin
ELISA	Enzyme-linked immunosorbent assay
EoP	Eosinophil-lineage committed progenitors
ESGP	Eosinophil secondary granule proteins
EPX	Eosinophil peroxidase
ES cells	Embryonic stem cells
fMLP	*formyl*-methionyl-leucyl-phenylalanine
GFP	Green fluorescent protein
GTP	Guanosine triphosphate

GM-CSF	Granulocyte macrophage-colony stimulating factor
HDM	House dust mite
HSC	Hematopoietic stem cells
IDO	Indoleamine 2, 3-dioxygenase
IFNγ	Interferon gamma
IHC	Immunohistochemistry
i.n.	Intranasal
i.p.	Intraperitoneal
i.t.	Intratracheal
IRES	Internal ribosomal entry site
MMP	Matrix metalloproteases
MCP	Monocyte chemoattractant protein
MBP	Major basic protein
NADPH	Nicotinamide adenine dinucleotide phosphate
NK cells	Natural killer cells
Neo	Neomycin resistance cassette
OVA	Ovalbumin
PAS	Periodic acid-Schiff stain
PAF	Platelet-activating factor
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDG2	Prostaglandin D2

PM	Plasma membrane
PMA	Phorbol 12-myristate 13-acetate
PMD	Piecemeal degranulation
RANTES	Regulated and normal T cell-expressed and secreted
ROS	Reactive oxygen species
SLO	Streptolysin-*O*
SNARE	Soluble NSF attachment protein receptor
TEM	Transmission electron microscopy
TF	Transcription factor
TGF	Transforming growth factor
TLR	Toll-like receptor
VEGF	Vascular endothelial growth factor
WT	Wild type
hE	Human eotaxin

Floxed VAMP-7 mice: B6; 129-Vamp-7<tm1>/RIKEN BRC

Zp3-Cre mice: C57BL/6-TgN(Zp3-Cre)93Knw/J mice; the germ-line *Cre* expressing mice

***Zp3Cre-V7* mice**: Ubiquitous VAMP-7 knockout mice; progeny of the C57BL/6-TgN(Zp3-Cre)93Knw/J mice and the B6; 129-Vamp-7<tm1>/RIKEN BRC mice

***eCre* mice**: B6.129P2-EPX ^{Cre1}/Lee Labs mice; the eosinophil-specific *Cre* expressing mice

***eCre-V7* mice**: Eosinophil-specific VAMP-7 knockout mice; progeny of the B6.129P2-EPX^{Cre1} /Lee Labs mice and the B6; 129-Vamp-7<tm1>/RIKEN BRC mice

***eCre-V7-IL5* mice**: *eCre-V7* mice crossed to C57BL/6-Tg(IL-5)NJ.1638/Lee Labs, which generate peripheral blood eosinophilia

***e-GFP* mice:** B6.Cg-*Gt(ROSA)26Sor*$^{tm6(CAG\text{-}ZsGreen1)Hze}$/J mice; high-throughput *Cre* reporter mice

***e-GFP-eCre* mice**: Eosinophil-specific GFP mice; progeny of the ROSA-GFP mice and the B6.129P2-EPX^{Cre1}/Lee Labs mice, which provide a means to measure *Cre*-mediated recombination events *in vivo*.

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Chapter One: Eosinophil Biology and Mouse Models of Asthma

1.1 Eosinophil biology

In 1879, Paul Ehrlich stained blood smears with the aniline dye eosin and observed a type of cell with magenta granules in the cytoplasm. He named these cells eosinophils (Figure 1.1). Tissue eosinophilia is classically associated with helminth infection (1-3) and inflammatory diseases most notably in allergic asthma (4, 5). Eosinophils have been considered as non-specific destructive effector cell types in disease as they manufacture, store, and release potent cytotoxic proteins with documented tissue-damaging capability (6, 7). Recent evidence from clinical and model-based research suggests, however, that eosinophils have a critical role in immune modulation (8) and tissue remodelling (9) in addition to their role in maintaining homeostasis in health (10). Targeting eosinophils in clinical treatments and animal models of allergic diseases has shown therapeutic and prophylactic effects (11-14). A better understanding of eosinophil biology would be beneficial in light of the recently nominated role they may play in inflammation.

1.1.1 Eosinophilopoiesis

In the bone marrow, pluripotent $CD34^+$ hematopoietic stem cells (HSC) are induced by cytokine systems to become eosinophil-lineage committed progenitors (EoP). $CD34^+$ progenitors also can be induced by local cytokine networks to become eosinophils in extramedullary tissues (15-18). Human eosinophils are thought to develop from human HSC through common myeloid progenitors (CMPs) (19-21). The mouse is the most commonly used modern animal model in experimental research on eosinophils, and mouse eosinophils are thought to be derivatives of CMPs through granulocyte macrophage progenitors (22-24) (Figure 1.2). EoPs participate in eosinophilopoiesis in the bone marrow as their number increases

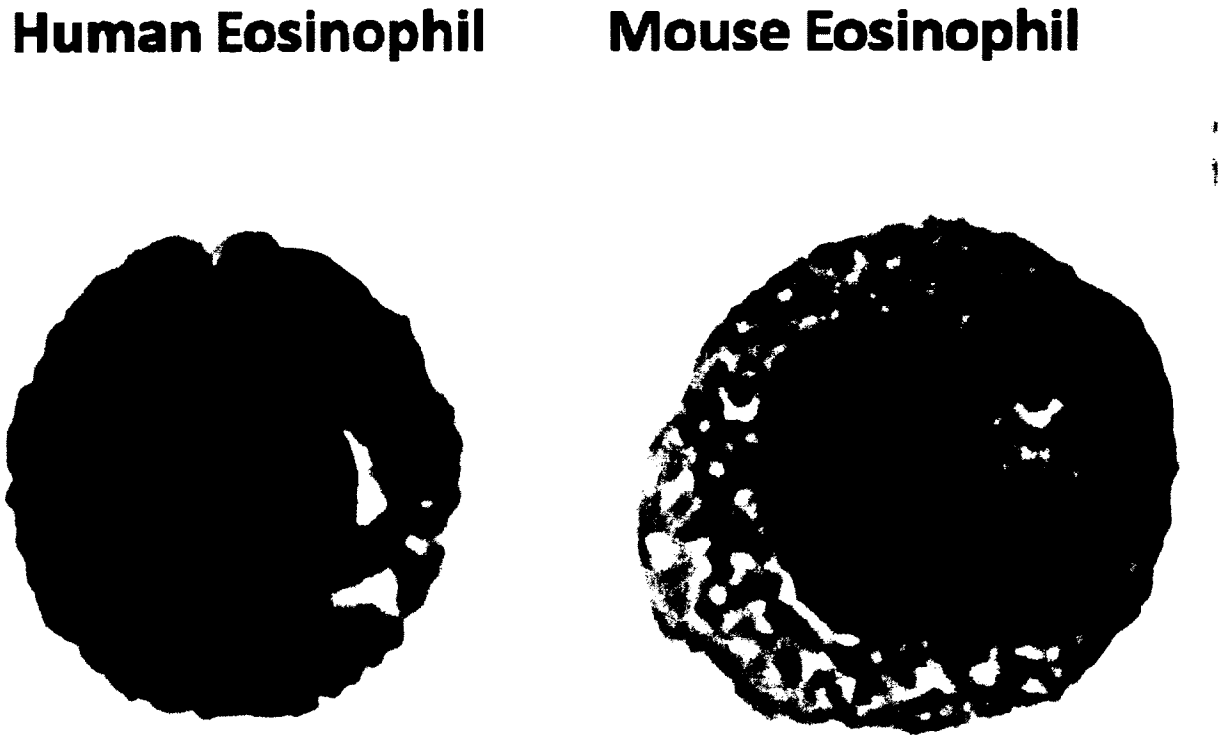

Figure 1.1. Human and mouse blood eosinophils stained with H&E. Human eosinophils (12-15 μM) and mouse eosinophils (9-12 μM) share remarkable similarities. Polymorphic nuclei are observed in both types of eosinophils. The nuclei of mature mouse eosinophils display a ring or figure eight shape. Human eosinophil granules are larger and more vibrant in colour. Eosinophils comprise 0-3% of blood leukocytes.

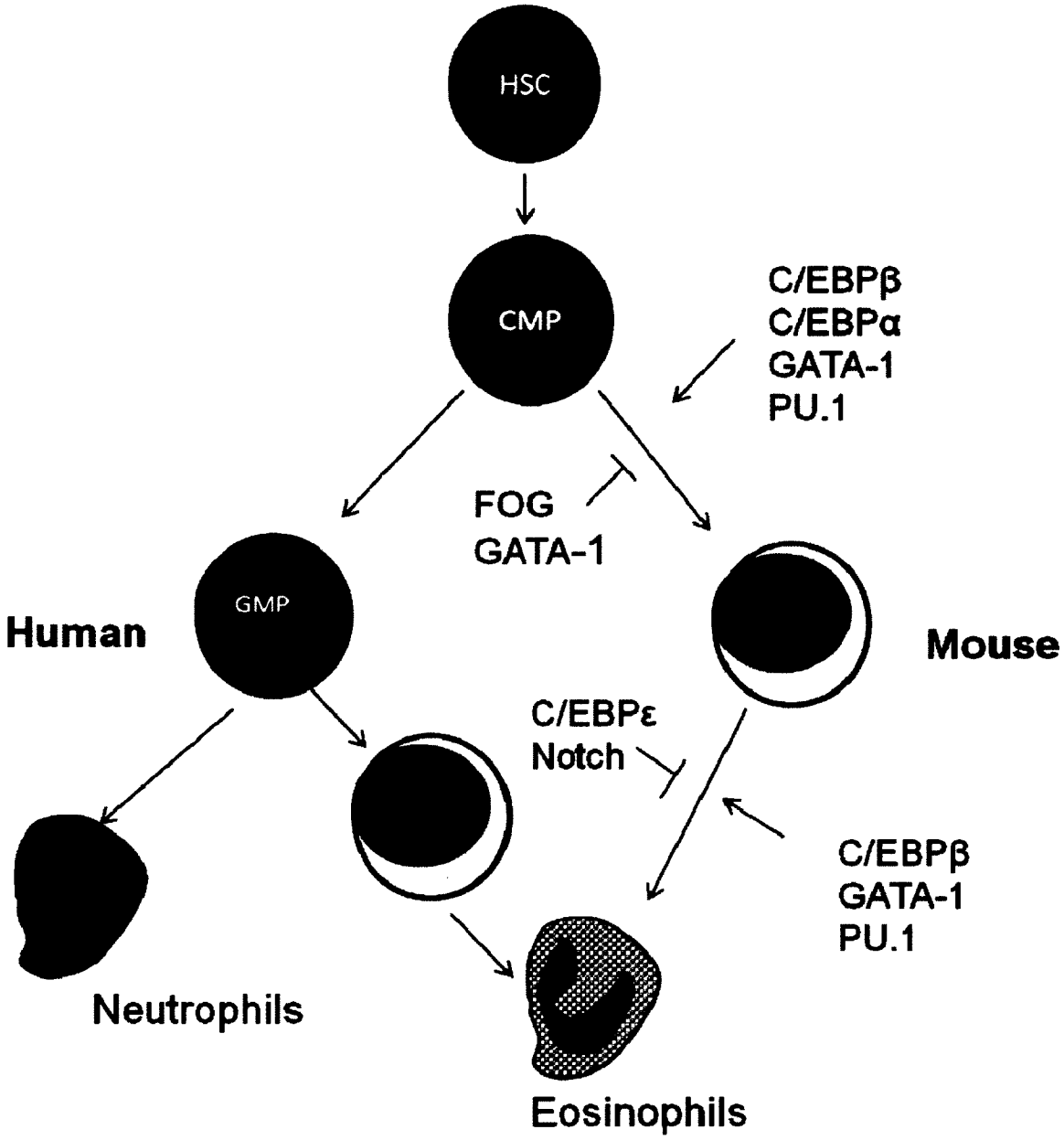

Figure 1.2. Eosinophil development as a result of a complex interplay between transcription factors. Modified from Uhm *et al.* (2012) (24).

during helminth infection and allergen exposure (25, 26). The proper identification of EoP can facilitate treatment in various inflammatory diseases associated with eosinophilia by preventing their development and maturation.

Eosinophil-specific protein production is critical in eosinophilopoiesis. Similar profiles of transcription factor (TF) modulation were shared by eosinophil-specific protein production (27). GATA-1, PU.1, and C/EBPα are three classes of TFs that are critical in eosinophil development (28-30). The consensus DNA-binding sequences of these TFs are present in the eosinophil-specific P2 promoter of the major basic protein (MBP), IL-5Rα, CCR3, and eosinophil-derived neurotoxin genes in human and mice (29-32). GATA-1 expression is three-fold higher in hEoPs than in hCMPs (19). *In vitro* enforced expression of TFs in CMPs suggest a cross antagonism between GATA-1/PU.1 and FOG-1/C/EBPα, providing modulation of eosinophilopoiesis (23, 33, 34).

1.1.2 Maturation

The cytokine milieu is essential to eosinophilopoiesis both in the bone marrow and tissue. IL-3, -5 and granulocyte macrophage-colony stimulation factor (GM-CSF) are critical cytokines influencing eosinophilopoiesis (26, 35-37). Mice deficient in the common β chain shared by IL-3, 5, and GM-CSF receptors have reduced circulating and tissue eosinophils at baseline (36, 38). IL-3/GM-CSF enhance the ability of eosinophils to adhere to epithelial cells and their response to chemokines (39). Airway eosinophilia is a hallmark feature of allergic asthma, and the levels of IL-3, -5, and GM-CSF are increased in asthmatic populations compared to healthy subjects (40).

IL-5 is distinct in its eosinophilopoietic potential as mice with defective IL-5 and /or IL-5 receptor (IL-5Rα) genes have impaired eosinophil development (41). IL-5 receptor expression in EoPs is a significant event in lineage commitment in mice eosinophilopoiesis (22). Increased

IL-5R mRNA and protein expression parallels the increase in IL-5-producing lymphocytes in mouse bone marrow (17, 42, 43). Overexpression of IL-5 by T cells in mice is associated with significant blood eosinophilia and extra-medullary eosinophilopoiesis (44). Bone marrow, blood, and tissue eosinophils respond to IL-5 neutralization differently, which suggests heterogeneous populations exist as the result of varying stages of development and activation (18, 45).

1.1.3 Granule protein expression

The focus of this section will be on prominent groups of secondary granule proteins, as majority of these proteins are toxic to airway epithelium, neurons, and connective tissues (46-54).

Major basic protein (MBP): MBP-1 and -2 are expressed in both human and mouse eosinophils (55-57). MBP-1 has the highest abundance among all eosinophil secondary granule proteins in both humans and mice (56, 57). Its highly cationic nature is maintained in both mouse MBP-1 and -2, but lost in human MBP-2 (58). MBP-1 is the predominately expressed form of the protein in both human and mice eosinophils, however, MBP-1 is also found in human basophils and mast cells (59, 60).

MBP has been associated with the development of pathology as a result of inflammation in systems such as the esophagus, dermis, and airways through direct interaction with the tissue by promoting scarring and remodelling (61-63). MBP may cause epithelial cell damage and mediate development of pathology such as epithelial desquamation (64, 65). The interaction of MBP and the nervous system has also been hypothesized to induce hypersensitivity in the airways by interfering with the M2 muscarinic receptor (61, 66, 67).

Eosinophil peroxidase (EPX): EPX appears to be the only eosinophil-specific protein with no evidence suggesting its expression in other cell types (68). EPX is, however, closely

related to myeloperoxidase (MPO). EPX is found in the electron-translucent matrix of the crystalloid granules in both human and mice (Figure 1.3). The presence of EPX as a result of degranulation or cytolysis has been observed in tissue samples isolated from patients with eosinophilic esophagitis (69), acute lung injury (ALI) (70), and allergic asthma (71).

Charcot-Leyden crystal (CLC) protein: CLC is found in primary granules of human eosinophils and is absent in mouse eosinophils (68, 72). CLC crystals were first detected in sputum samples of asthma patients (73). The expression of CLC is not limited to eosinophils; regulatory T cells have also been found to manufacture and release CLC (74). The activity and function of CLCs remain to be further studied.

Eosinophil-associated ribonucleases (Ears): Ears are a group of ribonucleases that are not necessarily eosinophil-specific, but they are the second most abundant protein group in eosinophils on a molar basis and are found in the electron-translucent matrix of the eosinophil secondary granules. In humans, eosinophil derived neurotoxin (EDN) and eosinophil cationic protein (ECP) are the two main homologous proteins for Ears with varying ribonuclease activities (75, 76). The mouse Ears family contain at least 13 genes (77), and 5 mouse Ears (Ear-1, -2, -6/7, -5/11) can be detected.

1.1.4 Granule types

Human eosinophils contain a minimum of four membrane-bound granule types: crystalloid granules, primary granules, small granules, and secretory vesicles (Table 1.1).

Crystalloid (secondary/specific) granules (CG): These granules (0.5-0.8 μm diameter) contain electron-dense cores and an electron-translucent matrix. EPX, ECP, and Ears are found

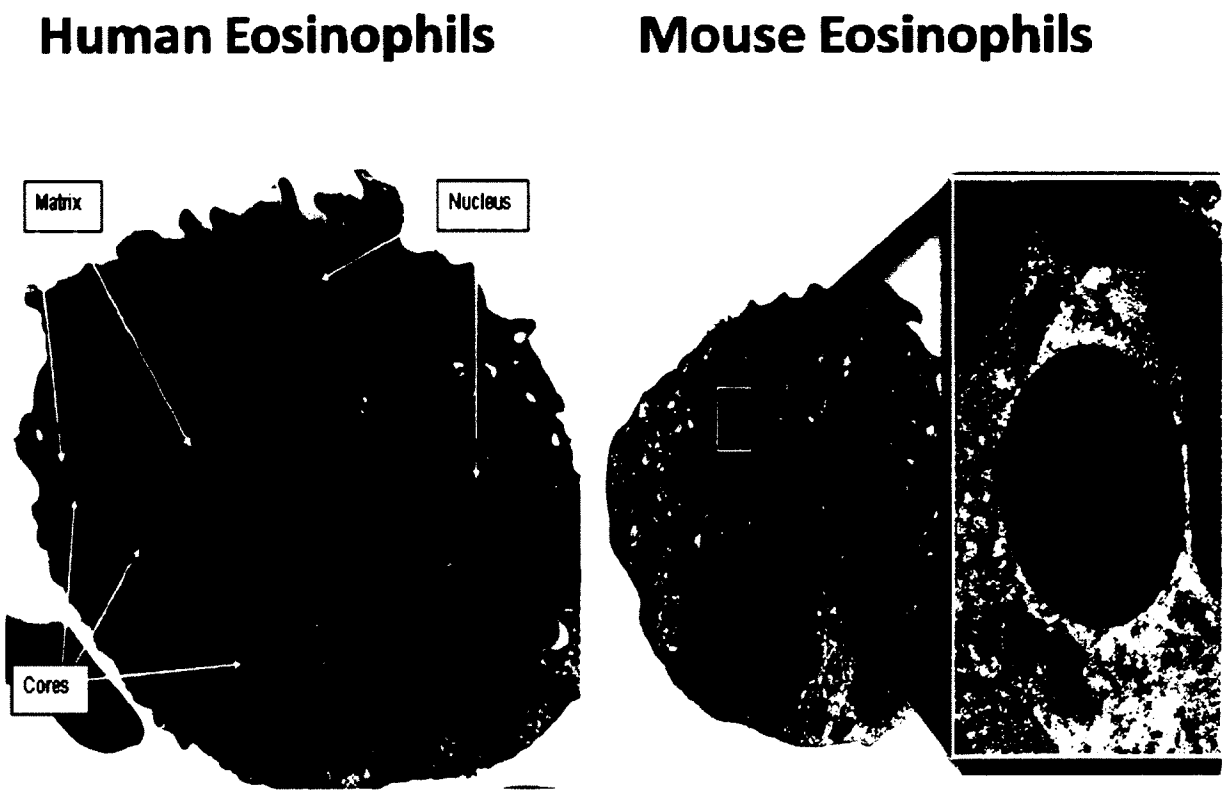

Figure 1.3. Human and mouse eosinophils visualized by transmission electron microscope (TEM). Eosinophils are characterized by their secretory granules with electron-dense cores of MBP and electron-translucent matrices containing EPX and cytokines such as IL-4. Micrographs were courtesy of Dr. Kita and Dr. Lee at the Mayo Clinic.

Crystalloid (secondary/specific granules)	Primary granules	Small granules	Secretory vesicles
Matrix			
MBP	CLC	Acid phosphatase	EPX
EPX		Arylsulphatase B (active)	Albumin
ECP		Catalase	
EDN		Elastase	
Ears (mouse)		ECP	
β-Glucuronidase			
Flavin adenine dinucleotide (FAD)			
Enoyl-CoA-hydrolase			
Cathepsin D			
Collagenase			
Elastase			
Catalase			
Acid phosphatase			
Acyl-CoA oxidase			
Arylsulphatase B (inactive)			
β-Hexosaminidase			
Lysozyme			
Nonspecific esterases			
Phospholipase A2			
3-Ketoacyl-CoA thiolase			
Acid β-glycerophosphatase			
Bactericidal permeability-increasing protein			
Core			
MBP			
Catalase			
β-Glucuronidase			
Enoyl-CoA-hydrolase			
Cathepsin D			

Table 1.1. Eosinophil granules and their contents. Modified from Moqbel, R. *et al.*, (2009) (78) and Lee, JJ. *et al.*,(2012) (68).

Mediators	Location	mRNA	Protein
IL-1α	unknown	√	√
IL-1β	unknown	√	√
IL-2	Crystalloid granules (core)	√	√
IL-3	unknown	√	√
IL-4	Crystalloid granules and small secretory vesicles	√	√
IL-5	Crystalloid granules (core/matrix)	√	√
IL-6	Crystalloid granules (matrix)	√	√
IL-8	Cytoplasmic	√	√
IL-9	unknown	√	√
IL-10	unknown	√	√
IL-11	unknown	√	unknown
IL-12	unknown	√	√
IL-13	unknown	√	√
IL-16	unknown	√	√
IL-17	unknown	√	√
Interferon γ	unknown	√	√
GM-CSF	Crystalloid granules (core)	√	√
TNFα	Crystalloid granules (matrix)	√	√
Eotaxin	Crystalloid granules	√	√
MIP-1α	unknown	√	√
LIF	unknown	√	√
MCP-1	unknown	unknown	√
MCP-3	unknown	√	unknown
MCP-4	unknown	√	unknown
RANTES	Crystalloid granules (matrix) and Small secretory vesicles	√	√
HB-EGF-LBP	unknown	√	unknown
NGF	unknown	√	√
PDGF-B	unknown	√	unknown
TGFβ	unknown	√	√
TGFα	Crystalloid granules (matrix) and Small secretory vesicles	√	√
VEGF	unknown	√	√
SCF	Cytoplasm and membrane	√	√

Table 1.2. Expression and location of eosinophil cytokines. Modified from Moqbel, R. *et al.*, (2009) (78) and Lee, JJ. *et al.*,(2012) (68).

in the matrix of CG (78). Immunoregulatory cytokines (e.g., IL-2, -4, -5,-6, -13, -17, TNF-α, TGF-β and GM-CSF) and chemokines (e.g., regulated and normal T cell expressed and secreted RANTES) have been shown to localize to CG (Table 1.2) (79).

Primary granules: These granules are packaged with Charcot-Leyden crystal protein, which share structural homology with the carbohydrate-binding galectin family (80). Genome sequencing has demonstrated that, unlike human eosinophils, mice do not have the CLC gene (81).

Small granules: These colorless granules contain acid phosphatase, catalase, NADPH oxidase components, arylsulphatase B, and cytochrome *b* (78).

Secretory vesicles: These highly abundant vesicles in the cytoplasm contain EPX (82-84), RANTES (85, 86), and IL-4 (84, 87, 88). Secretory vesicles are considered as a pool of "ready-to-release" cytokines and granule proteins. The rapid mobilization of vesicles and the release of IL-4 observed within 30 to 60 min of stimulation have been observed in human eosinophils (87). Using TEM and immunogold staining, IL-4 has been associated with crystalloid granules and small secretory vesicles (87).

1.1.5 Resident population and tissue recruitment of eosinophils

At baseline, eosinophils are present in the gastrointestinal tract (38), mammary gland (89), thymus (90), and the uterus (91) as tissue-dwelling cells. The recruitment of eosinophils to these tissues from circulation is not well-defined in health. Rather, the mechanisms of eosinophil migration to sites of Th2 inflammation, such as in asthma, is well characterized. Eosinophils are recruited to the circulation and tissues in response to cytokine and chemokine signalling. Residential cells such as mast cells, airway smooth muscle (ASM) cells, skin keratinocytes, airway epithelium, and macrophages (92) secrete eosinophil-specific eotaxin (-1, -2, -3 in

human, and -1, -2 in mice) of the chemokine C-C motif ligand (CCL) family (93). Airway and lung eosinophilia are significantly reduced in eotaxin-1 and -2 double knockout mice compared to single knockouts, which suggests that a synergism between chemokines is necessary for tissue eosinophil recruitment when exposed to allergens (94). Eotaxin-1 is thought to be important in eosinophil bone marrow egress (94). Eotaxin-2 is thought to be important for eosinophil tissue infiltration since bronchoalveolar lavage fluid from allergen-exposed mice is dominated by eotaxin-2 over eotaxin-1. Eotaxin-3 in humans specifically recruits eosinophils to the esophagus, as seen in patients with eosinophilic esophagitis (95), and is absent in mice.

IL-5 induces CCL chemokine receptor CCR3 expression on $CD34^+$ EoP to facilitate eotaxin-mediated egress from bone marrow into the circulation (96, 97). CCR3 is constitutively present on the surface of eosinophils and its expression increases in response to inflammatory stimuli (35). CCR3 and eotaxin interaction initiates actin polymerization, intracellular calcium flux, and chemotaxis for eosinophil mobilization and lung infiltration (94, 98, 99). Eotaxin signalling primes eosinophils by inducing Very Late Antigen (VLA)-4 expressions necessary for adhesion to endothelial cells (100, 101). Under inflammatory conditions, IL-4/13, and IL-5 (102-105) selectively induce endothelial expression of vascular cell adhesion molecule (VCAM)-1, which provides α4-integrin (e.g., VLA-4, 6)-mediated eosinophil recruitment to inflammatory foci (104, 106). Mouse eosinophils with defective CCR3 cannot penetrate venule walls in the lung and are prevented from being recruited to the airways and lung parenchyma in allergic airway inflammation (98, 99). Mice deficient in VCAM-1 and intercellular adhesion molecule (ICAM)-1 are also protected from lung eosinophil and lymphocyte infiltration following allergen exposure (104).

Nonspecific signals such as platelet activation factor (PAF), prostaglandin (PGD_2) and leukotriene (LTs) have documented effects on eosinophil chemotaxis and accumulation (107-

109). IL-5 is associated with enhanced migratory response from eosinophils to LTB_4, PAF and *formyl*-methionyl-leucyl-phenylalanine (fMLP) (110). Eosinophils express high levels of cell surface receptor for PGD2 (i.e., CRTh2) which has been shown to affect eosinophil recruitment and activation (107-109). Eosinophils express cysteinyl leukotriene receptor-1, and antagonist treatments decrease allergen-induced lung eosinophil infiltration in humans (111-113). Circulatory eosinophils are thought to be primed in asthma patients as they respond more readily to mobilization signals (114, 115) and show increased evidence of adhesion compared to eosinophils from healthy controls. Eosinophils isolated from the airways of asthmatics are more responsive to chemotaxis and adhesion signals than eosinophils isolated from peripheral circulation (116).

In the mouse model of allergic disease, eotaxin is also responsible for early recruitment of Th2 cells, which in turn provide Th2 cytokines that further induce the production of eotaxin from monocytes and macrophages (117). Inhibition of eotaxin function via single gene knockout or neutralizing antibody treatments have suggested a synergism is necessary among eotaxin, monocyte chemoattractant proteins (1,5) and IL-5 to induce pulmonary eosinophilia in a mouse model of allergic inflammation (118).

1.2 Eosinophil effector functions

1.2.1 Classical end-stage effector functions

The classical view of the role eosinophils play in mediating pathology in asthma and parasite killing has been mainly destructive (Figure 1.4). Eosinophil numbers in circulation and in tissue increase significantly in allergic diseases and the release of eosinophil secondary granule proteins (ESGP) at sites of inflammation is observed with or without intact cells (Figure 1.5). Biological samples from patients with eosinophilic inflammation and animal models display

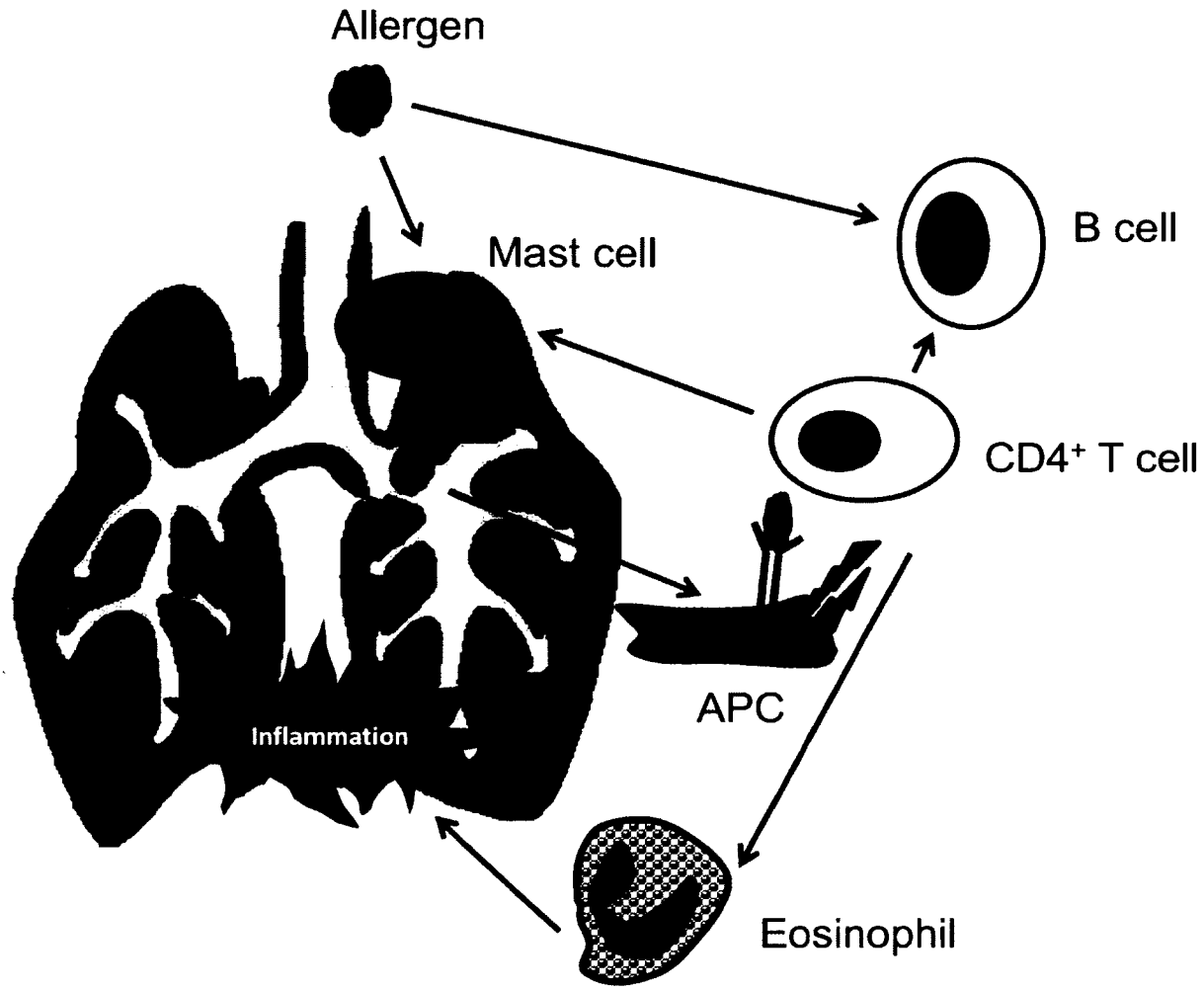

Figure 1.4. Eosinophil classical end-stage effector functions. In this model, in addition to other pathways, eosinophils are recruited to inflammatory sites as a result of T cell polarization and interaction. Once they reach the tissue, mature eosinophils release granule proteins and mediators to directly impact pathology.

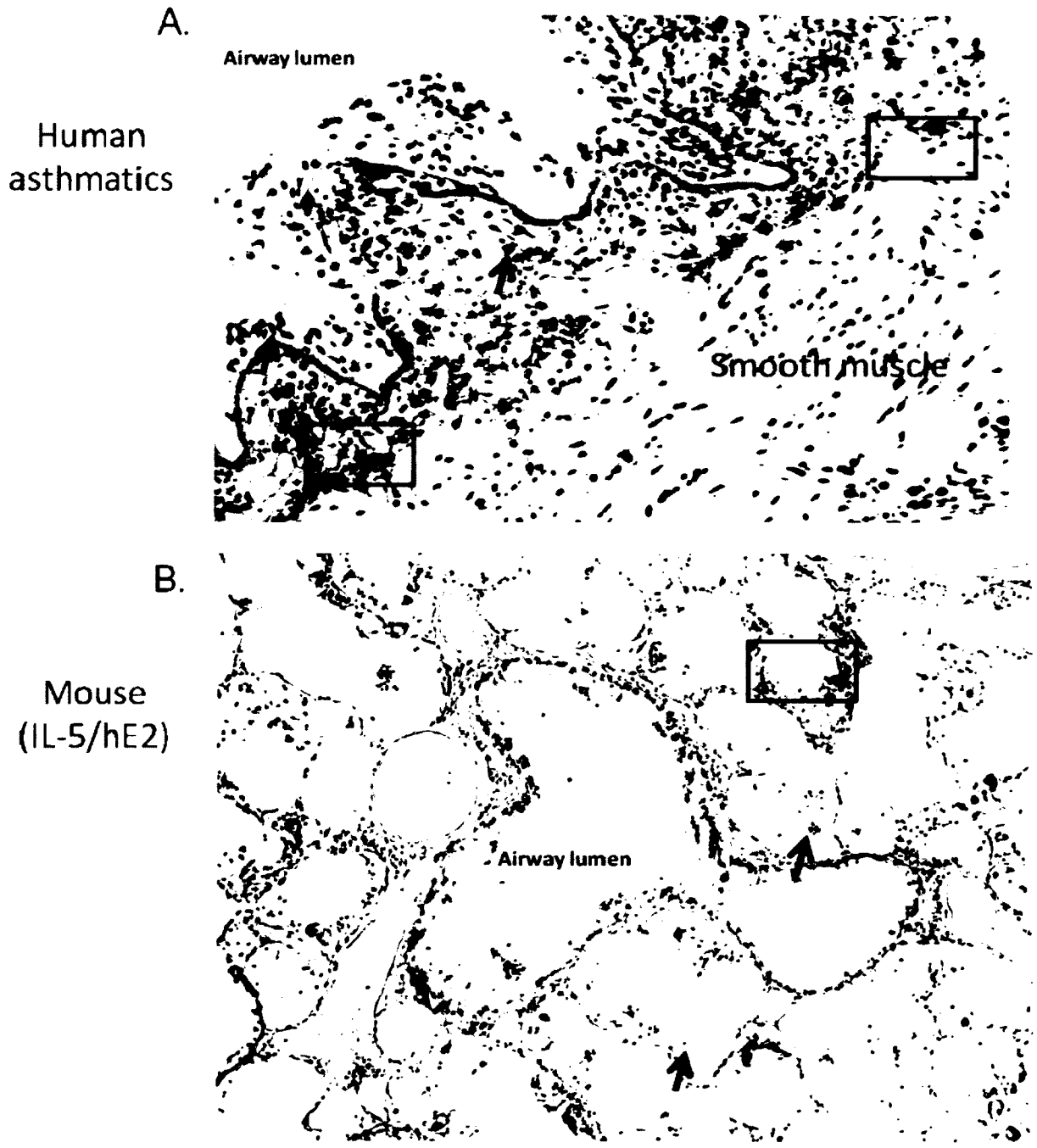

Figure 1.5. Eosinophil presence and degranulation in allergic airway inflammation. (A) EPX stain of human asthmatic airways stained by Cheryl Protheroe, Lee Labs, Mayo Clinic, Scottsdale, AZ. (B) MBP stained mouse lung from *IL-5/hE2* mice. The presence of eosinophils is indicated by a punctate staining pattern (black arrows). The evidence of degranulation is indicated by the lacy pattern of protein staining with or without intact cells (area in the black box).

evidence of eosinophil infiltration and degranulation (119-121). ESGP such as MBP, EPX, EDN, and ECP in humans, have documented toxicity and immune-regulating capability. MBP has toxic effects on human nasal sinus mucosa (47) and can also activate mast cells to release histamine and leukotriene *in vitro* (122). EDN is capable of activating dendritic cells (DC) and enhance a Th2 response through toll like receptor (TLR)-2 (123).

1.2.2 Immune modulation

Eosinophils have been implicated in acute and adaptive immunity. Eosinophils respond to and produce the proinflammatory cytokines TNFα, IL-1, IL-6 and IL-8, which have implicated these leukocytes in the acute phase of inflammation not associated with atopy (8). The presence of TLRs and receptors for Th1 cytokines on the surface of eosinophils has also suggested their participation in innate immune response that can lead to Th1 inflammation (124, 125). Eosinophils also express pattern-recognition receptors (PRRs) which are involved in innate immune activities (126-128).

Even though T cells have a dominating role under many inflammatory circumstances, eosinophils have also been thought to perpetuate and modulate inflammation through interactions with T cells (129) (Figure 1.6). Eosinophils are a rich repertoire of both Th1 (IFNγ and IL-12) and Th2 (IL-4, 5, 6, 13, 25) cytokines (130, 131) and are considered a major source of IL-4/13 at the site of inflammation (132). Eosinophils manufacture, store, and release Th1/Th2 modulating cytokines and thus can influence T cell activities such as recruitment, proliferation, and activation in mouse models of asthma (129). During inflammation, eosinophils have the potential to influence Th1 cell recruitment by releasing CXCR3 ligands such as CCL5/RANTES and IP10 (133, 134). Eosinophils are found in lung-draining lymph nodes of mice when exposed to allergen which suggests a dendritic cell-like activity to accomplish T cell education and activation (8).

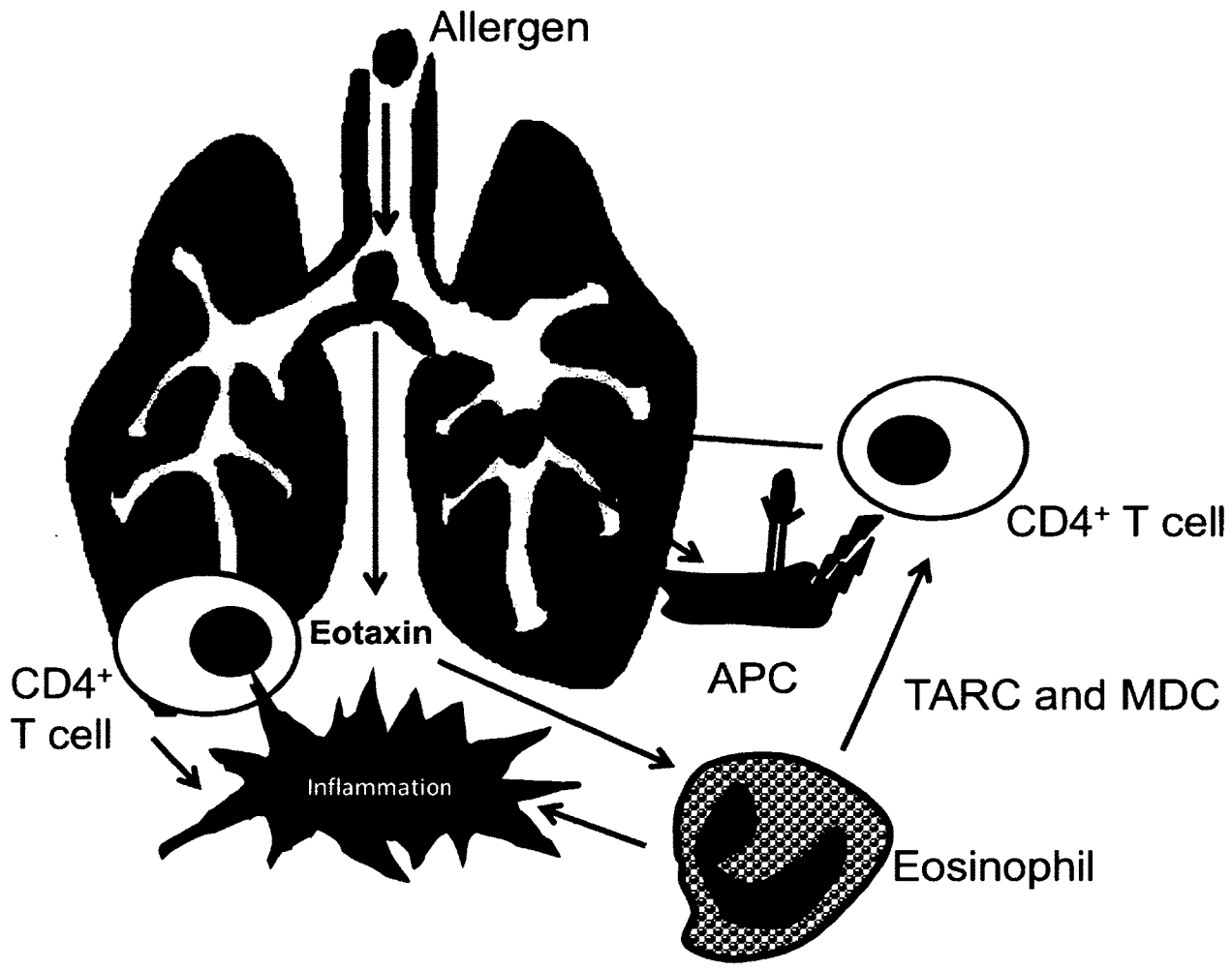

Figure 1.6. Immunoregulatory functions of eosinophils. T cell recruitment may depend on eosinophil activation. The recruitment of effector Th2 cells is dependent on chemokine release by eosinophils. TARC: thymus and activation regulated chemokine, MDC: macrophage-derived chemokine.

The immunomodulatory role of tissue eosinophils are reflected in their ability to communicate with other cell types (129, 135, 136) either directly as antigen-presenting cells (APCs) (137, 138) or indirectly via cytokine networks (Figure 1.7). Eosinophils also constitutively express indoleamine 2, 3-dioxygenase (IDO). Eosinophil-derived, IDO-mediated tryptophan catabolism generates kynurenines that may induce apoptosis preferentially in Th1 cells and further promote a Th2 bias (139-142).

Tissue-infiltrating eosinophils express IL-12 receptors (R) as well as IL-4R, IL-5R, IL-13R, and GM-CSFR which suggests eosinophils have the potential to respond to both Th1 and Th2 environmental cues and that certain cellular functions can be modulated by the Th1/Th2 balance. Tissue eosinophils have the potential to modulate the local Th1/Th2 balance as negative regulators as they express IL-10 and transforming growth factor (TGF) β, which are associated with T cell activation and down-regulation of inflammation (143).

1.2.3 Remodeling

Tissue (e.g., airway) remodelling is a major component of pathology that leads to clinical symptoms, and is thought to be a consequence of myofibroblast activation initiated by tissue damage and exaggerated repair (144). Fibrosis, angiogenesis, and metaplasia are some of the frequently observed clinical evidence of tissue remodelling as a result of inflammation. Eosinophils express and release TGFβ, vascular endothelial growth factor (VEGF), matrix metalloproteases (MMP), and platelet-derived growth factor (PDGF) in addition to secondary granules, eicosanoids, and leukotrienes, which have been shown to contribute to the development of clinically observed histopathology (8, 128, 145).

Eosinophil deficiency (e.g., ΔdbI-GATA) in a transgenic mouse model of allergic asthma has shown complete and/or partial protection from mucus-secreting epithelial cells,

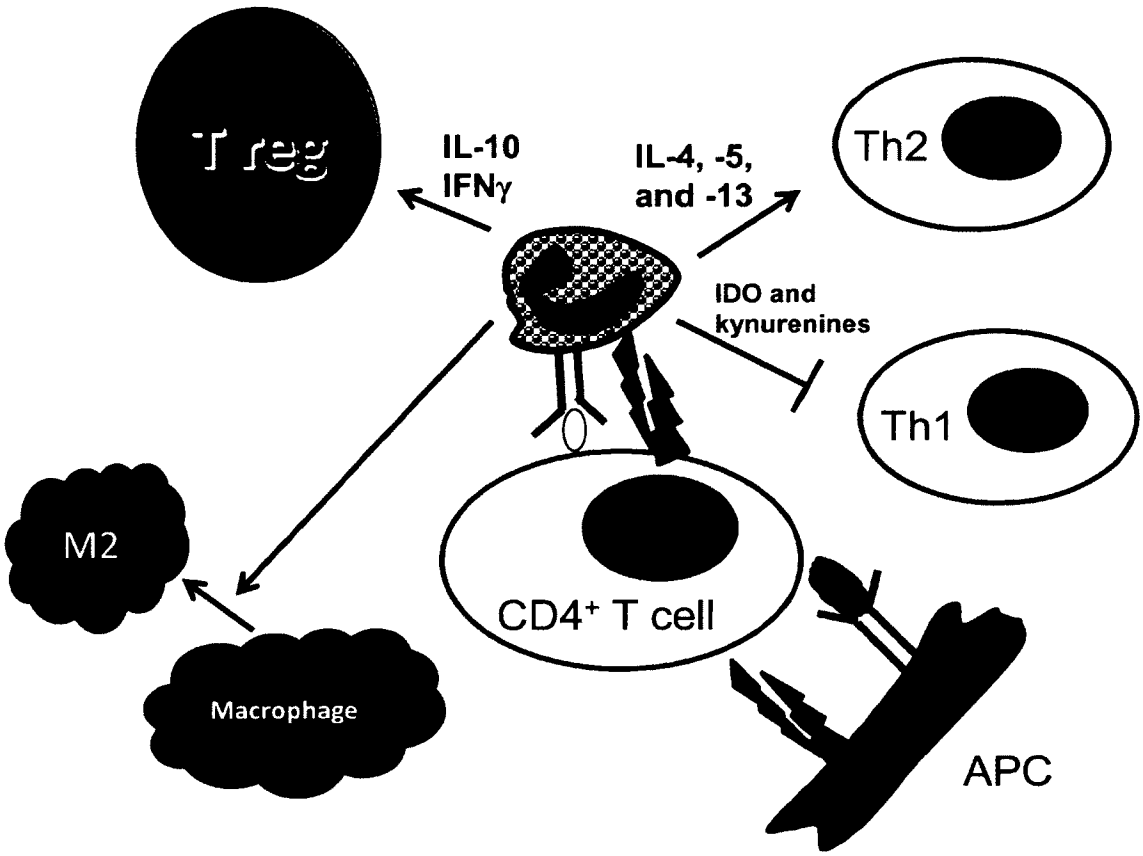

Figure 1.7. Other eosinophil effector functions discussed in current literature.

subepithelial collagen deposition, and ASM hypertrophy (12, 13, 146). IL-5 and IL-5R deficient transgenic mice developed less peribronchial fibrosis, ASM thickening, and TGFβ-positive cell infiltration compared to wild type mice following allergen exposure (147). Airway obstruction and airway hyperresponsiveness in part can be mediated by the downstream effect of eosinophil activities (66, 148). IL-1, IL-13 and TGF-β released from eosinophils are thought to initiate fibroblast migration and consequent tissue remodelling in inflammation (149).

Eosinophils can further promote T cell recruitment (150, 151) and sustain local responses by manufacturing LTB_4, lipid bodies (152), and extracellular DNA traps (153, 154) at inflammatory foci. Eosinophils produce survival signals such as stem cell factors and growth factors for mast cells, which can perpetuate local inflammation by releasing histamine and eicosanoids (155).

1.2.4 Homeostasis

It has been demonstrated that eosinophils in the bone marrow are co-localized with plasma B cells and secrete a proliferation inducing ligand (APRIL) and IL-6, which suggests eosinophil involvement in plasma B cell survival (156). Further studies by the same group have provided insight on the ability of eosinophils to modulate secondary immunization of plasma B cells, which enhanced their antibody production (157).

The interaction between eosinophils and macrophages has been thought to participate in various homeostatic functions of eosinophils. Eosinophils have been thought to aid T cell selection in the thymus via aiding macrophages in phagocytosis of apoptotic T cells (158). The polarization of adipose tissue macrophages to the alternative M2 phenotype (IL-10- and TGFβ-producing) from those produced by the classical M1 phenotype (IL-12-producing), via interactions with eosinophils has been suggested in glucose homeostasis (159).

The end-stage effector role with a potentially destructive ability is only part of what eosinophils do in both mediating inflammation and maintaining homeostasis. Recent evidence discussed above has initiated research into the immunomodulatory influence of eosinophils during both innate and adaptive immunity.

1.2.5 Role of eosinophils in airway inflammation

Since their first visualization in 1879, eosinophils have been observed in airways and in sputum samples from asthmatic patients. These observations have put eosinophils in the centre stage of the development of allergic airway inflammation for decades. Recently, however, two human patient-based clinical trials (160) (161) have put the role of eosinophils in the development of asthma to question. Despite more than 100 years of study and research, the causative link between eosinophils and allergic asthma has yet to be established.

Recent clinical evidence has re-introduced the role of eosinophils in mediating inflammation (11, 14) and has helped to re-define asthma phenotypes (162) according to the presence of infiltrating eosinophils. Airway remodelling and increased exacerbation rates are often associated with hyper-eosinophilia, and airway obstruction is more frequently associated with the absence of eosinophilia (163-165). In one landmark study, the specific role of eosinophils in asthmatic phenotypes associated with persistent eosinophil recruitment and activation was delineated in human patients treated with a combination of anti-IL-5 (mepolizumab) and corticosteroids (11). As a result of monitoring eosinophil numbers in sputum samples, prednisone-dependent asthmatics were able to taper their daily required steroid and decrease exacerbation frequency with concurrent anti-IL-5 treatment (11).

The patient population in studies conducted by Nair *et al.* and Haldar *et al.* represent a rather small percentage (~5%) of all asthmatic patients, and their asthmatic symptoms are considered to be severe (163). The lack of significant change in clinical outcomes in mild

asthmatics receiving anti-IL-5 treatment suggests eosinophils may have divergent roles in mediating inflammation in typical asthma (160).

Among all the parameters measured in studies conducted by Nair *et al.* and Haldar *et al.* (e.g., forced expiratory volume in 1 second, symptoms, disease control, and quality of life), the asthma exacerbation rate was the only parameter that improved consistently with anti-eosinophilic treatment. Significant improvement in other parameters measured was observed with the treatment of corticosteroids with general anti-inflammatory effects in the absence of anti-eosinophilic treatment with mepolizumab. These results illustrate the complexity of asthma pathology and suggest that the participation of eosinophils, albeit important in mediating inflammation, is not the only influence in the establishment of pathology.

To establish a link between the presence and function of eosinophils in the development of allergic asthma, *in vitro* and *in vivo* models to study eosinophil pathophysiology have been employed by researchers as laboratory tools. The following are some of the most frequently used *in vivo* models to elicit eosinophilic asthma in mouse models of allergic airway inflammation.

1.2.6 Mouse models of allergic airway inflammation

Allergen-mediated disease-like changes in mice may be referred to as models for a subtype of allergic asthma, since other factors such as obesity (166), psychosocial stress (167), viral infection (168), and exposure to pollutants (169) have also been associated with asthmatic symptoms clinically. Less than 50% of asthma in humans is attributed to a Th2-type pulmonary allergic response (170), and molecular and immunological mechanisms associated with the mouse model closely reproduce this particular phenotype.

Mouse strains commonly used in the laboratory have been inbred many generations to reduce genetic variability, as well as defined immunological and physiological features that are reproducible and reliable for research purposes. In contrast, humans are "out bred" and genetically unique as individuals, and asthma pathologies are loaded with environmental, genetic, psychological, and hormonal variables. Many approaches exist to elicit eosinophilia in mouse lungs in order to study the role of these cells in the development of the allergic airway inflammation. The following are the three most commonly used models:

Ovalbumin (OVA): OVA can induce robust eosinophilia in the mouse when sensitized systemically (i.e., intraperitoneal injection (i.p.)) and challenged locally (i.e., intranasal (i.n.) and intratracheal (i.t.)). Several variations of the protocol exist and can elicit different outcomes of pulmonary inflammation in terms of the percentage of eosinophils transmigrated into the airways. In the most commonly used OVA protocol, mice are sensitized with i.p. injections of OVA (20-100 μg of OVA with 2 mg alum) on days 1 and 14. On days 24, 25, and 26, mice are challenged with aerosolized OVA in saline. Varying versions of this OVA protocol can elicit 1-9 x 10^6 cells to the airways of which 10-40% are eosinophils in C57BL/6J and BALB/c mice (171, 172).

Aspergillus fumigatus (AF): AF is a type of fungus that can be used to generate a fungus-based mouse model of allergic pulmonary inflammation. Various versions of the protocol for *AF*-induced inflammation exist, and the most commonly used version consists of immunization with *AF* given intranasally to mice for 7 days or twice a week for 4 weeks without adjuvant. This protocol can elicit 35-40x10^4 eosinophils/ml of bronchoalveolar lavage BAL (173, 174).

House dust mite (HDM): HDM can be used to induce chronic and acute allergic inflammation in mouse models (175). In the acute model, HDM is administered without adjuvant

i.p. on day 0 and challenged on day 10. This model elicits about $7x10^5$ cells in the BAL and 20% are eosinophils (176). The percentage and total number of eosinophils in the BAL can be increased using a chronic protocol with i.n. instillation of 40 μg of HDM 5 days per week for 3 weeks, or 5 weeks (177).

Others: Multiple models exist to elicit allergic airway inflammation using the mouse model. Eosinophilia can also be elicited by various allergen treatments such as ragweed (178), *Alternaria alternata* spores (179, 180), and cockroach (181) with various combinations of provocation protocols.

1.2.7 Transgenic and gene knock-in/knock-out models of eosinophil-mediated airway inflammation

The most undeniable advantage of the mouse model is the use of genetically modified mice in experimental asthma conditions to validate the unequivocal link between a specific research target and airway inflammation and pathophysiology. Specific cell types, cytokines, cell surface receptors, transcriptional factors, and signalling machineries have been targets of research using transgenic mice with artful manipulation of the mouse genome. Pathologic features developed in mice exposed to allergens are comparable to clinical observations of asthma in humans (182). The relative ease of maintaining experimental colonies have increased the frequency of mouse model-based research, especially with the advent of transgenic technology (183).

Transgenic models of eosinophil-mediated airway inflammation can be categorized into hyper-eosinophilic and eosinophil-deficient models. In the hyper-eosinophilic category, two strains of transgenic mice (i.e., *IL-5* transgenic mice and *IL-5/hE2* double transgenic mice) are used frequently in studies related to eosinophilic diseases. IL-5 is known as the eosinophilopoietin that was acknowledged as eosinophil terminal differentiation factor (184).

Over expression of IL-5 systemically is associated with blood, spleen, liver, peritoneum, bone marrow, and intestine eosinophilia (185-189).

IL-5 overexpression alone in transgenic mouse model of allergic airway inflammation only elicited eosinophilia with little to no evidence of activation and degranulation; however, double transgenic mice with constitutive expression of IL-5 from T cells and human eotaxin-2 from lung epithelium (*IL-5/hE2*) develop disease-like changes that mimic human asthmatics with significantly increased airway remodelling (190). Targeting IL-5/eotaxin and IL-4/13 is associated with decreased airway inflammation and improved airway hyperresponsiveness (AHR) in mice (191, 192).

In eosinophil-deficient models, the most prominent link established between the presence of eosinophils and asthma was demonstrated in a mouse model with a congenital deficiency in eosinophils (12). The necessity of eosinophils in the development of AHR and airway mucin accumulation was observed in allergen sensitized and challenged eosinophil-deficient mouse model (PHIL). Using the same model, Jacobsen *et al.* have demonstrated the role of eosinophils in recruitment of T cells, which was critical in orchestrating Th2 inflammation in airways of mice treated with OVA (129).

The role of eosinophils and their communication with T cells were first observed *in vivo* in the IL-5 knockout (IL-$5^{-/-}$) mouse model (193). Adoptive transfer of eosinophils into OVA-treated IL-$5^{-/-}$ mice elicited airway inflammation (e.g., Th2 cytokine levels in airways, epithelial changes, and airway hyperresponsiveness) with a similar magnitude to that of OVA-treated wild-type mice. Mucin production, epithelial hyperplasia, and AHR in OVA-treated IL-$5^{-/-}$ was significantly reduced in mice that received anti-CD4 antibodies to deplete T cells, which suggests that communication between eosinophils and T cells is a necessary part of the development of pathology associated with allergic asthma in mouse models.

The use of mouse models of allergic airway inflammation also provides a means to evaluate histopathology and pathophysiology as the consequence of elicited allergic responses in mice. Lung samples, BAL, and AHR to cholinergic agonists may be evaluated in mouse models, which provide invaluable information regarding the magnitude of inflammation, tissue damage, mucin production, and airway function in response to allergen provocation.

1.2.8 Intrinsic advantages and limitations of mouse models of asthma

Anatomical differences between mice and men have impacts on airway responses to experimental stimuli. Persistent asthma is associated with pulmonary inflammation and tissue remodelling that is mediated, in part, by the epithelial–mesenchymal unit (194, 195). In addition to the lobe structure difference, mouse airway epithelium is less stratified and the airways have fewer generations of branching compared to humans (196, 197). Allergic inflammation elicited in mouse models is centred around the lung parenchyma and vascular tissue rather than the conducting airways seen in most cases of human asthma (198). Nevertheless, observation of pathological changes elicited by exposure to allergen allows investigators to evaluate parameters between experimental groups and controls in order to establish links between research targets and disease pathology *in vivo*.

AHR is identified in most asthmatics and correlates well with the severity and the control of the disease when therapeutic regimens are implemented (199) and can be independent of inflammation. Changes in AHR are usually measured as experimental parameters in the mouse model, but the relative lack of ASM and submucosal glands in the mouse lower airways, in contrast to human airways, makes mouse models less attractive (182). However, mouse airways do offer useful information. Mice are genetically limited in their ability to generate intrinsic AHR (200), which is argued to be a heritable trait (201, 202), as different strains of mice develop different levels of airway sensitivity to cholinergic agonists. AHR in nine

strains of laboratory mice were evaluated by Levitt *et al.* and significant differences were found among the strains (203). C57BL/6 had lower baseline pulmonary dynamics and cholinergic agonist-elicited AHR compared to BALB/c mice. If lung physiology and AHR are critical experimental parameters, then the strain of mice (AKR/J, BALB/c, C57BL/6, and C3H/He in decreasing order of AHR to agonists (204)) should be considered. C57BL/6 and BABL/c are two frequently used strains in developing transgenic mice, thus the balance between what the studies require and the availability of the transgenic strain is necessary for an optimum mouse model-based research design.

Tissue remodelling such as fibrosis, goblet cell hyperplasia, ASM thickening, and angiogenesis are observed in human asthmatic lungs (205). Structural changes in the airways have been observed early in life and can exist independently of airway inflammation, suggesting that pre-conditioning of the pulmonary environment is necessary to sustain the Th2 atopic response when exposed to allergen, virus, and environmental stimuli (144). Changes in fibroblasts, smooth muscle, nerves, and the epithelium have been postulated to contribute to the pre-conditioning processes. Wild-type mice do not have asthma unless exposed to allergens, and thus become less attractive for model-based studies involving airway environmental priming and intrinsic AHR.

Clinical observations such as AHR and inflammation are studied in mouse models of asthma to further the understanding of a cause-and-effect relationship in the disease to develop potential therapeutic agents for human asthma treatment. When addressing therapeutic potential of an agent, the effectiveness of the agent in controlling symptoms can be answered using mouse models, but improvements in the quality of life cannot be measured.

1.2.9 Eosinophil degranulation in human diseases and mouse models

Degranulation is defined as the release of granule products through exocytosis or cytolysis, which is a direct way to deposit secreted mediators towards a target. Eosinophils have been observed to undergo all 4 modes of exocytosis either *in vitro* or in tissue biopsies: 1). Classical exocytosis; 2). Piecemeal degranulation (PMD); 3). Compound exocytosis; and 4). Cytolytic release of intact granules (Figure 1.8).

Classical exocytosis describes the release of granule contents through fusion of individual granules with the plasma membrane. Using the patch clamp technique in the whole-cell configuration, classical exocytosis is associated with incremental stepwise increases in capacitance when whole granules are fused with the plasma membrane in response to stimulation. Classical exocytosis is only seen *in vitro* with purified eosinophil preparations (206).

Classical exocytosis is further divided into constitutive and regulated categories (206) (207). All cells, including eosinophils, can constitutively release proteins (e.g., extracellular matrix proteins) in the endoplasmic reticulum, which are processed and released through the Golgi apparatus via vesicular trafficking. In regulated exocytosis, receptor-mediated stimulation is necessary for granule mobilization and degranulation of pre-stored granule proteins and mediators. A heterogeneous group of granules are found in the cytoplasm of eosinophils, which suggest eosinophils can undergo both constitutive and regulated exocytosis (208, 209).

Compound exocytosis describes homotypic fusion of multiple granules prior to the fusion with the plasma membrane. Eosinophils undergo compound exocytosis when treated with opsonised larvae of the helminth parasite *Schistosoma mansoni* (210, 211). In cases of parasite defense, general all-directional degranulation is less efficient and can cause more collateral damage in tissues compared to focal release of cytotoxic granule proteins. Granule

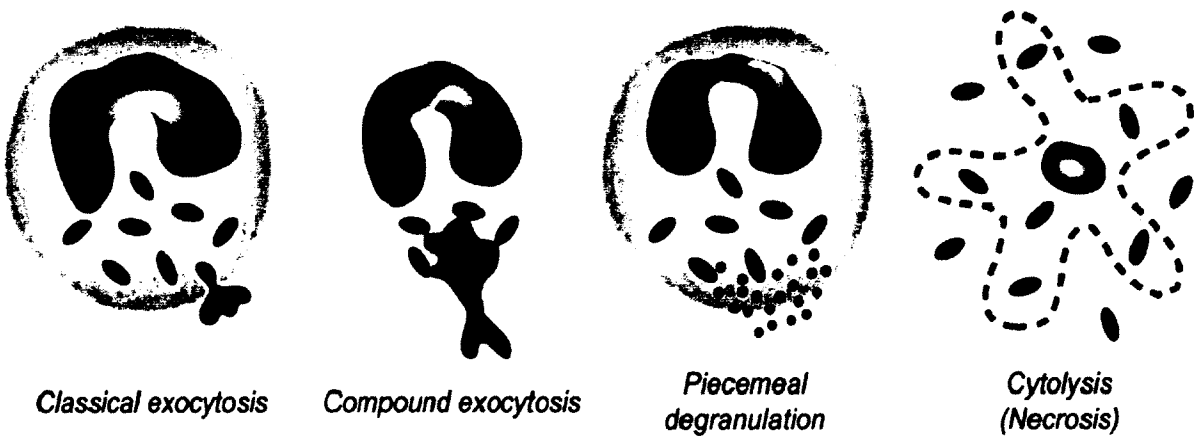

Figure 1.8. Modes of degranulation in eosinophils. From Moqbel and Lacy, CIHR Research Proposal, 2008.

membranes contain most of the machinery necessary for homotypic fusion. Granule-granule fusion in horse eosinophils was observed at high concentrations of GTPγS, whereas, classical individual granule-PM fusion occurred using low concentrations of GTPγS (211).

It is important to note that more than one secretory pathway may be activated simultaneously. Imaging of degranulating eosinophils attached to parasites *in vitro* has demonstrated that compound exocytosis focuses the release of cytotoxic granular proteins directly onto targets and elicits maximal killing with minimum damage to host tissue.

Piecemeal degranulation (PMD) is characterized by a gradual hollowing of crystalloid granules via secretory vesicle budding, mobilization, and fusion with the plasma membrane. Under transmission electron microscopy, human eosinophils in allergic tissues undergo PMD and are reflected by a reduction in electron density in secretory granules that gradually empty their contents, as well as the appearance of electron-lucent secretory vesicles budding off the granules (Figure 1.9). PMD and cytolysis are the most commonly observed physiological modes of exocytosis in human diseases (212-218). Tissue eosinophils undergoing PMD is evident in nasal polyposis (212, 215), severe asthma (219), rhinitis (220), ulcerative colitis, Crohn's disease (215), cutaneous allergic reaction (221), and cancer (222).

During PMD, human eosinophils form large tubulovesicular structures composed of small membrane-bound sombero vesicles (87, 208) (223), in conjunction with small secretory vesicles to facilitate the trafficking and rapid release of assorted proteins (RANTES/CCL5, MBP, IL-4) in response to environmental stimuli (85).

Mouse eosinophils isolated from blood, airway, and bone marrow after OVA provocation manifest minimal alterations in their secondary granule morphology. Half of the mouse tissue eosinophils examined have marginal irregular cytoplasmic membrane processes,

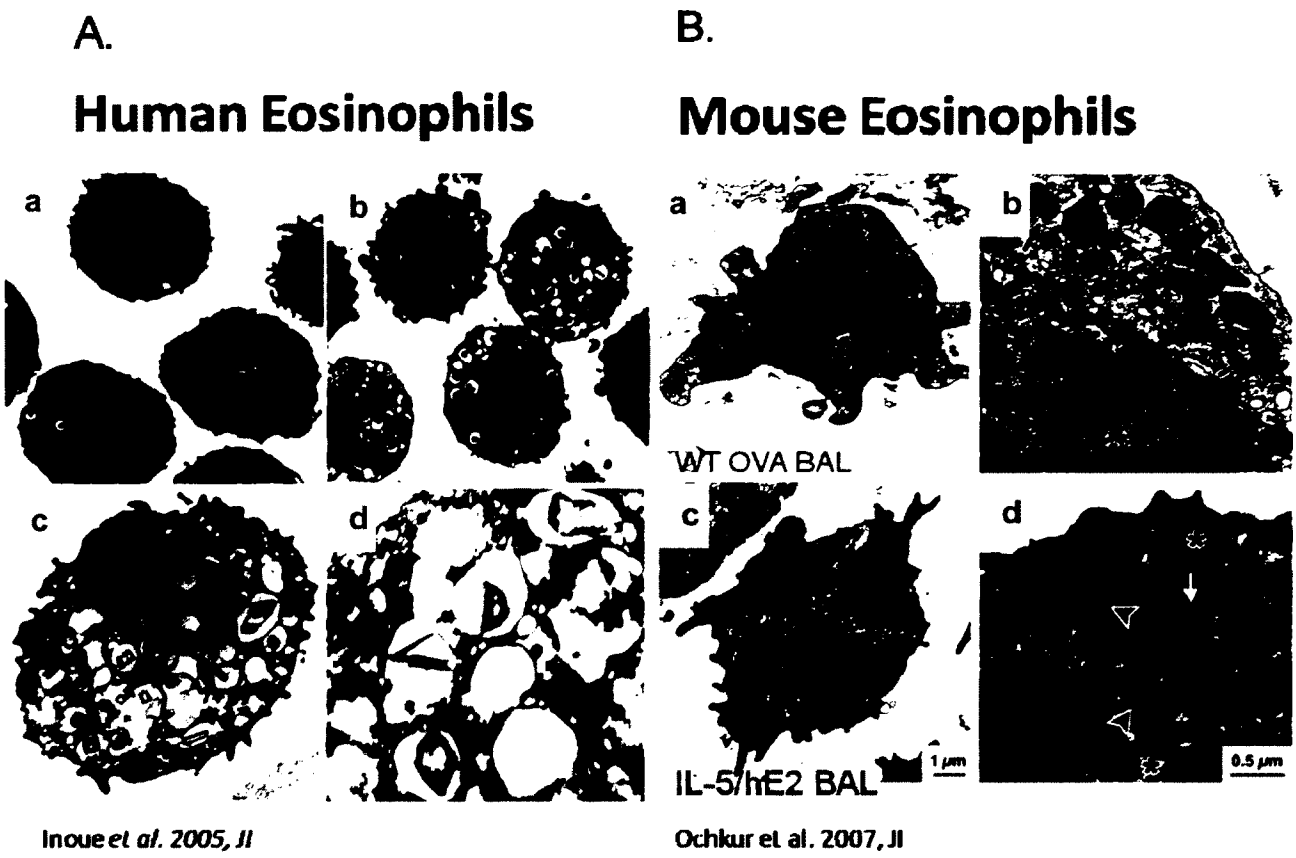

Figure 1.9. Eosinophil piecemeal degranulation detected by TEM. The hollowing of granules as a result of degranulation is observed in human and transgenic mouse eosinophils. (A) Human eosinophils at resting state (a); and activated by *Alternaria* extract (b). Activated eosinophils with hollowed granules (c); eosinophil secondary granules appeared to be electron-translucent within activated eosinophils (d). (B) Mouse eosinophils isolated from the airways of wildtype mice treated with OVA (a); secondary granules within mouse airway eosinophils appear to be intact with electron-dense cores (black arrows) (b); eosinophils collected from *IL-5/hE2* mice BAL in the absence of allergen provocation (c); mouse eosinophil secondary granules with hollowed appearance that is similar to activated human eosinophils (d).

however, the "ghost-like" crystalline core and presence of small secretory vesicles seen in human eosinophil PMD are not detectable (224). Mouse eosinophils activated by CCL11 have ultrastructural changes that suggest PMD and degranulation of RNases, EPX, β-hexosaminidase and MBP (225). Phorbol 12-myristate 13-acetate (PMA) and fMLP are two potent stimuli that readily induce degranulation in human eosinophils, but fail to induce morphological changes and granule protein release from mouse eosinophils except for mouse Ear release (225), which suggests species-specific differences in eosinophil degranulation (224).

Eosinophils isolated from *IL-5/hE1* mice were observed to have marginal degranulation that was limited to the airway lumen of the mice (226). Eosinophils isolated from *IL-5/hE2* transgenic mice BAL show extensive evidence of degranulation as partial and complete loss of electron-dense secondary granule crystalline core accompanied by the loss of electron opacity within the granule matrix is observed (190). These different phenotypes associated with *IL-5/hE2* versus *IL-5/hE1* transgenic mice may suggest differential regulation of eosinophil activation and degranulation.

PMD also represents a mechanism for differential and/or directional release of granule proteins (ECP and EDN) and cytokines (IL-4, -6, -13) dependent on the local micro-milieu (227-230). In human eosinophils, vesicular transport proteins such as v-SNAREs (*i.e.*,VAMP-2) are co-localized with RANTES/CCL5 containing crystalline granules and is involved in PMD upon interferon γ (IFNγ) stimulation (231). VAMP-7 in human eosinophils has been associated with the selective release of EPX (232).

Cytolysis describes the release of intact or ruptured cytoplasmic granules during necrotic cell death. Cytolysis is the second most common physiological mode of exocytosis observed in tissue eosinophils. At sites of inflammation, eosinophils undergo necrotic cell death.

The lack of membrane integrity leads to dispersal of intact and ruptured secretory granules and granule contents into the surrounding extracellular matrix, which likely affects the normal physiological function of tissues.

Human eosinophil cell-free granules are readily detectable in tissues from allergic asthma, dermatitis, rhinitis, eosinophilic esophagitis, helminth infections, and urticaria (221, 233-237). Intact eosinophil granules are capable of receptor-mediated secretion autonomously as they express a spectrum of functional receptors (IFNγ, GPCR for eotaxin) on the surface membranes and release their contents in response to stimuli in the absence of intact cells (238). Agonist-dependent release of cytokines from cell-free granules is also selective as observed in the stimulation of intact eosinophils. This further suggests the immunoregulatory ability of eosinophils and their granule proteins (238). Cell-free granules from mouse eosinophils have been observed to release RNases with CCL11 stimulation *in vitro* (225).

1.3 Degranulation/exocytosis mechanism of vesicular trafficking

Receptor-mediated exocytosis is one form of degranulation from activated eosinophils, and is a highly regulated process. Receptors (GPCR ligand C5a, PAF, fMLP, Ig-binding, FcγRII, FcαR, IFNγ, CCL11, CCL5) amalgamate various signalling pathways to induce intracellular calcium change and change the phosphorylation status of serine/ threonine kinases, leading to the release of granular contents (206).

Soluble *N*-ethylmaleimide sensitive factor attachment protein (SNAP) receptors (SNAREs) are membrane-associated proteins that consist of core components of the membrane fusion machinery necessary for exocytosis (239-242). SNARE-mediated degranulation is postulated as a regulated process in eosinophils (232, 243-245), neutrophils (243, 246), mast cells (247-256), and platelets [32-36]. The minimal machinery hypothesis

describes the process of fusion between vesicular and plasma membranes as the result of a mechanical force generated by physical interaction between SNARE proteins anchored in opposing membranes (257).

Cells express SNARE isoforms with distinctive subcellular localizations which can be categorized into vesicle associated membrane proteins (VAMPs or v-SNAREs) and target membrane-associated SNAREs (t-SNAREs) (257). To initiate fusion pore formation in the event of degranulation, the functional domain of v-SNAREs interacts spontaneously with the functional domain of its cognate t-SNAREs to form a four-α-helix bundle called the trans-SNARE complex (Figure 1.10). The formation of the trans-SNARE complex docks the vesicle and creates a mechanical force that brings the vesicular and plasma membrane into close proximity and catalyze fusion (239) with the assistance from GTPases (258-261), Sec/Munc proteins (257) (262-264), (Figure 1.11) and calcium-dependent kinases (259, 265).

Fusogenic SNARE combinations are limited despite the fact that there is ubiquitous expression of numerous types of SNARE isoforms (266-268). Human eosinophils express core components of SNARE complexes, such as VAMP-2, -7, -8, syntaxin-4, and SNAP-23 (232, 269), and the treatment of tetanus toxin and botulinum toxin inhibits granule docking and subsequent fusion and degranulation events (270). Mouse eosinophils express v-/t- SNAREs and their accessory proteins but their involvement in exocytosis needs further invesitigation.

Cytokine secretion from eosinophils mainly occurs through PMD in a differential manner from crystalloid granule exocytosis (230). This differential secretion is associated with IL-6, IL-4, and RANTES/CCL5, which are found in both CG and small secretory vesicles (84, 85, 271). In addition to locations that cytokines versus granule proteins are stored, the secretion kinetics of cytokines (e.g., RANTES/CCL5) and granule proteins (e.g., β-hexosaminidase) in

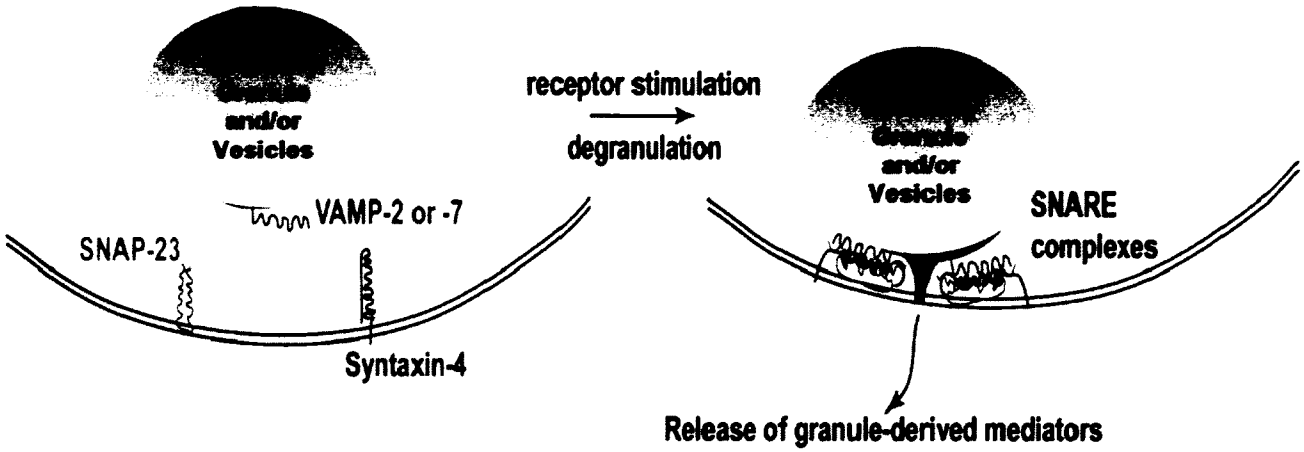

Figure 1.10. Scheme showing VAMP-2 and VAMP-7 facilitating granule or vesicle membrane fusion with the plasma membrane. The interaction between v- and t-SNAREs generates mechanical force to bring the granule or the vesicle membrane in close proximity of the plasma membrane to allow fusion to occur. From Moqbel and Lacy, CIHR research proposal, 2008.

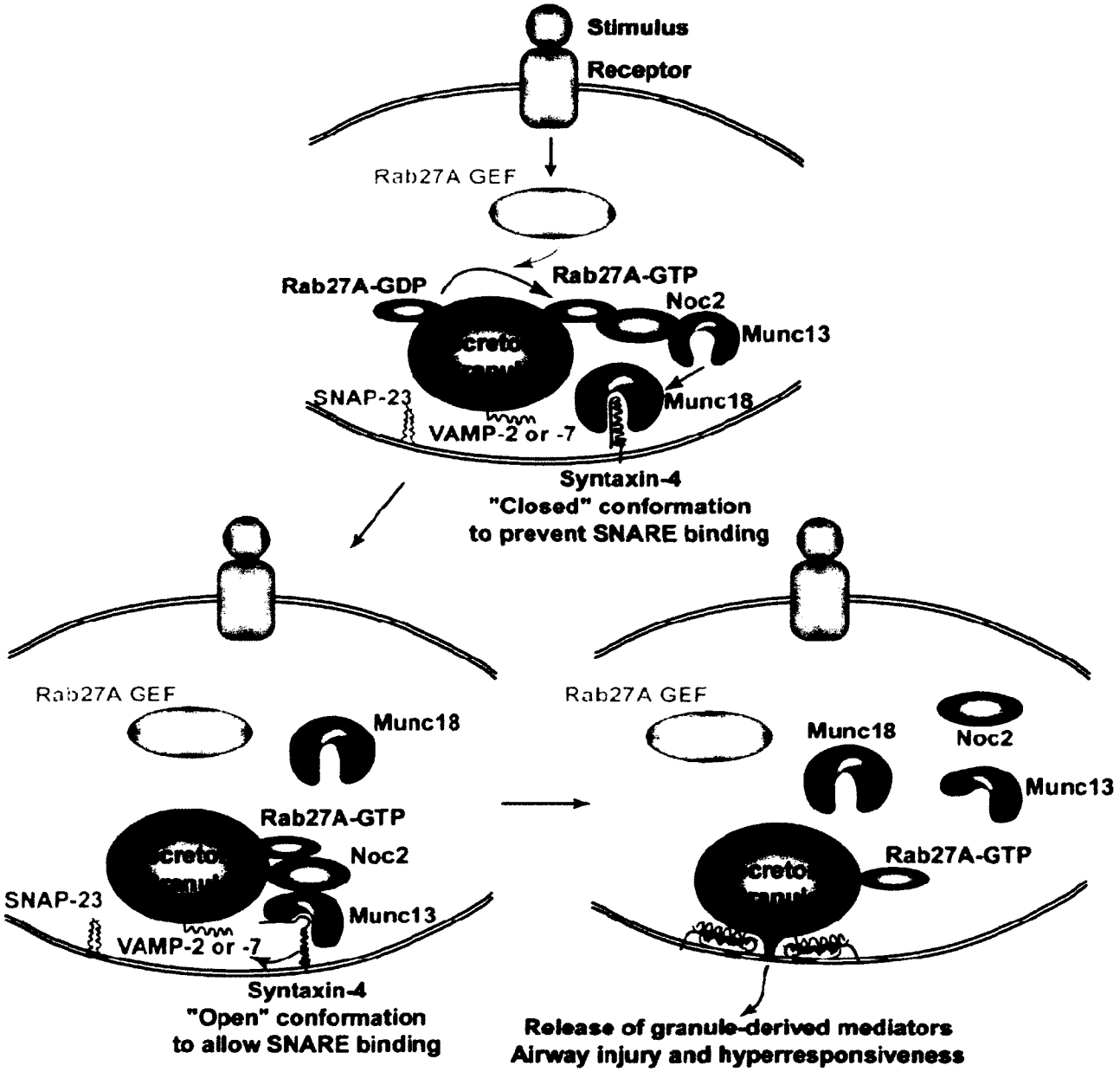

Figure 1.11. Scheme showing SNARE-mediated membrane fusion regulated by accessory proteins. Receptor signalling to Rab27A leading to Noc2/Munc13 activation to cause the release of Munc18 from syntaxin-4, thus allowing SNARE binding for granule docking and fusion. From Moqbel and Lacy, CIHR Research Proposal, 2008.

response to IFNγ stimulation differ in human eosinophils (85). An important observation that membrane-bound IL-4 receptor α chain localizes to secretory vesicles in human eosinophils has suggested a mechanism for selective release of eosinophil cytokines. It was postulated that receptor-mediated (i.e., CCL11/eotaxin and IFNγ) stimulation is associated with differential recruitment of cytokine receptors (e.g., IL-4R) within the granule to mobilize small vesicles as well as the movement of secretory vesicles independently of CG. Fusion of these small secretory vesicles with the plasma membrane thus results in the selective secretion of cytokines from stimulated eosinophils.

Differential expression of v-SNARE proteins is observed in CGs and secretory vesicles. Cell-free granules stimulated by LTC_4, LTD_4, and LTE_4 elicited differential release of ECP within the CGs, but not eosinophil-derived cytokines or chemokines (272). VAMP-2-positive small secretory vesicles exist as a discrete pool within the cytoplasm that rapidly fuse and release their contents independently of CG (e.g., RANTES/CCL5) in response to specific stimuli (i.e., IFNγ) (85). Small secretory vesicles have been postulated to act as shuttling compartments in CG release. VAMP-7 has been found in the same fractions as EPX, which localizes to CG and small secretory vesicles in human eosinophils (84, 232). VAMP-2 is thought to regulate eosinophil small secretory vesicle release, while VAMP-7 is thought to be involved in the release of cationic proteins from crystalloid granules (230, 273). VAMP-7 and VAMP-8 are also found in human eosinophils, and inhibition of VAMP functions using neutralizing antibodies against VAMP -7, and -8 in human eosinophils have been associated with varying degrees of down-regulation in release of granule proteins (232). The release of EPX and EDN was significantly reduced with low concentrations of anti-VAMP-7 treatment; however, release was unaffected by high concentrations of anti-VAMP-8 treatment in human eosinophils, suggesting that VAMP-7 is critical for eosinophil granule exocytosis. Therefore, the main focus of this thesis

is to understand more about the role of VAMP-7 in eosinophil degranulation and how this could affect airway physiology and function in allergic inflammation.

Ubiquitous VAMP-2 and VAMP-7 knockout mouse strains exist as the result of either targeted mutations or the *Cre-LoxP* binary system (274-276). No significant morphological differences in synaptic properties were observed between WT and heterozygous VAMP-2 knockout mice; however, the catalyzing of fusion pore formation and the stabilization of fusion pore in neurons were affected by the absence of VAMP-2 (275). In the absence of VAMP-7, neurons were unable to extend axons to their fullest extent in mice, while epithelial cell polarity and lysosomal exocytosis were intact (274). VAMP-7-deficient mice were also observed to have enlarged third ventricles, and increased anxiety compared to WT mice (276). The phenotypic properties observed in the VAMP-7 ubiquitous knockout mice suggest that VAMP-7 may play a role in higher brain function, thus generating complicating variables in the study of the role that VAMP-7 plays in other parts of the body. Using the *eCre-LoxP* binary system as described in this thesis, the specificity of VAMP-7 deletion is limited to eosinophils.

1.4 Thesis hypothesis and objectives

Evidence from clinical observations and hypothesis-driven research has indicated a potential role for eosinophils in allergic airway inflammation. Observations of eosinophil degranulation in tissue damage and pathophysiology associated with airway inflammation have prompted research into the role that eosinophils play in disease development. The role of eosinophil degranulation as a critical effector mechanism in asthma remains elusive. It is anticipated that the release of granule proteins from mouse eosinophils may share a similar SNARE-based mechanism as human eosinophils, and the inhibition of protein release may be associated with decreasing levels of histopathology and function in mouse models of allergic asthma. Therefore, the research objectives of this project were three-fold: 1) To develop mouse models of VAMP-7 deficiency; 2) to define the role of VAMP-7 in receptor-mediated eosinophil degranulation and cytokine release using isolated cells and *in vitro* assays; and 3) to demonstrate that VAMP-7-mediated eosinophil degranulation contributes to the pathophysiology of allergic airway inflammation.

Central Hypothesis:

The v-SNARE (VAMP-7) is critical for eosinophil degranulation. In turn, allergen provocation of mice with eosinophil-specific VAMP-7 deficiency will result in loss of one or more effector functions that contribute to pathophysiological changes in allergic airway inflammation.

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Chapter Two: Methods

2.1 *Cre-Lox P* Recombination

In constitutive gene knockout mouse strains, ubiquitous gene ablation occurs in all cells of a mouse, including zygote stage embryos. Thus, many knockout mice strains have complex phenotypes, and embryonic lethality may result with the loss of a particular gene that is critical to embryogenesis. To overcome problems associated with ubiquitous gene ablation and to address complex phenotypes associated with the loss of one gene, tissue-specific genetic recombination can be achieved by the *Cre-Lox P* recombination system (1), which relies on the recognition of target recombination sequence elements (*Lox P*) by cognate recombinase (Cre, ***C***ause ***re***combination).

The *Cre-Lox P* system consists of the virus (P1 bacteriophage)-derived Type I topoisomerase, *Cre* recombinase (38kDa), which catalyzes the site-specific recombination of DNA between two target *Lox P* (locus of crossing over) sequences, with no additional supporting sequence or protein required (2). During the normal life cycle of the P1 bacteriophage after viral DNA is injected into bacteria, *Cre* recombinase circularizes the viral linear genome to gain access to the bacteria genome by mediating recombination events between endogenous *Lox P* sequences (3). This naturally occurring process has been adapted as a genetic manipulation tool to induce conditional knockout in mice since basic *Cre-Lox P* strategy recombination pathways are absent in mammals (1). The *Cre* recombinase can be expressed in many different organisms such as plants, bacteria, yeast, and mouse (4, 5) using traditional transgenic technology or gene targeting technology (1). For *Cre* recombinase to function in mice, exogenous *Lox P* sequences must be engineered into the gene of interest (i.e., the mouse VAMP-7 gene), thereby mediating excision of the gene when *Cre* is present in the same cell as the *Lox P* sequences. A characterized promoter (i.e., the EPX promoter) that is

specific to a cell type (i.e., eosinophils) can be used to drive the expression of the *Cre* recombinase cDNA, restricting expression of the enzyme in the cell type with the chosen promoter.

The *Lox P* recombination sequence element is a 34-base pair (bp) sequence that consists of two 13-bp inverted repeats flanking an 8-bp spacer region that confers the direction of the sequence (2). The direction of two *Lox P* sequences determines the outcome of the recombination event. DNA sequences can be deleted, inverted or translocated, depending on the directions of the *Lox P* sequences. DNA between directly repeated *Lox P* sequences will be excised in a circular form by the Cre recombinase thereby generating the null allele in the knockout mice offspring. The *Lox P* sequences are engineered to flank either the entire gene of interest or functionally essential exons of the gene using embryonic stem (ES) cell manipulation (1). DNA sequences flanked by two *Lox P* sites are said to be "floxed". Mice expressing the *Cre* recombinase cDNA driven by the cell-specific promoter and mice carrying *Lox P* sequences flanking the gene of interest (the floxed mice) are crossed to each other to generate cell lineage specific knockout mice in which gene targeting only occurs in cells expressing the *Cre* recombinase.

2.2 Mouse strains

2.2.1 B6.129P2-EPX $^{tm1(Cre)}$ /Lee Labs/Ozgene (*eCre* mice)

The *eCre* mice were generated by a joint effort from the Lee Labs, Mayo Clinic, Scottsdale, AZ, USA, and Ozgene, Australia. In this strain of mice, the *Cre* recombinase is expressed from the endogenous *EPX* locus (Figure 2.1). The transgenic construct contains the *Cre* recombinase cDNA following the *EPX* regulatory sequence. Exons 1-6 of the *EPX* gene were also ligated into the knock-in targeting construct following the internal ribosomal entry site

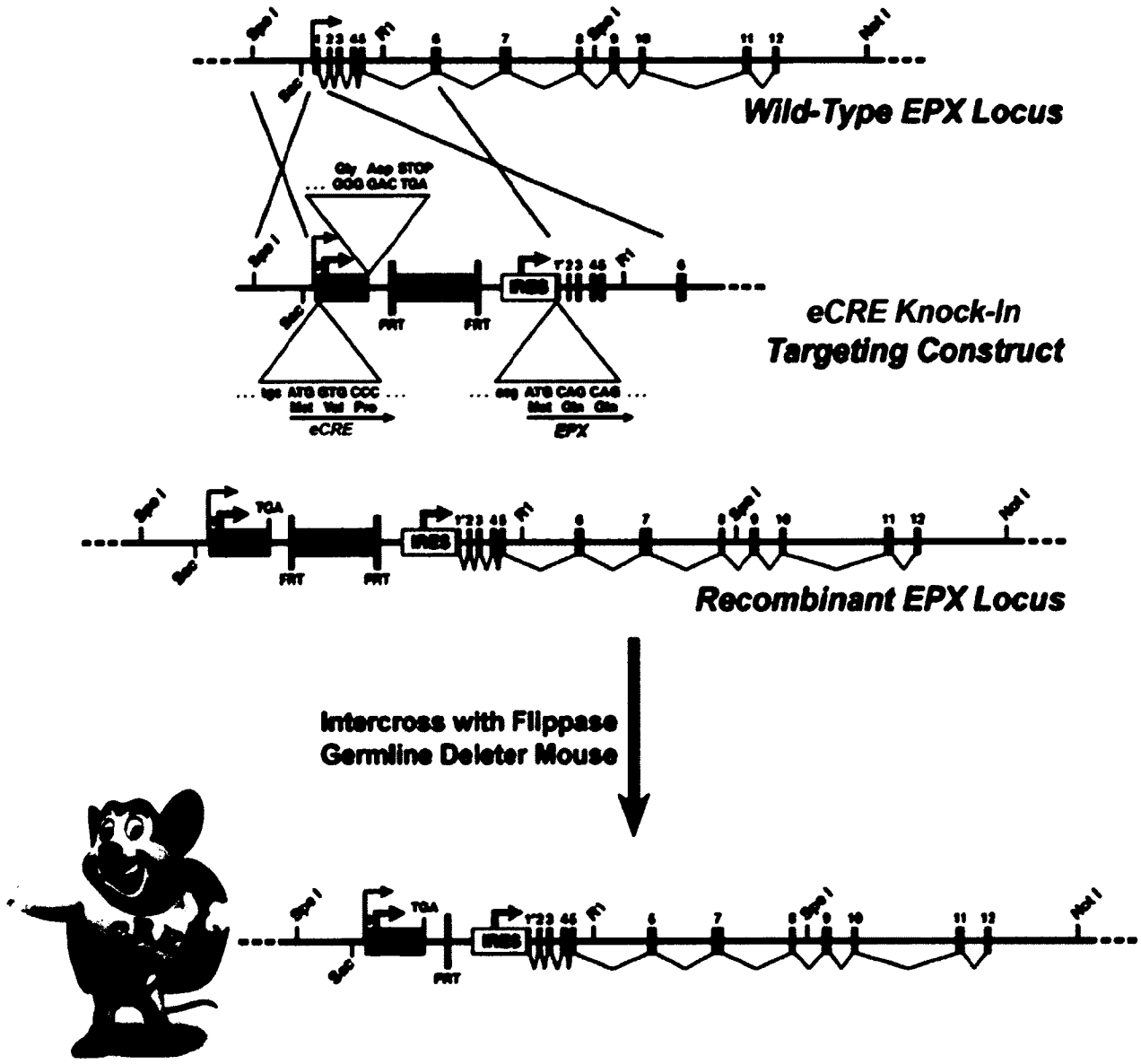

Figure 2.1. Generation of *eCre* mice. *eCre* knock-in targeting construct contains the *eCre* cDNA driven by the *EPX* regulatory sequence and partial *EPX* DNA following the IRES element. The targeting construct was electroporated into ES cells. Homologous recombination events switch the targeting construct and a portion of wild-type EPX locus. The final progeny carrying the *eCre-EPX* locus were crossed to floxed VAMP-7 mice to induce eosinophil-specific VAMP-7 deficiency.

(IRES) sequence to ensure expression of the *EPX* in the recombinant gene. The neomycin resistance cassette (Neo), a drug selection marker for ES cells with the recombinant *EPX* allele, was flanked by two flippase recognition target (FRT) sequences. The neo cassette was removed in cells of the germ-line by crossing ES-cell derived mice to a mice strain that expressed DNA recombinase (flippases) that recognize the FRT sequences. The phosphoglycerate kinase (PGK)-Neo-IRES combination flanked by FRTs was ligated into a site that is adjacent to the *Cre* recombinase cDNA to generate the knock-in targeting construct. The knock-in targeting construct was linearized and electroporated into ES cells. Homologous recombination events occurring naturally in the ES cells mediate a one-to-one replacement of the endogenous gene with the targeting construct, generating the recombinant *EPX* locus.

Mice generated using this knock-in strategy conformed to Mendelian inheritable traits and exhibited no gross abnormalities. The lifespan of *eCre* mice was similar to that of control wild-type mice.

2.2.2 C57BL/6-TgN(Zp3-Cre)93Knw/J /*Zp3-Cre* mice

The *Zp3-Cre* mice were purchased from Jackson Laboratory, MA. In this transgenic mice strain, the *Cre* recombinase expression is driven by regulatory sequences from the mouse *zona pellucida* 3 (*Zp3*) gene (6). The C57BL/6-TgN(Zp3-Cre)93Knw/J strain is useful for expressing *Cre* recombinase in female germ-line cells and mediating gene deletion ubiquitously in the progeny of the *Cre-Lox P* binary system. Hemizygous *Zp3 Cre* expressing females that are also homozygous or heterozygous for the floxed alleles are crossed to wild-type males. *Cre*-mediated recombination events occur during oogenesis and the progeny from the cross express the desired recombined allele. This is important for use with floxed VAMP-7 mice, since the VAMP-7 gene is found on the X chromosome.

To generate this strain, a transgenic construct containing the 6000-bp sequence of the mouse *Zp3* promoter and the *Cre* recombinase cDNA coupled with metallothionein-1 poly (A)

tail was linearized and injected into C57BL/6J zygotes to produce founders that were positive for the *Cre* recombinase expression at the *Zp3* locus.

2.2.3 B6; 129-Vamp-7<tm1>/RIKEN BRC/ Floxed VAMP-7 mice

The floxed VAMP-7 mice were purchased from RIKEN BioResource Center, Ibaraki, Japan. As described above, the VAMP-7 gene is located on the X chromosome. The flanking of VAMP-7 gene by the two *LoxP* sequences was achieved in homologous recombinant ES cells using a gene targeting strategy as ES cells have only one X chromosome. To maintain the expression of VAMP-7 in ES cells and to obtain viable homologous recombinant cells, partial VAMP-7 cDNA sequences (exons 5 to 8) were inserted into the targeting vector after the splice acceptor of exon 5 (Figure 2.2). The IRES sequence and neomycin resistance gene were inserted after the partial VAMP-7 cDNA, and these sequences were flanked by two FRT sequences for Flp-mediated deletion of the Neo drug selection marker. This cassette, together with exons 3 and 4, can be deleted using the *Cre-Lox P* recombination system (7).

2.2.4 IL-5 Transgenic mice (C57BL/6-Tg(IL-5)NJ. 1638/Lee Labs)

Our understanding of the SNARE involvement in eosinophil exocytosis has been limited by the paucity of these granulocytes (<3% of peripheral blood in wild-type mice). In the NJ.1638 strain of IL-5 transgenic mice, mouse interleukin 5 (IL-5), which is critical to promoting the proliferation of cells committed to eosinophil lineage, is constitutively expressed by peripheral T cells by fusion with the CD3δ promoter. The targeted construct contains a cDNA and genomic IL-5 DNA fusion gene (pIL-5.Exp) (8). The fusion gene contains a genomic IL-5 sequence and a 170-bp fragment from the 5' end of the IL-5 cDNA pSPGK.m.TRF23, which encodes all introns

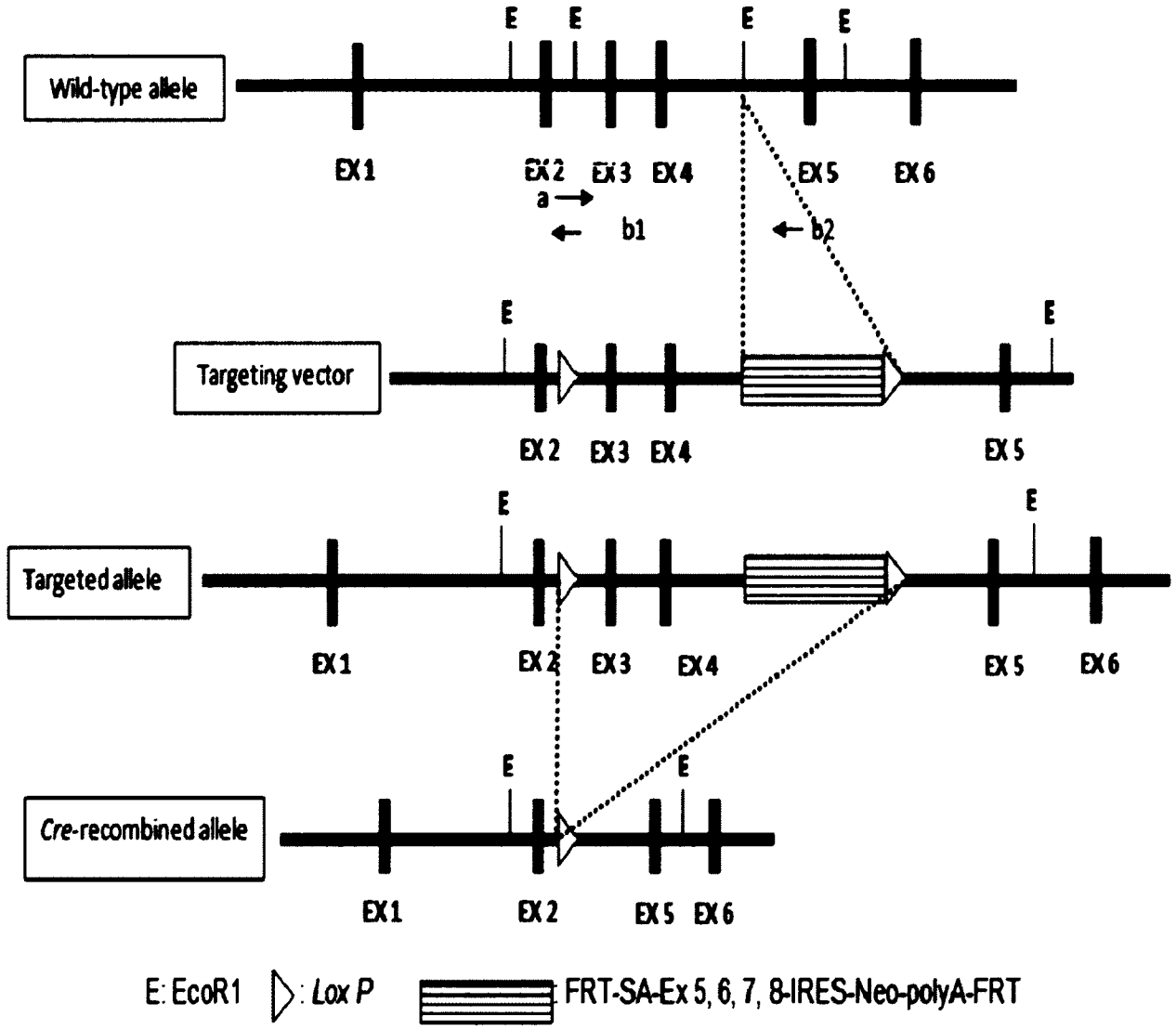

Figure 2.2. Generation of floxed VAMP-7 mice. Targeting vectors containing *Lox P* sequences flanked exons 3 and 4 and an IRES-neo-poly A sequence followed by the partials sequence of the VAMP-7 gene were electroporated into the ES cells to generate the floxed VAMP-7 targeted allele carrying mice. Three primers (a, b1, and b2) were designed based on sequences in the wild-type allele (P-a is in exon 2, P-b1 is in exon3, and P-b2 is in exon 5) to detect the deletion of exons 3 and 4 from the VAMP-7 gene. Mice carrying the targeted allele were crossed to *eCre* mice. Modified from Sato, M., *et al.*, 2011(7).

of the IL-5 gene as well as a 1.2 kb of 3' flanking sequence (9). Without the upstream regulatory sequence, the fusion gene maintains the structural features of the genomic IL-5 gene and is devoid of known endogenous regulatory elements.

Individual mice from this line (NJ.1638) exhibited dramatically elevated white blood cell counts and peripheral blood eosinophilia (8). Crossing IL-5 transgenic animals with mice that are positive for the targeted VAMP-7 allele and *eCre* gene will allow amplification in the number of VAMP-7-deficient eosinophils for *in vitro* degranulation studies.

2.2.5 *IL-5/hE2/EPX*$^{-/-}$ (Lee Labs)

IL-5 transgenic mice (NJ.1638) were generated as described earlier. A human eotaxin-2-overexpressing transgenic mouse was constructed by cloning the open reading frame of human eotaxin-2 gene (InvivoGen) into a lung-specific shuttle vector downstream of a 2.3-Kb BamHI fragment of the rat Clara cell secretory protein CC10 promoter (a gift of J. Gitlin, Washington University School of Medicine, MO). This gene was upstream of a 2-Kb fragment of the human growth hormone gene, which contains a poly(A) tail, additional signal sequences, and exon-intron splicing motifs for efficient expression in transgenic mice (10). The resulting construct was subsequently injected into embryos derived from a cross of F1 (CBA/CaJ X C57BL/6J) females and C57BL/6J males. Mice used in this study were hemizygous for both IL-5 and eotaxin-2 transgenes.

EPX knockout (*EPX*$^{-/-}$) mice were generated using a targeting vector consisting of 129/SvJ-derived genomic DNA flanking a 3.9-kb fragment containing exons 6, 7, and 8 of EPX gene (11). The targeting vector replaces these exons with a neomycin resistance cassette (PGK-neo) and the diphtheria toxin *A* gene as a negative selection marker for ES cells. The targeting construct was linearized and electroporated into ES cells (12). ES cells positive for the targeted construct were injected into C57BL/6J blastocysts to generate chimeric animals that

were bred to 129/SvJ mice. IL-5/hE2 double transgenic mice were crossed to *EPX*$^{-/-}$ mice to generate the *IL-5/hE2/EPX*$^{-/-}$ mice.

Mice with the double transgenic background display evidence of eosinophil activation, particularly degranulation. Double transgenic mice also display eosinophil-dependent histopathology and lung dysfunction (13), providing a unique environment to study the activation and role of eosinophils in remodelling and lung function. Having the double transgenic mice on an *EPX*$^{-/-}$ background, we can evaluate the ability of VAMP-7-deficient eosinophils to release EPX in an *ex vivo* setting by intratreacheal installation of the experimental cell population into the airways of double transgenic mice.

2.2.6 B6.Cg-*Gt(ROSA)26Sor*$^{tm6(CAG-ZsGreen1)Hze}$/J

The *Cre*-reporting *ROSA* mice were purchased from Jackson Laboratory, MA. This strain of mice is designed to be a high-throughput *Cre*-reporter, which evaluates the expression of *Cre* recombinase by inducing fluorescent protein production in tissues designed to express the enzyme (14). The targeting vector used to generate this strain of mice contains the CMV-IE enhancer/chicken β-actin/rabbit β-globin hybrid promoter, which target the construct insertion into the *Gt(ROSA)26Sor* locus. The *Cre* reporter unit in the targeting vector contains a *Lox P*-flanked STOP cassette, ZsGreen1 sequence (Clontech), the enhanced green fluorescent protein engineered a woodchuck hepatitis virus post-transcriptional regulatory element, a PolyA signal, and the PGK-*FRT*-Neo-PolyA cassette for ES cell drug selection (Jackson Laboratory). When crossed to mice expressing *Cre* recombinase, cells expressing *Cre* will induce the excision of the STOP cassette flanked by the *Lox P* sequences in the reporter mice, initiate the expression of the ZsGreen1 sequence, and commence the production of green fluorescent protein (GFP).

2.2.7 Eosinophil-specific VAMP-7 knockout/(B6;129S4-Tg(Epx-cre)/Vamp7<tm1Aha>)

Mice carrying VAMP-7-deficient eosinophils were generated by crossing the **B6.129P2-EPX ^{Cre1}/Lee Labs** strain to the **B6; 129-Vamp-7<tm1>/RIKEN BRC** mice. Female mice that were homozygous for the VAMP-7 targeted allele or male mice that were hemizygous for the VAMP-7 targeted allele were crossed to mice expressing *eCre* to generate eosinophil-specific knockout mice (Figure 2.3). The progeny of the first generation were crossed to each other and 12.5% of the second generation progeny were eCre$^{+/-}$ and VAMP-7 floxed males, which were used as experimental animals in the characterization of *eCre-VAMP-7* mice and the *in vivo* section of this project. Knockout mice were born at the expected Mendelian frequency and exhibited no gross abnormalities until death. Their lifespan was similar to that of control mice.

2.2.8 Germ-line VAMP-7 knockout/(B6;129S4-Tg(Zp3-cre)/Vamp7<tm1Aha>)

This ubiquitous VAMP-7 knockout mice strain is generated by crossing the **C57BL/6-TgN(Zp3-Cre)93Knw/J** mice to the **B6; 129-Vamp-7<tm1>/RIKEN BRC** mice. The *Zp3* promoter is transiently activated in growing oocytes prior to the completion of the first meiotic division. Female mice heterozygous for the *Zp3 Cre* allele and heterozygous for the targeted VAMP-7 allele were crossed to wild-type C57BL/6J males. Progeny of the first generation were crossed to each other in the second generation, and 100% of the second generation progeny were VAMP-7 null (Figure 2.4).

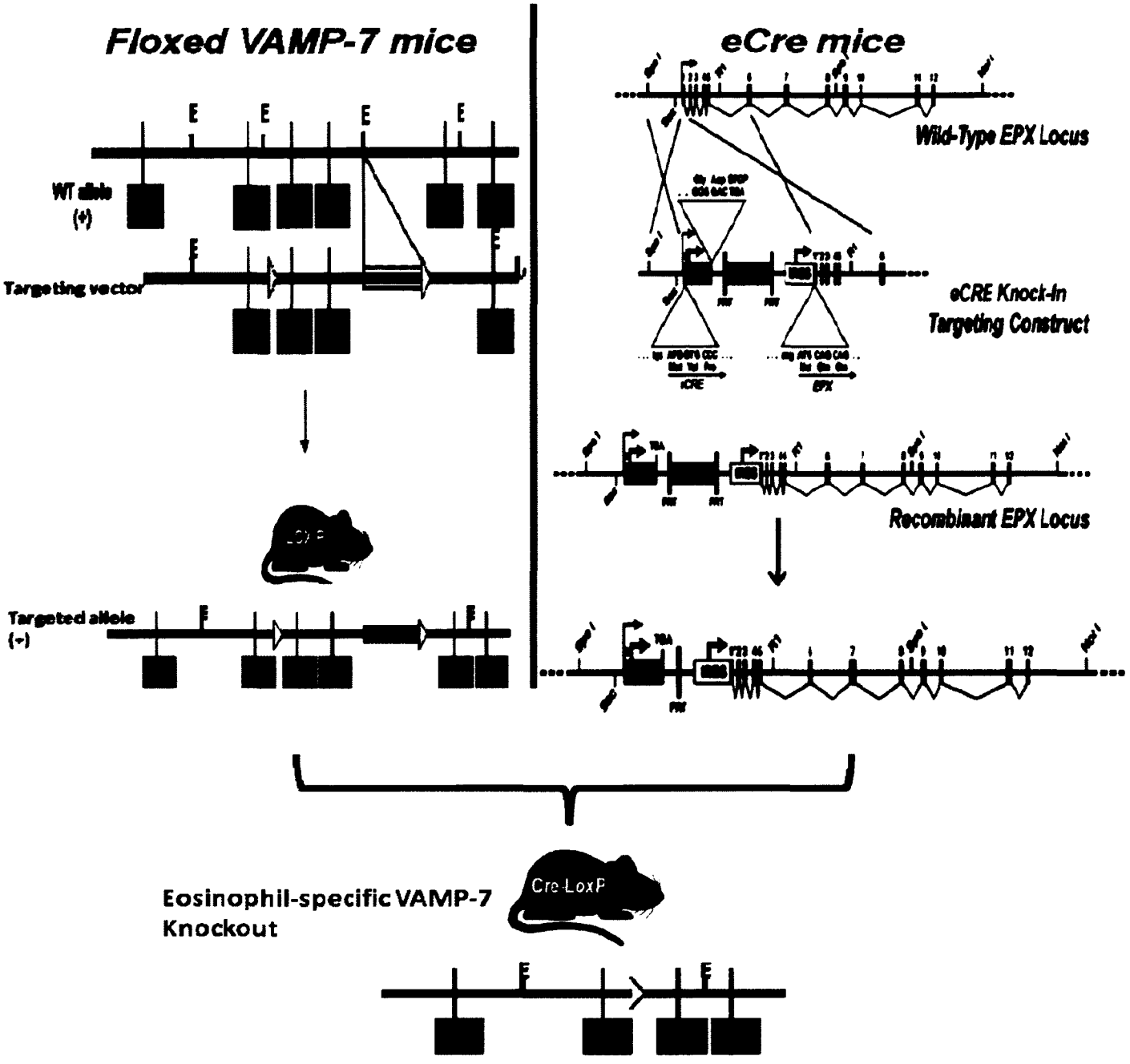

Figure 2.3. Generation of *eCre-V7* mice. *eCre* expressing mice were crossed to floxed *VAMP-7* mice. Male progeny that were heterozygous for *eCre* and hemizygous for the targeted VAMP-7 allele were used as the experimental group. E= restriction digest sites.

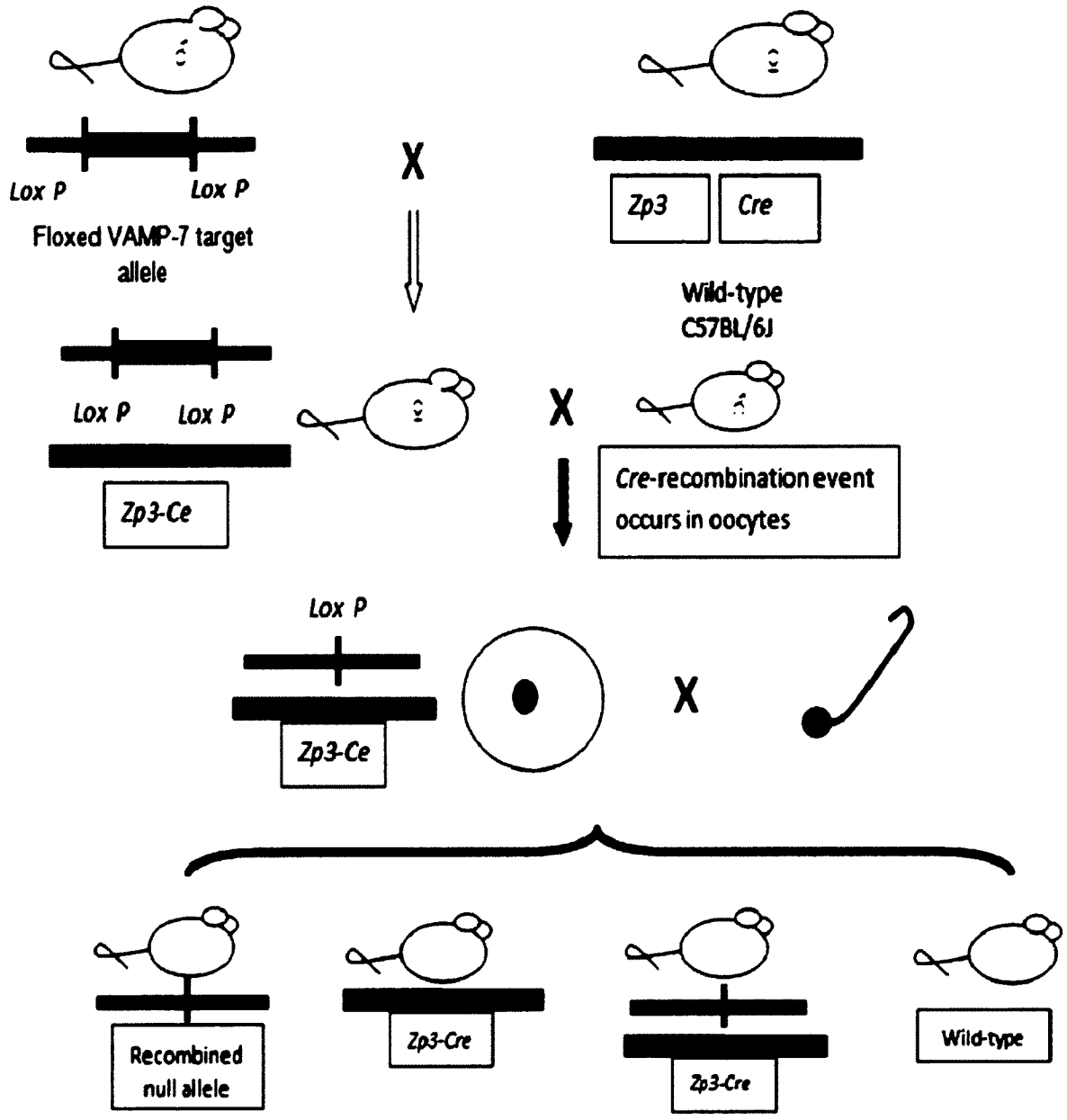

Figure 2.4. Generation of the *Zp3Cre-V7* mouse. The *Zp3* gene directs expression of the *Cre* recombinase exclusively in the growing oocyte prior to completion of the first meiotic division. Mature mammalian oocytes are transcriptionally inactive. *Cre*-mediated recombination events occur in the female germ line cells. For the *Cre* recombinase to mediate the deletion of DNA sequences in the floxed allele, female mice hemizygous for the *Zp3-Cre* gene and the floxed VAMP-7 targeting allele were crossed to wild-type mice. 25% of the progeny are germ line knockout mice carrying the recombined VAMP-7 allele. Modified from de Vries, W.N., *et al.*, 2000 (6).

2.3 DNA isolation for genotyping

Tail biopsies (0.4-0.6 cm) were cut and placed in Eppendorf tubes labeled with the animal identification number. A volume of 200 µl of tail lysis buffer (1M Tris pH 8.0, 5M NaCl, 0.5M EDTA pH 8.0, 10% SDS and dH_20) containing 50 µg/ml of Proteinase K (Roche, Cat #3115879001) was added to each tube. Tail biopsies were vortexed occasionally and incubated at 55°C overnight. Tail samples were centrifuged at 20,000 x *g* for 5 min to remove debris. DNA from the digested tails was then purified using the DNeasy Blood and Tissue kit (Qiagen, Cat #69506). Briefly, the supernatant was transferred to a new Eppendorf tube and 200 µl of Buffer AL was added and mixed thoroughly by vortexing. Then 200 µl of 96-100% ethanol was added to each sample and mixed thoroughly by vortexing. This mixture was transferred into a DNeasy Mini spin column placed in a 2 ml collection tube. The samples were spun at ≥ 6,000 x *g* for 1 min. and flow-through was discarded along with the collection tube. Spin columns were inserted into a new set of collection tubes and 500 µl of Buffer AW1 was added to each and spun at ≥ 6,000 x *g* for 1 min. The flow-through was discarded along with collection tubes. The spin columns were then inserted into a new set of collection tubes and 500 µl of Buffer AW2 was added to each spin column and spun at 20,000 x *g* for 3 min. The spin columns were then transferred into new Eppendorf tubes and 100 µl of Buffer AE was added to each spin column and incubated for 1-2 min at room temperature to elude the DNA. The tubes were spun at ≥ 6,000 x *g* for 1 min to collect eluted DNA from the spin column. Samples were stored at 4°C until analyzed by identification using polymerase chain reaction (PCR).

2.4 Polymerase chain reaction identification for *eCre*

DNA recovered from tail biopsies was used as a template in PCR to genotype animals carrying the *eCre* gene (Figure 2.1). Animals were identified as wild-type +/+ (no *eCre* allele), heterozygous (mutant (M)/+) (one *eCre* allele), or homozygous (M/M) (two copies of *eCre* allele) mice using a four-primer strategy. The internal control was identified as a 166-bp PCR amplicon using the following primers:

OZEPO F2: 5'-GAA GAA AGA AAC CAT CAC AGG ACC TC-3'

OZEPO R1: 5'- GGG TGA GGA TGA GTG TGG CTA AG-3'

The eCre locus was identified as a 582-bp PCR amplicon using the following primers:

eCre F2: 5'- CTG CTG AAC CTG AGG ATG TGA GG-3'

eCre R2: 5'- GGG TGG ACA GTT GGG AGG TG-3'

PCR reactions were combined using the following reagents: 2.5 U Taq (Roche, Cat# 11647687001), 0.1 μM of dNTP and 2 μM of each primer. The PCR cycling strategy used was 1 cycle of 94°C for 5 min, 10 cycles of 94°C (1 min), 62°C and decrease 1°C/cycle to 53°C (30 sec), 72°C (90 sec), 25 cycles of 94°C (1 min), 53°C (30 sec), 72°C (90 sec), and a final extension of 72°C for 7 min. PCR amplicons were analyzed using 1% agarose gel electrophoresis with ethidium bromide staining of the DNA.

2.5 Polymerase chain reaction identification for the targeted VAMP-7 allele

DNA recovered from tail biopsies was used as a template in PCRs to genotype potential animals carrying the targeted VAMP-7 allele. Animals were identified as wild-type (+/+, no targeted VAMP-7 allele), heterozygous (M/+, one targeted allele), or homozygous (M/M, two targeted allele) mice using a three-primer strategy. The wild-type allele was identified as a 2,430-bp PCR amplicon using the following primers (7):

P_1: 5′- TTT TGA GCG TTC TCG AGC G -3′

P_2: 5′- GAC TTA CTA GGA AGG GGG ATA GGG G -3′

The targeted VAMP-7 allele was identified as a 1,600-bp PCR amplicon using P_2 and a primer derived from the neomycin-resistance gene:

P_3: 5′- TGG ATT GCA CGC AGG TTC TC -3′

PCR reactions were combined using the following reagents: 1 U of TaKaRa LA Taq with GC buffer (Cat# RR02AG), 0.1mM of dNTP, and 0.4 μM of each primer. The PCR cycling strategy was 1 cycle of 94°C for 3 min, 30 cycles of 94°C (30 sec), 60°C (30 sec) and 72°C (3 min), and a final extension of 72°C for 5 min. PCR amplicons were analyzed using 1% agarose gel electrophoresis with ethidium bromide staining of the DNA.

2.6 Polymerase chain reaction identification of the IL-5 transgene

DNA recovered from tail biopsies was used as a template in PCRs to genotype potential animals carrying the transgene. Animals were identified as wild-type (+/+, no transgene) or transgenic (+/-, one copy of the transgene) mice using a two-primer strategy. The primers used for PCR identification were derived from the CD3δ regulatory sequences of the IL-5 transgene (9) and the first exon of the IL-5 gene (15):

Sense primer: 5'-ACC CCA CAC CTA GCC CAC TG-3'

Antisense primer: 5'-TGG CAG TGG CCC ACA CAC AGC-3'

PCR reactions were combined using the following reagents: 1.25 U of Taq in Gene Amp PCR System 9600 (Perkin-Elmer), 0.2 μM of dNTP mix and 0.2 μM of each primer. The PCR protocol used was 1 cycle of 94°C for 5 min, 30 cycles of 94°C (1 min), 72°C (2 min) and 72°C (10 min), and a final extension of 72°C for 5 min. PCR amplicons were analyzed using 1% agarose gel electrophoresis with ethidium bromide staining of the DNA.

2.7 Polymerase chain reaction identification for *Cre*-recombined VAMP-7 allele

DNA recovered from tail biopsies was used as a template in PCRs to genotype potential animals carrying the *Cre*-recombined VAMP-7 allele (Figure 2.2). Animals were identified as positive for the *Cre*-recombined VAMP-7 null allele using a three-primer strategy. The wild-type allele was identified as a 328-bp PCR amplicon derived from exons 2 and 3 using the following primers:

P-a: 5'- GCA TTA CCT GCC CCA GGC AAA ACT G-3'

P-b1: 5'- GGG ACA CAG AGG AAG CAG GTA ACG G-3'

The *Cre*-recombined allele was identified as a 326-bp PCR amplicon using primer P-a and -b2 primer derived from exon 5:

P-b2: 5'- GAG AGA TCA GGG AAT TGG TAC CGG A -3'

PCR reactions were combined using the following reagents: 0.5 U of Taq in 5X Green Go Taq reaction buffer (Promega, Cat # M7911), 0.1 µM of dNTP, and 0.2 µM of each primer. The PCR protocol used was 1 cycle of 94°C for 2 min; 35 cycles of 94°C (30 sec), 58°C (30 sec) and 72°C (1 min); and a final extension of 72°C for 5 min. PCR amplicons were analyzed using 2% agarose gel electrophoresis with ethidium bromide staining of the DNA.

2.8 Polymerase chain reaction identification for human eotaxin-2 transgene

DNA recovered from tail biopsies was used as a template in PCRs to genotype mice positive for the human eotaxin-2 transgene. Mice were identified as positive for the transgene using a two-primer strategy. The human eotaxin-2 transgene was identified as a 475-bp amplicon derived from eotaxin-2 open reading frame and the human growth hormone gene using the following primers (13):

P-forward: 5'-CAC CAC CAA GAA GGG CCA GCA GTT-3'

P-reverse: 5'-ACA GAG GGA GCC GGA GAG CAA GAG-3'

PCR reactions were combined using the following reagents: 0.2 U of Tap from the GeneAmp XL-PCR kit (Applied Biosystems), 0.1μM of dNTP, and 0.2 μM of each primer. The PCR protocol used was 1 cycle of 94°C for 5 min, 30 cycles of 94°C (1 min), 60°C (2 min), 72°C (2 min), and a final extension of 72°C for 7 min. PCR amplicons were analyzed using gel electrophoresis with ethidium bromide staining of the DNA.

2.9 Polymerase chain reaction identification for EPX knockout

DNA isolated from tail biopsies was used as the template in PCR reactions to identify mice carrying the EPX targeted allele. Mice were identified as wild-type (+/+, no EPX targeted allele), heterozygous (+/-, only one copy of the targeted allele), and homozygous (-/-, both copies of the targeted allele) using a three-primer strategy. The wild-type allele was identified as a 750-bp amplicon using the sequence information based in exon 8 (P1) and exon 9 (P2):

Primer 1 (P1): 5′-TGAAACCCCCAAACTGACGG-3′

Primer 2 (P2): 5′-ACAGAGCTAAGCGGGACGTG-3′

The targeted *EPX* locus was identified as a 1000-bp amplicon using P2 and P3, which is based on the sequence information in the neomycin-resistance gene:

Primer 3 (P3): 5′-CATCGAGCGAGCACGTACTC-3′

PCRs were assembled using the GeneAmp XL-PCR kit (PerkinElmer Applied Biosystems) with 0.2 U of Taq, 0.2 μM of dNTP and 0.2 μM of each primer. The PCR cycling strategy used was 94°C for 5 min followed by 30 cycles of 94°C (1 min), 57°C (2.5 min), 72°C (2 min), and a final extension of 72°C for 7 min (16).

2.10 Bone marrow isolation

The femur and tibia were removed surgically after mice were euthanized with CO_2. The epiphyses of the long bones were removed with a scalpel. Femurs and tibias were flushed into Eppendorf tubes with RPMI 1640 (Life science, Gibco 11835) using a 3 ml syringe and a 25G needle. The marrow solution was transferred into a 15 ml conical tube filled with 12 ml of RPMI

1640 and centrifuged in a large bench top centrifuge at 500 x *g* for 10 min at 4-8°C. Erythrocytes were removed by re-suspension of the cell pellet with 1 ml cold sterile dH_2O followed by 100 µl of 10XPBS.

2.11 Bone marrow derivation of mouse eosinophils

Bone marrow progenitors were isolated as previously mentioned and the *in vitro* derivation was carried out as described earlier (17). A total of ~1.5-2x10^7 per femur and ~0.8-1x10^7cells per tibia were isolated and resuspended in RPMI (Invitrogen, Cat #11875-093) supplemented with: 100 IU/mL penicillin and 10 µg/mL streptomycin (Cellgro), 2 mM L-glutamine (Invitrogen), 25 mM HEPES, 1x non-essential amino acids, 1 mM sodium pyruvate (Gibco), 50 µM β-mercaptoethanol (Sigma-Aldrich). Cells were filtered through a 40 µm cell strainer (BD Falcon, Cat # 352340) into a 50 ml conical tube. Filtered cells were transferred into a new 15 ml conical tube and brought up to 12 ml with 0.1% BSA in PBS. The suspension was centrifuged at 90 x *g* for 10 min at 4-8°C and re-suspended to 1x 10^6cells/ml in RPMI. Recombinant Murine stem cell factor (100 ng/ml, PeproTech, Cat# 250-03) and recombinant Murine Flt-3 ligand (100 ng/ml, PeproTech, Cat# 250-31L) were added into the cell suspension. The culture was incubated in a humidified atmosphere of 5% CO_2 at 37°C.

On day 4, 15 ml of culture was removed and transferred to a 50 ml conical tube. The culture was centrifuged at 90 x *g* for 10 min at 4°C. The cells were re-suspended in RPMI supplemented with 10 ng/ml of recombinant mouse interleukin-5 (rmIL-5; R&D Systems) and replaced in the incubator at 37°C. Cells (1 x 10^6) were sampled from the culture for cytospin and DNA extraction, and PCR identification of the *Cre* re-combined allele.

On day 6, another sample of 1 x 10^6 cells was removed for DNA extraction. The culture was then returned to the incubator without changing the media. On day 8, another 1 x 10^6 cells were removed, and the culture was transferred into a 15 ml conical tube and centrifuged at 90*g*

for 10 min at 4°C. The cells were resuspended to a final concentration of 1x 10^6 cells/ml in RPMI supplemented with 10 ng/ml of rIL-5 in a new flask and returned to the incubator at 37°C.

On day 10, the old media was replaced by new RPMI supplemented with 10 ng/ml rIL-5. The culture was re-suspended to a final concentration of 1x 10^6 cells/ml. Cells (1 x 10^6) were taken from the culture for cytospin and DNA extraction. Supernatant from the culture was collected for ELISA analysis of granular protein (EPX). The culture was returned to the incubator. Prior to terminating the cell culture on day 12, 1 x 10^6 cells were sampled for cytospin and >90-100% eosinophils was determined by visual inspection of Diff-Quik-stained cytospin preparations.

2.12 White blood cells isolation by lysis of red blood cells

Mice were warmed under a heat lamp and placed in a holder to expose the tail for collecting peripheral blood. A volume of 100-800 µl of tail blood was collected in Eppendorf tubes filled with 40 µl-100 µl of heparin. The tubes were inverted to mix the heparin and blood collection. The tubes were then filled with 1x lysis buffer (diluted from 10X Pharm Lyse™, BD and water). The cell suspension was spun at 500 x *g* for 5 min at 4°C after 1 min of lysis. The supernatant was removed by aspiration. The cell pellets were resuspended by tapping or gentle vortexing. The lysis process was repeated up to 10 min or until a clear white pellet was found at the bottom of the Eppendorf tubes. The white blood cell pellets were subsequently washed in 0.25%BSA in PBS with 2 mM EDTA (Sigma Aldrich, Cat #T6025).

2.13 Sorting of eosinophils by flow cytometry

Single-cell suspensions were stained for 25 min at 4°C with antibodies against CCR3-FITC, SiglecF-PE, Gr-1-PE Cy 7, B220-APC, and CD4-Alexa 700 in 3 ml of 0.5% BSA, 2 mM EDTA in 1x PBS. Individual cells were hydro-dynamically focused to pass in front of a laser as a stream of single droplets. Each drop was given an electronic charge depending on the fluorescent cell surface staining in the droplet. The charged cells were either attracted or

repelled into collection tubes by the deflection plates. Cells were sorted using a Becton Dickinson FACS Aria Cell Sorter with 488 nm and 635 nm lasers. Sorted eosinophils were CCR3+Siglec-F+Gr1+B220-CD4- cells. Sorted lymphocytes were pooled and collected as CCR2-Siglec-F-Gr1-B220+CD4+.

2.14 Real-time PCR analysis for VAMP-7 exons 3 and 4 excision

Approximately 20-30 ng of DNA isolated from sorted eosinophils of eCre-V7 mice or from the bone marrow culture was used to perform real-time PCR using iQ SYBR Green Spermix (BioRad) and the following primer sets (P-C and P-D) designed based on sequence in exon 1, the un-excised control region (54-bp):

P-C: 5'-GAA GCT TGC GGC GTC AGG TC- 3'

P-D: 5'-GGG TCG CCC ACT GCC TGA AA- 3'

Two primers (P-A and P-B) were designed based on the sequence immediately up-stream of the first *LOX* P sites in exon 3 (104-bp):

P-A: 5'- AAC TCC TGG CTG ACT CTT TGC ATC T- 3'

P-B: 5'-GGA CAC AGA GGA AGC AGG TAA CGG- 3'

The efficiency of VAMP-7 excision was evaluated using the standard $\Delta\Delta$Ct method and comparing the results of the qPCR from the excised region of Vamp-7 to that of exon 1; the lower the ratio, the more *Cre*-recombined allele present (Figure 2.5).

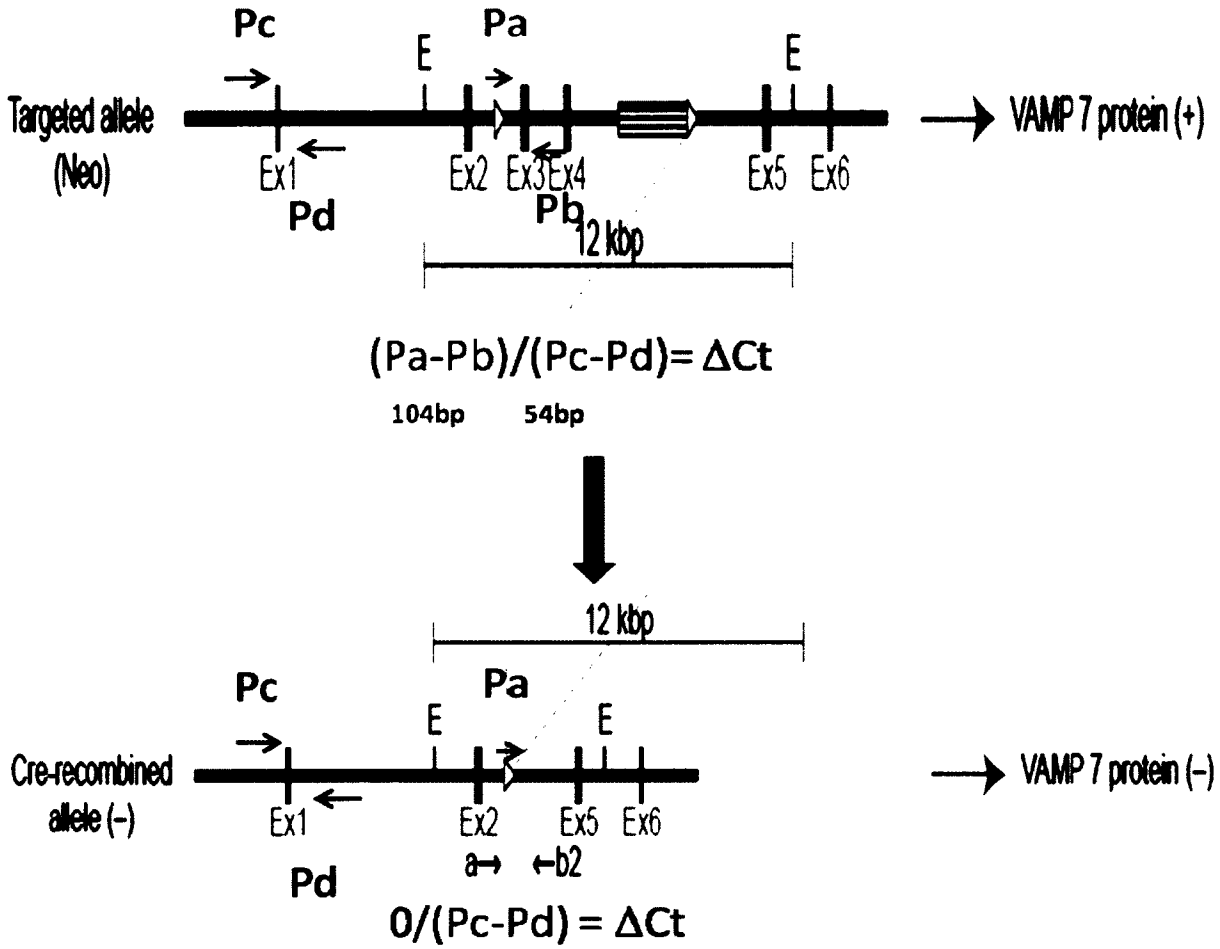

Figure 2.5. VAMP-7 deletion efficiency confirmed by real-time PCR (qPCR). qPCR data were collected using the standard ΔΔCt values. The qPCR data for the excised region was compared to that of the control region to generate ΔCt. The ΔCt value for the *cCre*-V7 mice were compared to that of the floxed VAMP-7 mice to generate the ΔΔCt ratio. Modified from Sato, M., *et al.*,2011(7).

The samples were run on a BioRad My iQ Single Color Real-Time PCR Detection System (PCR Cycles: 95°C 3 min, 95°C 10 sec (40x), 55°C 30 sec, 95°C 1 min, 55°C 1 min, followed by a melting cure up to 95°C at 10-second intervals) and analyzed using BioRad iQ5 software. The relative ratio of excised to un-excised VAMP-7 DNA was normalized to 1 relative unit for the controls with experimental groups represented as the fold changes compared to the control group. Statistical analysis was performed using a paired *t*-test.

2.15 Flow cytometry identification of eosinophils isolated from progeny of B6.Cg-*Gt(ROSA)26Sor*$^{tm6(CAG-ZsGreen1)Hze}$/J and B6.129P2-EPX $^{tm1(Cre)}$ /Lee Labs/Ozgene (*eCre* mice)

A total of 1 x 10^6 cells were placed in FACS tubes and pelleted at 500 *x g* for 5 min at 4°C.The cell pellets were re-suspended by vortexing for 5 sec. The cell suspension was incubated in 50 µl of FACS buffer (PBS +0.5% BSA (freshly filtered and contains no azide) + 2mM EDTA) with 1 µg/µl of Fc Block (CD16/32, eBiosciences) for 5 min at 4°C. A total of 50 µl of FACS buffer with 1 µg/ml of rat anti-mouse IL-5 receptor α/CD125 (PE channel, BD Pharmingen™, cat# 558488); rat anti-mouse Ly-6G/Gr-1, clone RB6-8C5 (PE-Cy7 channel, eBiosciences, cat #25-5931); Mouse CCR3-fluorescein mAb, clone 83101 (APC channel, BD Pharmingen™, cat# 557974), was added to each sample and incubated for 25 min at 4°C in the dark. The cell suspension was washed by adding 2 ml of cold FACS buffer and subsequently pelleted at 500 *x g* for 5 min at 4°C. The cell pellets were re-suspended in 300 µL of 0.2% paraformaldehyde in FACS buffer and stored in the dark at 4°C. The FITC channel (FL 1) was left open to see all GFP^+ cells.

2.16 Eosinophil isolation

Eosinophils used in *in vitro* experiments were obtained at > 98% from peripheral blood of 6 to 8 week old *eCre*-VAMP-7-expressing mice on the IL-5 background (NJ.1638) as previously described (8). Briefly, whole blood pooled from mice was layered on top of Histopaque 1119 (Sigma Aldrich, Cat # 11191) and centrifuged at 800 x *g* for 30 min at room temperature. White

blood cells pooled at the plasma and Histopaque interface were transfered to a new 50 ml conical tube. Cells were washed once with cold PBS and pelleted at 500 x *g* at 4°C for 5 min. Contanminating erytherocytes were removed from the cell pellet by hypotonic lysis with 5 ml of cold dH2O for 30 sec with gentle mixing. The lysis was terminated by adding 45 ml of cold PBS into the cell suspension. The cell suspenstion was subsequently filtered through a 40 µm filter and centrifuged at 500 *g* for 5 min at 4°C and resuspended in MACS buffer (0.5% BSA, 2mM EDTA in 1x PBS). To obtain pure eosinophils, the white blood cells in a single–cell suspension were incubated with MACS® MicroBeads conjugated with CD45R/B220 antibodies (B cells) (Miltenyi Biotec, Cat. #130-049-501), and CD90/Thy1.2 antibodies (T cells) (Miltenyi Biotec, Cat. # 130-049-101) for 25 mintues at 4°C. The mixture of white blood cells and magnetic beads were passed through a MACS Column (1-2 x10^8 cells/column) filled with ferromagnetic spheres placed in a MACS Magnetic Separator. The lymphocyte population (T and B cells), labeled by antibody-conjugated microbeads, were retained within the column while unlabeled eosinphils were filtered out of the white blood cell population by gravity flow. The purity of the eosinophil population was determined by Diff-Quik stained cytospin samples of isolated cells.

2.17 *In vitro* Eosinophil Stimulation

Mouse peripheral eosinophils were isolated at >98% from *eCre* -VAMP-7 expressing mice on the IL-5 transgenic background (NJ. 1638) as described previously. Eosinophils were stimulated *in vitro* as previously described (18). Purified eosinophils were collected by centrifugation and resuspended in phenol red-free RPMI 1640 (Life Technologies, Cat #11835) at 10^6cells/ml. Aliquots of 2 x 10^5 cells were incubated with PAF (200ng/ml, Alexis Biochemicals, Cat # ALX-301-008) and/or ionomycin (50ng/ml, Sigma-Aldrich, Cat #10634), and DMSO as vehicle control (Sigma-Aldrich, Cat #D5879) for 30 min at 37°C in an atmosphere of 5% CO_2 and 95% humidity. The supernatants were collected by spinning the culture at 500 x *g*

for 5 min at 4°C. Supernatants were centrifuged again at 16,000 x *g* for 5 min to remove cells, organelle, and debris. Supernatants were stored at -80°C for further analysis.

2.18 Intratreacheal instillation of VAMP-7-deficient eosinophils into *IL-5/hE2/EPX*$^{-/-}$ recipient mice

Purified eosinophils were resuspended in 1x PBS at a concentration of 1 x 10^7cells/25-30 µl and rested on ice. Recipient mice (*IL-5/hE2/EPX*$^{-/-}$) were anaesthetized with isoflurane and were suspended by their front teeth to expose the trachea through the oral cavity. A volume of 25-30 µl of eosinophil suspension or vehicle control (PBS) was installed into the trachea of the mice. Mice were allowed to recover from anesthesia post-transfer. BAL samples were collected from the recipient mice 24 h later. BAL samples were spun at 500 x *g* for 5 min at 4°C, and supernatants were spun again at 16,000 x *g* for 10 min to remove cellular debris prior to storage at -80°C.

2.19 EPX Enzyme-linked immunosorbent assay (ELISA)

The release of EPX was measured by ELISA as described earlier (19). A microtitre plate (Nunc-Immuno™ Plate MaxiSorp, Thermo Scientific, Cat #439454) was coated with 100 µl of mouse anti-EPX monoclonal antibody, clone MM25-429.1.1 (capturing antibody), at a final concentration of 2 µg/ml, in a 1/10 dilution of Coating Solution (KPL Cat# 50-84-00) at 4°C overnight. Coated wells were cleared of unbound capturing antibodies by three rinsing cycles with Wash Solution (KPL Cat# 50-63-00, 1/10 diluted in distilled H_2O). Non-specific binding was reduced by incubating wells with 300 µl of BSA Diluent/Blocking solution (KPL Cat# 50-61-00) at room temperature for 30 min. After Blocking Solution was removed from wells, 100 µl of sample (or standard) was added and incubated at room temperature for 1.5 h. Unbound target antigens were cleared from wells by three cycles of washes. Following the wash, 100 µl of the 0.8 µg/ml biotinylated rat anti-EPX monoclonal antibody, clone MM25-82.2.1 (detection antibody), was added to each well and incubated at room temperature for 1.5 h. After unbound detection

antibody was cleared by three washing steps using Wash Solution, 100 µl of 1/500 Streptavidin-Alkaline Phosphatase (R&D Systems, Cat. # AR001, in 1% BSA, 0.05% Tween 20, 0.025 M Tris, 0.5 M NaCl (pH 7.4)) was added to the wells and incubated at room temperature for 20 min. After three washes, 100 µl of BluePhos Microwell Phosphatase Substrate System (KPL, Cat # 50-88-00) was added to each well, and the plate was incubated at 37°C for 1 h with gentle rotation. To terminate the enzyme reaction, 100 µl of Stop Solution (KPL, Cat # 50-89-00) was added to each well. The absorbance of each sample (or standard) was determined at a wavelength of 610-630 nm with Bio-Tek Quant Microplate Spectrophotometer with KC4™ Data Analysis Software from Bio-Tek (Winooski, VT). EPX standards were prepared by isolating peripheral blood eosinophils from NJ.1638 mice. Standards were made from a purified eosinophil suspension with a concentration equivalent to 14.6 x 10^6cells/ml. Cell suspension (250 µl) was spun for 10 min at 950 *g*. Supernatant (200 µl) was removed and 250 µl of 0.22% hexadecyltrimethylammonium bromide (CTAB, Sigma, Cat. # H6269) in 0.3 M sucrose solution was added to lyse cells. The lysate was vortexed for 1 min and subsequently flash-frozen in liquid nitrogen and stored at -80°C. For standards, lysates were thawed on ice and pulse-spun at 16,000 *g*.

2.20 MBP/Ears Dot Blot ELISA

The release of MBP and Ears were detected using single-dimension ELISA as described previously (13). Nitrocellulose membrane (Bio-Rad, Cat #162-0147) was pre-soaked in PBS for 15 min before placing in a Bio-Dot Microfiltration Apparatus (Bio-Rad, Cat #170-6545). PBS (100 µl) was used to re-hydrate the membrane and filtered by vacuum. Samples and standards (100 µl) were transferred to the Microfiltration Apparatus and left at room temperature for 90 min to allow it to filter through the membrane by gravity to facilitate binding of antigen. This was followed by a wash with 100 µl of PBS, which was left at room temperature for 5 min, then pulled through the membrane under vacuum. The membrane was removed from the

Microfiltration Apparatus and blocked with 1% casein (Pierce, Cat #37528) at room temperature for 1 h on a slow shaker. The membrane was subsequently incubated with biotinylated rat-anti mouse MBP (MM20 220.1.2, 1 µg/ml) antibody or biotinylated rat-anti mouse Ears (MT3 25.1.1, 1 µg/ml) in 1% casein solution for 1 h at room temperature on a slow shaker. The membrane was then washed three times for 5 min each with 0.05% Tween-20 (Pierce, Cat #2832) in PBS (PBST). Following the wash, the membrane was incubated with streptavidin (Roche, Cat #1089161, 1:1000) in 1% casein solution on a slow shaker for 30 min at room temperature. The membrane was subsequently washed five times with PBST at room temperature. In the dark, chemiluminescent substrate, Lumi-Phos solution (Pierce, Cat #34150), was added to the membrane and incubated for 3 min at room temperature. Excess substrate was drained from the membrane and the edge of the membrane was blotted lightly onto filter paper. The membrane was wrapped with clear plastic wrap and placed in a photo cassette for exposure onto autoradiography film (MIDSCI, Cat #21700-03). Experimental samples were compared to a standard curve of protein lysates derived from known numbers of eosinophils.

2.21 IL-4, -13 ELISA

ELISA for detecting IL-4 and -13 from biological fluid were assessed using immunoassay kits (R&D Systems) according to manufacturer's instructions.

2.22 Induction of allergen airway inflammation/acute OVA protocol

Experimental mice were sensitized and challenged with chicken OVA as described previously (16). Male mice (6-14 weeks old) were sensitized on days 0 and 14 by intraperitoneal injection of 20 µg of OVA (Sigma, Cat #A5503) and 2.25 mg adjuvant Imject® Alum ($Al(OH)_3$-$Mg(OH)_2$, Thermo Scientific, Cat #77161) resuspended in 100 µl of 0.9% sodium chloride (Hospira, Cat #0409-7984-06). On days 24, 25, and 26, in a PIE nebulizer at 30 mg pressure, sensitized mice were exposed for 25 min to an aerosol of 1% (wt/vol) OVA dissolved in 0.9% sodium chloride. Control mice received 25 min of aerosol challenge of 0.9% sodium chloride

alone. Mice were rested on day 27, and then assessed for pulmonary infiltrate, histopathology, and lung function two days following the last OVA challenge on day 28.

2.23 Preparation and quantification of bronchoalveolar lavage (BAL) fluid

BAL fluid was collected as previously described (20). Mice were euthanized with sodium pentobarbital. Tracheotomy was performed to expose to the trachea. An 18-gauge catheter (1.3 X 30 mm, BD Angiocath, Cat #381147) was inserted into the trachea. The lungs were lavaged with 1 ml aliquots of ice-cold PBS with 0.2% fetal calf serum (Life Technologies, Cat #12483-020). Typically, 0.7-0.9 ml of instilled lavage fluid was recovered. BAL samples collected were centrifuged at 500 *g* for 5 min at 4°C to pellet cells for cytospins and differential counts. All supernatants were centrifuged again at 16,000 *g* for 10 min to remove lung debris. BAL supernatants were stored at -80°C for further analysis. If red blood cells needed to be removed at this step, 70 µl of 1x Pharm Lyse™(BD, Cat #555899) was added to the cell pellets, followed by 500 µl of 5% BSA in PBS. Total BAL cell counts were carried out using a hemocytometer. All pellets were prepared for cytospin onto slides.

2.24 Cytospin and staining for differential cell counts

ColorFrost Plus slides (ThermoScientific- in associated with Fisher #99-910-0) were pre-coated with 10% FBS before adding cells. Samples (100,000 cells/ml) were loaded onto pre-coated slides and spun at 500 RPM for 5 min at room temperature in a cytocentrifuge (Thermo Scientific Cytospin 4, A78300003). The slides were completely air-dried at room temperature prior to differential staining with modified Wright Stain technique (Diff-Quik Stain set, SIEMNS, Cat #B4132-1A) and coverslipped with xylene and with Consul-Mount (Thermo Scientific, Cat# 9990441). A total of 300 cells/sample were counted to obtain differential cell counts for BAL samples.

2.25 Assessments of pulmonary histopathology: Collagen deposition, goblet cell metaplasia, and airway smooth muscle hyperplasia

Lungs were inflated *in situ* with 1 ml 10% formalin and fixed before being embedded in paraffin. Sections of 4 μm were cut and stained with hematoxylin and eosin for general assessment of histopathology such as inflammatory cell infiltrates as well as epithelial and airway smooth muscle hypertrophy and/or hyperplasia. Lung sections were also stained with Masson's Trichrome for evaluation of collagen deposition and fibrosis. Goblet cell metaplasia and airway mucin accumulation were assessed by periodic acid-Schiff (PAS) staining. Mayo Clinic Arizona Histology Core sectioned and stained the slides.

2.26 Immunohistochemistry using rat-anti mouse MBP monoclonal antibody (mMBP)

Slides containing 4 μm lung sections were stained with mMBP as described previously (14). Briefly, paraffin was removed by incubating slides in a 55°C oven for 10-15 min in a vertical position and immersed in the following solution: xylene (3 baths) for 5 min each; 50% xylene / 50% ethanol (1 bath) for 2 min; absolute ethanol (2 baths) for 2 min, 95% ethanol (1 bath) for 2 min; 70% ethanol (1 bath) for 2 min; and in water bath for 1 min.

Slides were mounted onto coverplates and secured into the slide rack. Slides were incubated with 200 μl of Dual Endogenous Block (Dako Cytomation, Cat #S2003) for 10 min at room temperature, and then rinsed 3 times with wash buffer (Dako, Cat# S3006). Pepsin Solution (200 μl, Invitrogen, Cat# 00-3009) was added to the slides and incubated for 10 min. Slides were subsequently washed and blocked with 200 μl of the Blocking Solution (1.5% Normal Rabbit Serum (NRS, Vector Labs, Cat# S-5000), Avidin D solution (Vector Labs, Cat# SP-2001) in Tris-buffered saline (TBS, Dako, Cat# S3001) and incubated at room temperature for 20 min. Then mMBP antibody (MM20 220.1.2, 200 μl of 2 μg/ml (diluted in 1.5% NRS)) or negative control (Rat IgG, Vector Lab, Cat# I-4000) were added to slides and incubated at room temperature for 40 min. Unbound antibodies were washed away by three rinses with wash

buffer. To detect bound anti-mMBP, anti-rat IgG biotinylated mouse adsorbed reagent (Vector Labs, Cat# BA-4001, 200 µl of 0.4 µg/ml) was added to the slides and incubated for 30 min as the secondary antibody. Vectastain ABC-AP (Vector Labs, Cat# AK-5000, 200 µl) was added to the slides and incubated for 30 min. Slides were washed with wash buffer three times before adding 200 µl of Permanent Red substrate (Dako, Cat# K0695) and incubated for up to 30 min at room temperature. Slides were rinsed with distilled water, removed from the slide rack, transferred onto a slide holder, and immersed in distilled water. To counterstain, slides were then immersed in 0.1% Methyl Green solution (Sigma, Cat# M-8884) for 15 sec and rinsed with distilled water. The slides were air-dried prior to coverslipping with Consul-Mount (Thermo Scientific, Cat# 9990441).

2.27 Assessment of AHR in response to methacholine challenge

Functional airway phenotypes in mice were evaluated using a previously described method (21-23). Briefly, mice were assessed by inducing airflow obstruction with aerosolized methacholine 48 h after the last OVA challenge. Before each animal was prepared for the assessment, the computer controlled ventilator (Flexivent-, SCIREQ) was calibrated as described previously (24). Mice were anesthetized by an intraperitoneal injection of 90 µg/g body weight pentobarbital sodium (Abbott Laboratories) diluted 1:5 in saline. Tracheotomy was subsequently performed on anesthetized mice, and a fire-polished glass endotracheal cannula (18G) was inserted into the trachea and secured by sutures. The endotracheal cannula was attached to a pneumotachograph (model 8410, Hans Rudolph) and an ultrasonic nebulizer (Porta-Sonic model 8500C, DeVillbiss Health Care) for methacholine delivery. Mice were paralyzed with 0.5 µg/g body weight pancuronium bromide (Sigma Aldrich, Cat# P1918) and placed on a 37°C heating station to maintain body temperature during the procedure. Ventilation for the mice was provided by the Flexivent apparatus with a tidal volume of 8 ml/kg and a frequency of 2.5 Hz. A positive end-expiratory pressure of 2–3 cmH_2O was applied by

submerging the ventilator expiratory line under water. Baseline airway functions were collected for each animal with 7 breaths of regular ventilation. Mouse airway responses to methacholine were assessed with the delivery of increasing concentrations (0, 3, 6, 12, 25, and 50 mg/ml in sterile saline) of methacholine aerosol (Sigma Aldrich Chemical, Cat #A2251) into the trachea at 20 breaths/min for 30 sec with the ventilator piston delivering a tidal volume of 0.8 ml. Before each aerosol challenge, the lungs were inflated to total lung capacity (30 cmH_2O) to re-establish a standard volume. After each aerosol exposure, 7 breaths were collected for analysis of airway resistance data at the peak of the response.

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Chapter Three: Characterization of VAMP-7 Conditional Knockout Mice and the Generation of VAMP-7-Deficient Eosinophils

3.1 Introduction

The contribution of SNAREs to exocytosis in the degranulation process has been demonstrated in human neutrophils, eosinophils, platelets, and mast cells (1-5). Human circulatory eosinophils permeabilized and treated with a neutralizing antibody against VAMP-7 showed a dose-dependent decrease in EPX and EDN release (6). In this study, it was hypothesized that VAMP-7-mediated membrane fusion is critical in eosinophil degranulation and the release of secondary granule proteins in mouse peripheral blood eosinophils. In turn, the ablation of VAMP-7 gene expression is hypothesized to result in the augmentation of one or more effector functions in mouse eosinophils such as receptor-mediated degranulation.

To define the role of VAMP-7-mediated events in eosinophil degranulation, interference with VAMP-7 expression is necessary. Many researchers evaluated the role of v-SNAREs in immune cells (e.g, eosinophils, neutrophils, NK cells, and mast cells) using cells treated with neutralizing antibodies and/or small interfering RNA, which are introduced into isolated cells via chemical or electrical permeabilization (6-9). Critical information regarding the function of SNARE proteins has been provided using *in vitro* models of down regulation of protein functions. Nevertheless, receptor-mediated degranulation responses from intact cells genetically devoid of SNARE expression in a specific cell type have not been reported.

Genetic ablation of target protein using transgenic mouse models is a unique translational tool, which is routinely used to study the function of target proteins. To generate knockout mice, gene targeting and gene trapping are two methods used to insert artificial DNA constructs into the pronuclei of developing zygotes or chromosomes in the nuclei of ES cells

(10). Gene trapping is useful when the DNA sequence is unknown, and designed DNA constructs with selection markers are randomly integrated into the mouse genome. Phenotypes of the founders are evaluated to determine which gene's expression is altered as the result of the integration. Gene targeting is preferred as a high-throughput method to introduce gene silencing throughout the entire genome when the DNA sequence for the gene of interest is known (e.g., VAMP-7).

In gene targeting, an artificially designed DNA construct (also known as the targeting vector), which shares homologous sequences to the gene of interest, is introduced to the nuclei of ES cells. The cell's machinery recognizes homologous sequences and switches the portions of the existing gene with designed DNA constructs as a result of homologous recombination. Gene targeting allows researchers to knock out a gene at high efficiency and to generate ubiquitous gene knockout in mice when used alone. However, when ubiquitous gene deletion is associated with an embryonic lethal phenotype or when tissue specificity is required for hypothesis-driven research, the *Cre-Lox P* binary is commonly used as it provides sophisticated control over when and where the gene deletion occurs.

In this study, two strains of mice deficient in VAMP-7 were generated using the *Cre-Lox P* binary system. The first is a ubiquitous knockout strain in which mice are congenitally devoid of VAMP-7 expression in all tissues, including peripheral blood eosinophils. The second is an eosinophil-specific knockout strain, in which mice have the VAMP-7 gene deleted only in their eosinophils.

The ubiquitous VAMP-7 knockout strain was generated by crossing the floxed VAMP-7 mice to the *Zp3-Cre* mice. Female progeny of this cross ($Zp3^{+/-}$ and homozygous for floxed *VAMP-7* targeted allele) were subsequently crossed to wild-type C57BL/6J male mice. The *Zp3* gene is active during oogenesis, thus mediating VAMP-7 deletion in germ line cells of mice and generating a ubiquitous knockout in the cross to the wild-type male mice (Figure 2.2).

Eosinophil-specific VAMP-7 gene deletion was achieved by crossing the floxed *VAMP-7* mice to *eCre* expressing mice. The progeny of this cross expresses *Cre* recombinase in eosinophil lineage-committed cells only, thereby mediating the deletion of the VAMP-7 gene in eosinophils. The functionality of the *EPX* gene in *eCre* mice was evaluated to ensure the integrity of the knock-in gene. The baseline EPX expression in white blood cells of heterozygous and homozygous *eCre* mice was measured and compared to that of the wild-type mice.

The effectiveness of the e*Cre-Lox P* binary system to delete the VAMP-7 gene was reflected by levels of *Cre*-recombined VAMP-7 null allele in eosinophils, determined by RT-PCR and qPCR analyses. The paucity of eosinophils in healthy animals has been a difficulty associated with *in vitro* research. In order to obtain sufficient eosinophils for evaluation of *eCre-Lox P* binary system in VAMP-7 deletion, two different methods were used to generate eosinophil populations. First, mouse eosinophils were derived from *ex vivo* culture of unselected bone marrow progenitors. Second, eosinophils were sorted from the peripheral blood of the *eCre-V7-IL5* mice. Finally, a high-throughput *Cre* reporting system that enables direct visualization of native green fluorescent protein (11) as the result of Cre expression and interaction with the *LoxP* sites was used to evaluate the effectiveness of eosinophil-specific *Cre*-mediated recombination events in mouse eosinophils.

The focus of this project is the role of eosinophil-specific VAMP-7-mediated events in receptor mediated degranulation and its implication in mouse models of allergic airway inflammation. The ubiquitous VAMP-7 knockout mice were used as negative controls for *eCre-VAMP-7* mice, and were not included as an experimental group in any of the *in vitro* and *in vivo* analysis.

3.2 Results

eCre mice have EPX expression levels comparable to wild-type *C57BL/6J* mice

Endogenous EPX expression was measured to ensure that the expression of EPX had not been altered by genetic manipulations to create *eCre* mice. The endogenous EPX levels in heterozygote and homozygote *eCre* expression mice were confirmed by EPX ELISA (12) using lysed white blood cells. EPX complete knockout ($EPX^{-/-}$) (13) and wild-type C57BL/6J mice were used as negative and positive controls, respectively. Equivalent levels of EPX were detected from heterozygote *eCre* mice and wild-type C57BL/6J mice. However, endogenous EPX levels detected from white blood cell isolated from homozygous *eCre* mice were ~50% less compared to wild-type and heterozygote *eCre* littermates (Figure 3.1). This is likely due to the effects of introducing Cre recombinase into the promoter region of the EPX gene, causing shifts in the open reading frame of the gene.

Therefore, these results indicate that one copy of unaltered EPX gene is necessary for normal levels of protein production. Consequently, male mice that were hemizygous for the floxed VAMP-7 allele and heterozygous for the *eCre* gene were used in all experiments.

Eosinophils derived from *eCre-V7* mice have comparable growth patterns to eosinophils derived from wild-type mice in *ex vivo* cultures

The paucity of cells in wild-type naïve mice has always been a difficulty to overcome when studying eosinophils. An *ex vivo* culture was generated in order to obtain sufficient eosinophils to evaluate their physiology. *Ex vivo* culture of unselected bone marrow progenitors were generated in media supplemented with recombinant murine (rm) SCF, rm Flt-3, and rm IL-5. Usually, > 90% purity of phenotypically mature eosinophils was generated both from ubiquitous and eosinophil-specific VAMP-7 knockout mice on day 12 as determined by Diff Quik staining (Figure 3.2). Eosinophils sampled from day 10 (Figure 3.2 A.) and 12 (Figure 3.2 B.) had appearances characteristic of eosinophil secondary granules and "ring-shaped" nuclei.

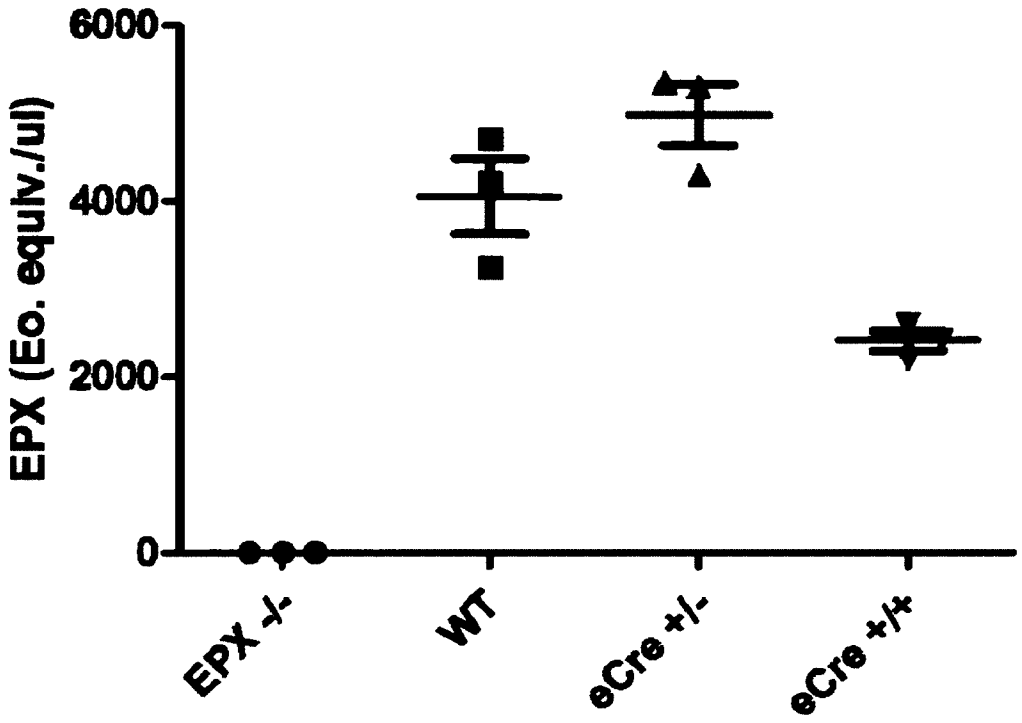


Figure 3.1. Baseline EPX expression levels in *eCre* mice were comparable to that of wild-type mice. To test of the functionality of the knockin EPX gene in *eCre* mice, the level of EPX expression was evaluated in WBC isolated from heterozygous and homozygous *eCre* mice. EPX complete knockout mice (*EPX*$^{-/-}$) and wild-type mice were used as negative and positive controls, respectively. The amount of EPX in WBC was analyzed by ELISA-based detection system (n=3).

After a 12-day culture with sampling on days 0, 4, 6, 8, 10, and 12, all groups evaluated (floxed *VAMP-7* mice, *eCre*, *eCre-V7*, and wild-type *C57BL/6J* mice) had similar growth patterns. As indicated in Figure 3.3, total cell numbers of all groups (~2.0x 10^7 cells at day 0) decreased in the first 4 days (to ~1.0x10^7 cells) of culture with rm SCF and rm Flt-3 as non-granulocytic progenitors in the bone marrow became depleted. As expected, with the removal of rm Flt-3 and rm SCF, and the addition of rm IL-5 on day 4, total cell numbers of all groups recovered to ~ 2.0x 10^7 cells. A linear expansion of cells was observed over days 8-10 that reached a plateau from days 10 to 12. Cells with terminally differentiated eosinophil characteristics also increased in number as observed in Diff Quik stained cytospin samples.

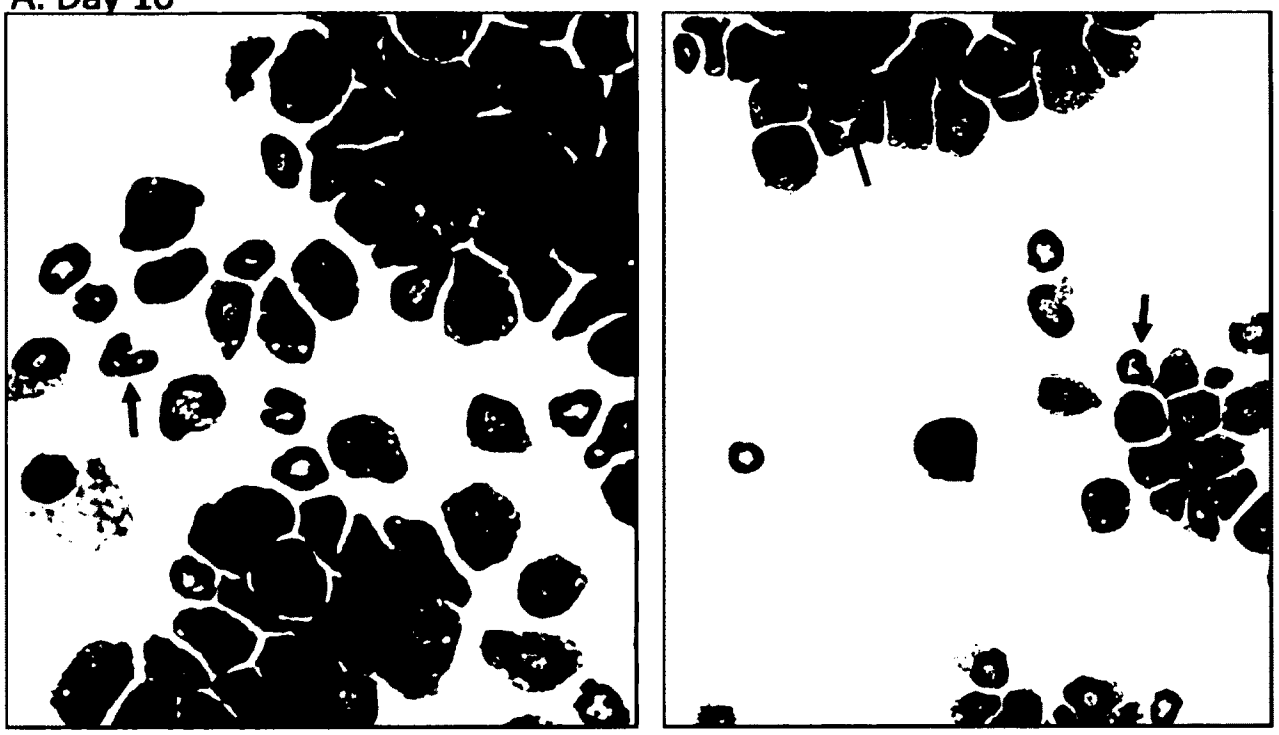
Eosinophils in late anaphase were observed in the *ex vivo* culture on day 10 (as represented by the red arrow in Figure 3.2). As indicated in the cytospin samples, eosinophils harvested on day 10 and 12 lacked the blue hue as the result of cytoplasmic basophilia, which is a part of normal eosinophil development. The cytoplasm of cells was occupied by distinctive granulation that appeared magenta/orange/pink when stained with eosin. No difference in growth patterns and morphology was observed between eosinophils derived from the bone marrow of *eCre-V7*, floxed VAMP-7, *eCre* mice, and their wild-type littermates.

Figure 3.2. Bone marrow-derived eosinophils on day 10 and 12 indicate eosinophilic characteristics. Diff Quik-stained cytospin samples of bone marrow derived-eosinophils from *ex vivo* cultures collected on day 10 (A) and 12 (B). Eosinophil-specific characteristics, such as the ring-shaped nucleus and the magenta/pink/orange-granulated cytoplasm, were observable in eosinophils obtained from days 10 and 12. Most cells lost their cytoplasmic basophilia as expected. Eosinophils in late anaphase were observed in day 10 cultures, as indicated by the red arrow. Approximately 2-3% of neutrophils were also observed in the culture on day 10 as indicated by black arrows.

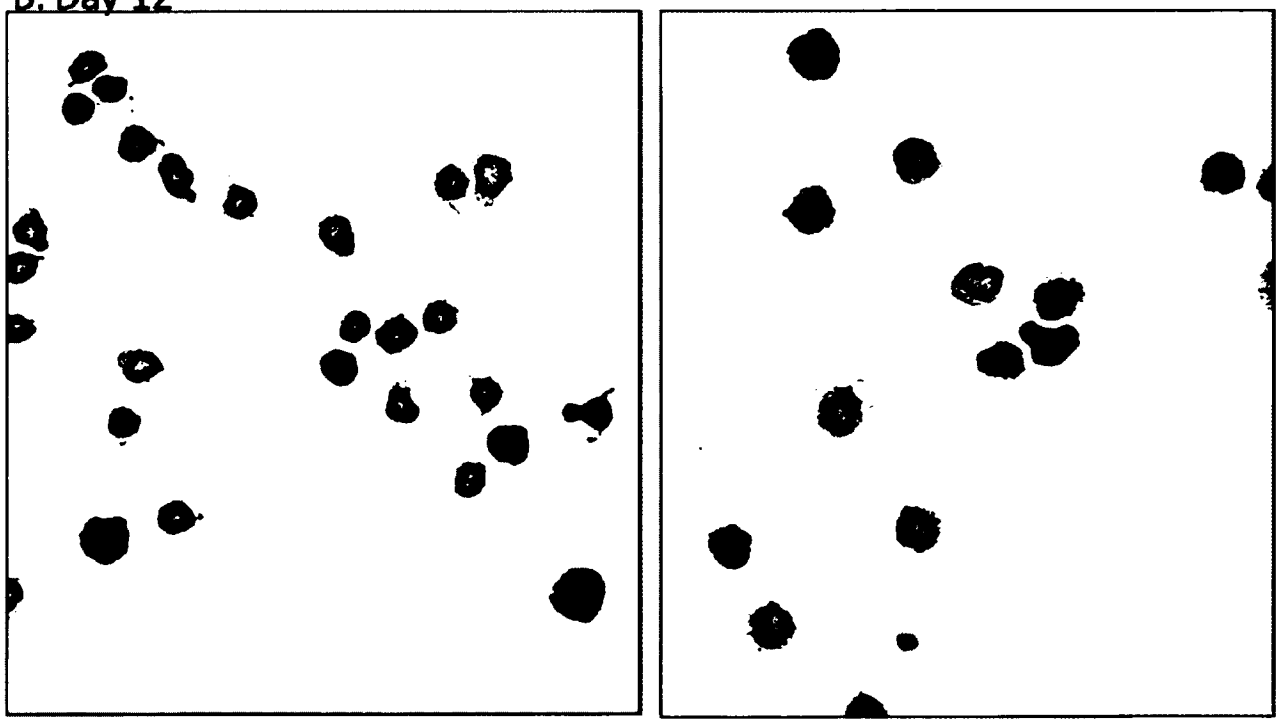
Wild-type BM eosinophils

eCre-V7 BM eosinophils

A. Day 10



B. Day 12



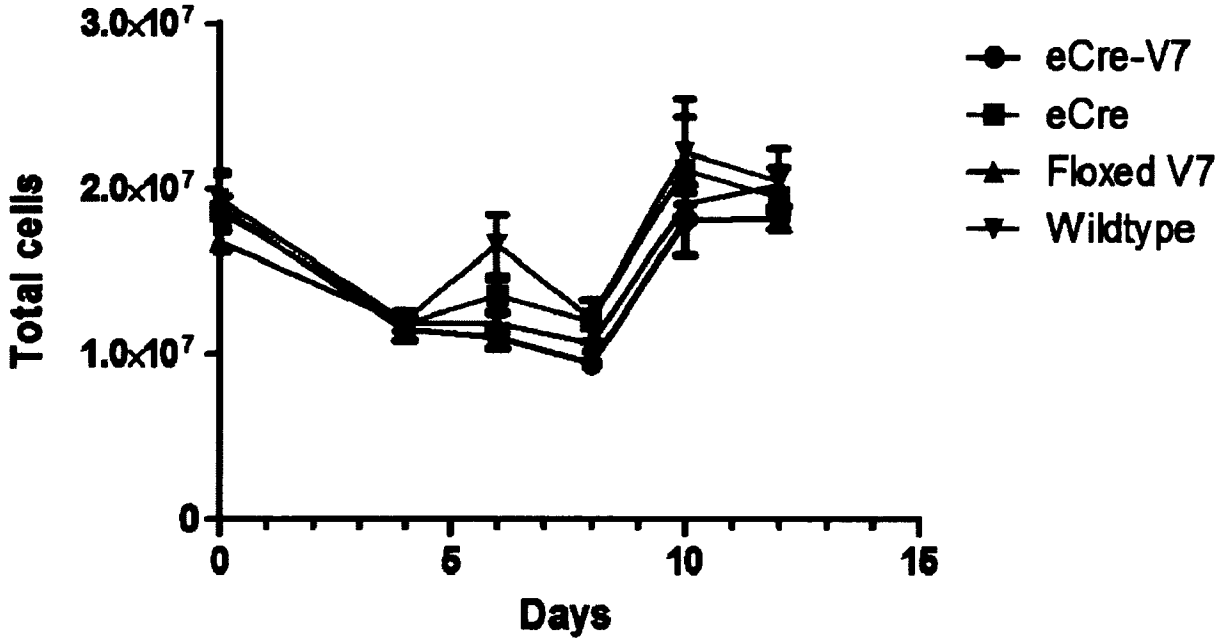


Figure 3.3. Eosinophils derived from *eCre-V7* mice bone marrow have comparable growth patterns to those of wild-type mice bone marrow. Unselected bone marrow progenitors were isolated from *eCre-V7*, *eCre*, floxed V7 and wild-type mice and stimulated with rm SCF (100 ng/ml) and rm Flt-3 (100 ng/ml) for 4 days and rmIL-5 thereafter for up to 8 days. Culture samples were collected on days 0, 4, 6, 8, 10 and 12, and total numbers of cells in the culture were calculated from the haemocytometer based count in 15 ml of the culture (n=3).

VAMP-7 gene deletion in bone marrow derived eosinophils was confirmed by PCR

To evaluate if VAMP-7 gene deletion was successful in *eCre-V7* mice, traditional PCR was used with a two-primer (a-b1 and a-b2, Figure 2.2) strategy. Primers were designed based on sequences in the floxed VAMP-7 targeted allele to distinguish mice carrying the targeted allele from mice carrying the *Cre*-recombined VAMP-7 null allele (14). In wild-type and floxed VAMP-7 mice, the size of the amplicon between primers Pa and Pb2 was ~21,000-bp. When exons 3 and 4 of the VAMP-7 gene were deleted from the genome, the size of the amplicon between primers Pa and Pb2 was reduced to 326-bp.

Results from traditional PCR revealed that the *Cre*-recombined allele, indicated by the 326-bp amplicon, was present only in eosinophils derived from unselected bone marrow progenitors isolated from mice that were genotyped as *eCre-V7* mice. Wild-type, *eCre*, and floxed VAMP-7 mice had no amplification for the *Cre*-recombined null allele using the primer set Pa and Pb2. As indicated in Figure 3.4, 40 ng of DNA extracted from eosinophils isolated from the *ex vivo* bone marrow culture on days 0, 4, 6, 8, 10, and 12 were used as templates for PCR reactions to identify the *Cre*-recombined VAMP-7 null allele. The *Cre*-recombined allele was detected in the *eCre-V7* mice bone marrow culture in all sampling days (lane 2 in Figure 3.4). As expected, the *Cre*-recombined VAMP-7 null allele was absent from eosinophils derived from the floxed VAMP-7, *eCre*, and wild-type mice as indicated in lanes 1, 3, and 4.

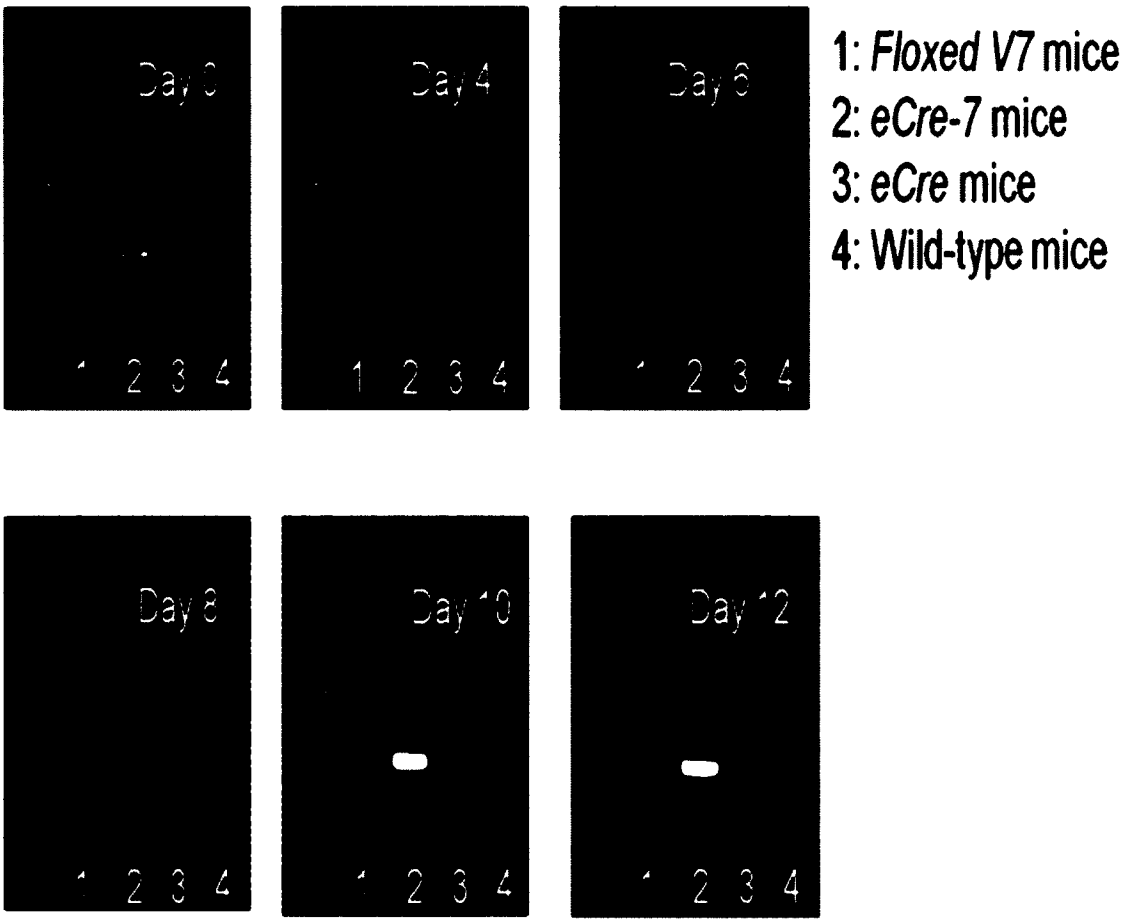


Figure 3.4. VAMP-7 gene deletion in bone marrow derived eosinophils as confirmed by traditional PCR. The *Cre*-recombined allele was amplified and used as the indication of successful VAMP-7 deletion. DNA (40 ng) was used as the template for a two-primer PCR. The presence of the *Cre*-recombined allele was restricted to eosinophils derived from bone marrow progenitors isolated from *eCre-V7* mice (lane 2). This allele was absent in eosinophils from the parental strains (floxed *VAMP-7* and *eCre* mice) of the *Cre-Lox P* binary system and in wild-type controls (lanes 1, 3, and 4), suggesting normal expression of VAMP-7 (n=3).

Efficiency of eosinophil-specific VAMP-7 gene targeting assessed by quantitative PCR (qPCR) in bone marrow-derived eosinophils

The level of *Cre*-recombined allele was assessed using qPCR with a four-primer strategy. The amount of product amplified by qPCR was reflected by the standardized ΔΔCt value generated by the fluorescent intensity of double-stranded DNA as the result of PCR amplification. Pa was designed based on the sequence between exons 2 and 3. Pb was designed based on nucleotide sequences in the targeted region (exon 3) (Figure 2.5). The product amplified by qPCR using Pa and Pb indicates the deletion of exons 3 and 4 of the VAMP-7 gene by *Cre*-mediated recombination. The ΔΔCt value of the amplicon generated by primers Pc and Pd, designed based on the sequence in the un-excised region (exon 1), was used as the constant. The (Pa-Pb) ΔΔCt value was divided by the constant (Pc-Pd) ΔΔCt value, thus providing a ΔΔCt value ratio for each experimental group. The decrease in reporter fluorescent signal is directly proportionate to the number of amplicons generated. A lower ΔΔCt ratio indicates decreased copies of the targeted region as the value of Pa-Pb approached 0 when exons 3 and 4 were deleted.

Eosinophils derived from unselected bone marrow progenitors isolated from *eCre* and floxed VAMP-7 mice have comparable ΔΔCt ratio of excised/un-excised VAMP-7 DNA, indicating an intact VAMP-7 gene (Figure 3.5). The qPCR data was normalized to floxed VAMP-7 mice. DNA isolated from the WBC of ubiquitous VAMP-7 knockout mice (*Zp3Cre-V7*) was used as the negative control with 100% of cells containing the *Cre*-recombined VAMP-7 null allele and a ΔΔCt value of 0. As indicated in Figure 3.5, eosinophils derived from unselected bone marrow progenitors isolated from *eCre-V7* mice had ~ 77% of cells containing the *Cre*-recombined VAMP-7 null allele, indicating a 77% efficiency of the *eCre* binary system. Diff Quik-

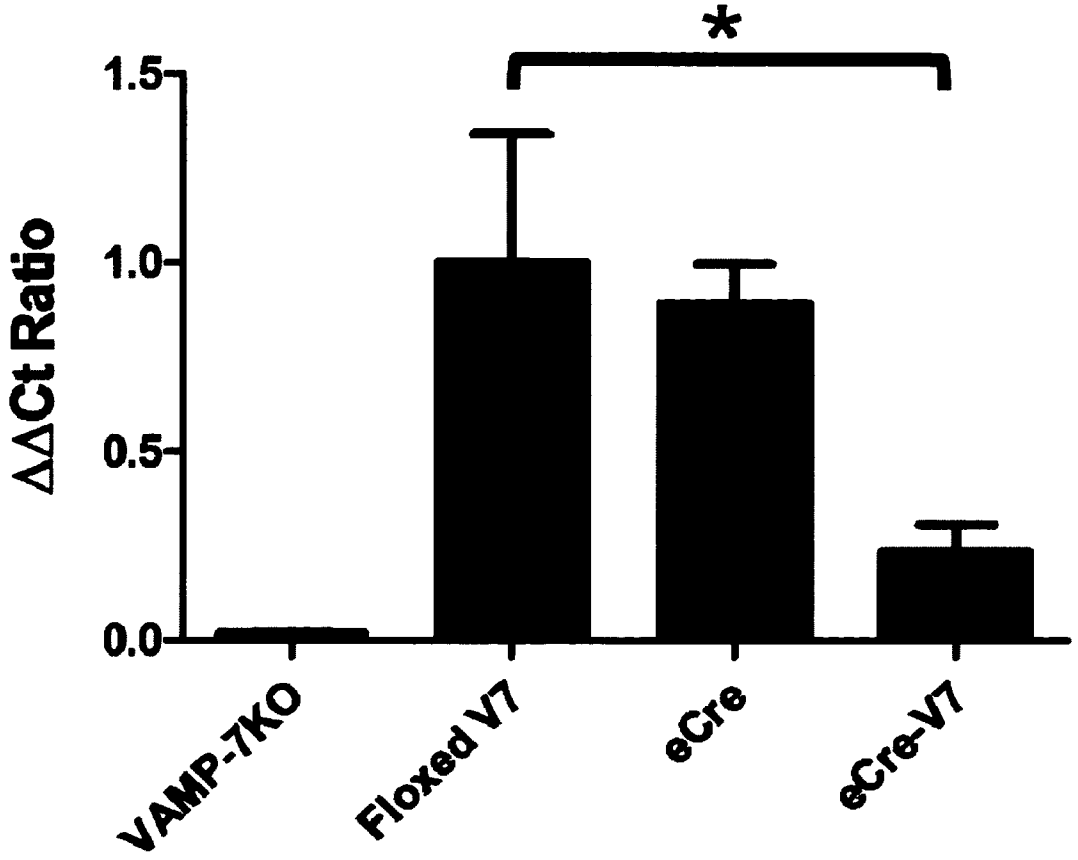


Figure 3.5. VAMP-7 gene deletion in bone marrow derived eosinophils quantified by qPCR. A total of 25 ng of DNA was used as the template for the four-primer qPCR strategy. All qPCR data were reflected by the standardized ΔΔCt value. A low ΔΔCt value indicates the deletion of the VAMP-7 gene (*n*=3).

stained cytospin samples indicated the majority (> 90%) of the cells were eosinophils and a small percentage (~2-3%) were neutrophils.

Efficiency of eosinophil-specific VAMP-7 deletion as assessed by qPCR in eosinophils sorted from peripheral blood

Eosinophils isolated from the peripheral blood of mice were defined and sorted by flow cytometry using a combination of cell surface markers (CCR-3$^+$, IL-5R$^+$, Gr-1$^{mid\text{-}hi}$, CD4$^-$, B220$^-$). DNA isolated from eosinophils and lymphocytes (CD4$^+$, B220$^+$, CCR-3$^-$, IL-5R$^-$, Gr-1$^-$) sorted from peripheral blood of *eCre-V7-IL5* mice was used as a template for qPCR analysis to calculate the percent of eosinophils with the *Cre*-recombined null VAMP-7 allele. Ubiquitous VAMP-7 knockout mice were used as the negative control with a $\Delta\Delta$Ct ratio value of 0. All data were normalized to the $\Delta\Delta$Ct value of the lymphocytes population. Eosinophils sorted from peripheral blood of *eCre-V7-IL5* mice had ~ 86% *Cre*-recombined VAMP-7 null allele, indicating an 86% efficiency of expression of the *eCre* binary system (Figure 3.6).

These results were further confirmed by traditional PCR for the detection of *Cre-recombined* VAMP-7 *null allele as shown in Figure* 3.7. The traditional PCR results were consistent with the qPCR data shown above, where the *Cre*-recombined allele amplicon was present only in sorted eosinophil populations and absent in lymphocyte populations as well as WT eosinophils. This confirms that VAMP-7 deletion was restricted to eosinophils. The purity of eosinophil populations and the maturation state of blood-derived eosinophils are more homogenous than *ex vivo* derived eosinophils from unselected bone marrow progenitors. Therefore, the percentage of eosinophils deficient in the VAMP-7 gene is higher in sorted blood eosinophils.

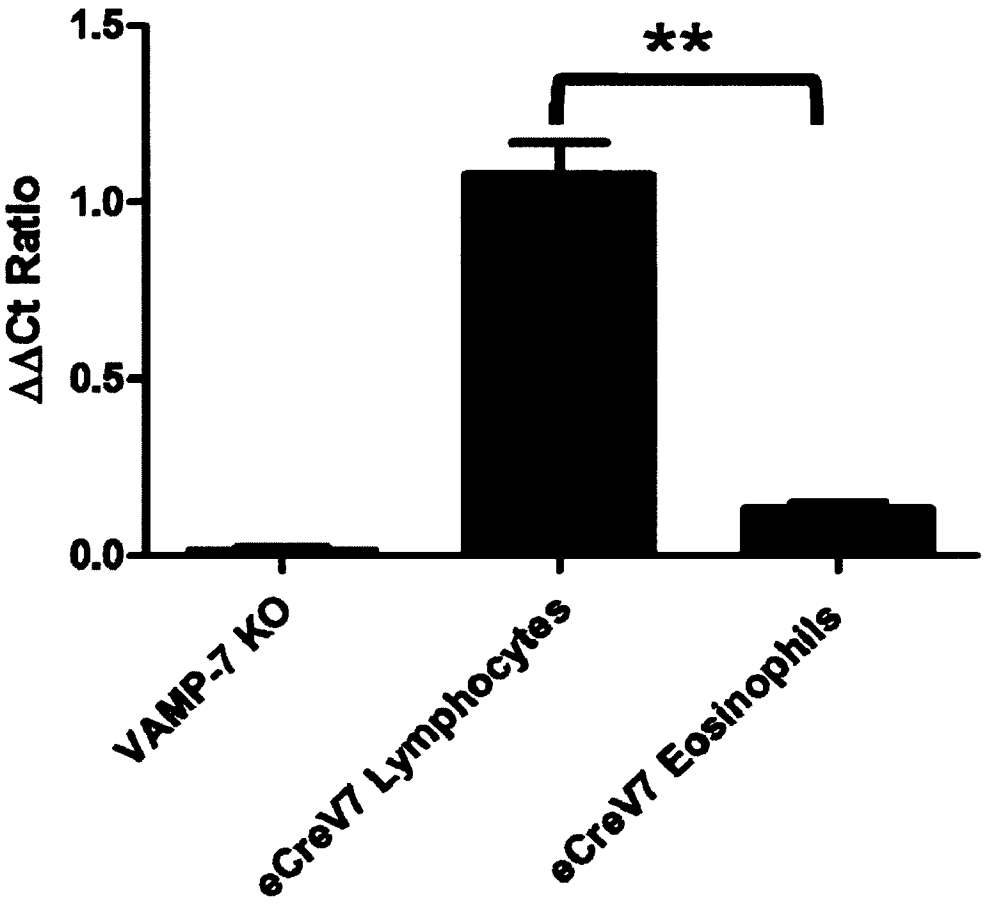


Figure 3.6. Efficiency of eosinophil-specific VAMP-7 excision in samples of whole blood. Eosinophils ($CCR3^+$, $IL\text{-}5R^+$, $Gr\text{-}1^+$, $CD4^-$, and $B220^-$) and lymphocytes ($CCR3^-$, $IL\text{-}5R^-$, $Gr\text{-}1^-$, $CD4^+$, and $B220^+$) were separated from peripheral blood. A total of 25 ng of DNA extracted from eosinophils and lymphocytes were used in the four-primer qPCR quantification strategy to detect *Cre*-recombined allele expression. All qPCR data was normalized to the lymphocyte data (n=3).

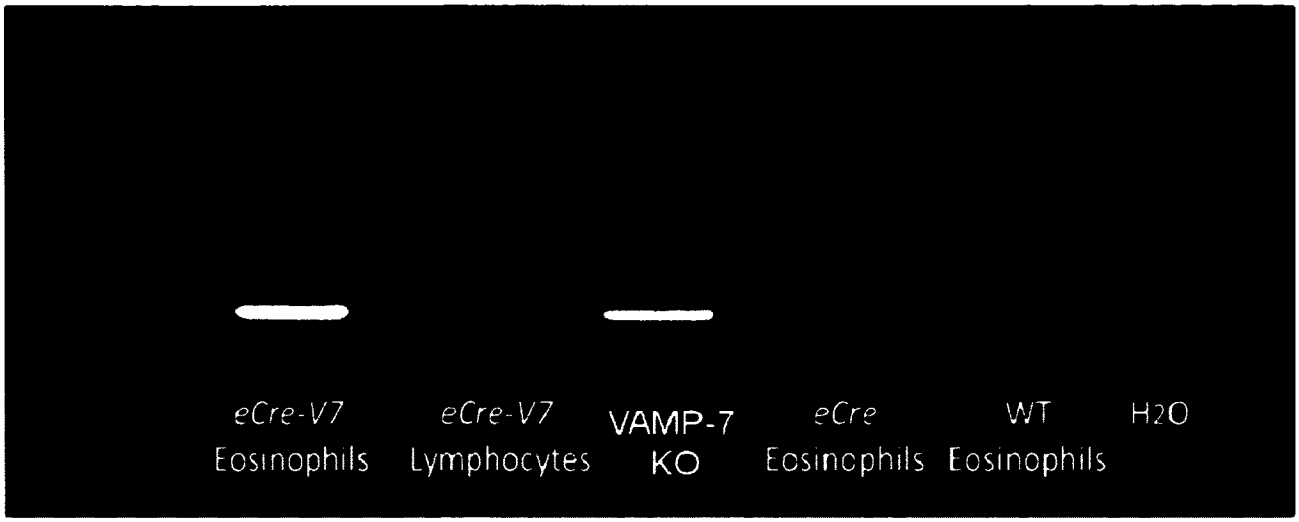


Figure 3.7. VAMP-7-specific targeting was restricted to eosinophil populations. DNA was extracted from eosinophils ($CCR3^+$, $IL\text{-}5R^+$, $Gr\text{-}1^+$, $CD4^-$, and $B220^-$) and lymphocytes ($CCR3^-$, $IL\text{-}5R^-$, $Gr\text{-}1^-$, $CD4^+$, and $B220^+$) sorted from *eCre-V7* mice. A total of 40 ng of DNA was used in a two-primer PCR. The *Cre*-recombined VAMP-7 null allele (326-bp) was present in *eCre-V7* eosinophils and VAMP-7 ubiquitous knockout WBC, and absent in lymphocyte populations as well as WT eosinophils.

Eosinophil-specific *Cre*-mediated gene arrangement was confirmed using an enhanced green fluorescent protein (*e-GFP*) expressing *Cre*-reporter system

The quantification of *eCre-LoxP* mediated VAMP-7 deletion using population-based detection methods, such as PCR, has been limited by the purity of eosinophils used to provide DNA templates. Cre "mosaicism" can also lead to inefficient recombination events. Cre "mosaicism" can result in two ways: first, the Cre recombinase might not be expressed in every eosinophil. Second, the Cre recombinase might be expressed, but fail to recombine the floxed VAMP-7 targeted allele in every eosinophil. In order to characterize e*Cre*-mediated events, a strain of *Cre* reporter mice (*e-GFP* mice) was used as a surrogate to floxed VAMP-7 mice. Failure to observe robust reporter activity throughout the entire eosinophil population is a good indication that the Cre line will also fail to recombine the floxed VAMP-7 allele.

The resulting *e-GFP-eCre* system enabled direct visualization of *Cre*-mediated recombination events using single-cell based detection methodology, such as flow cytometry. The progeny of the *e-GFP-eCre* cross produced GFP in cells that express the *Cre* recombinase as a result of *eCre-Lox P*-mediated recombination. Using flow cytometry, 95% of eosinophils (SSC^{hi}, CCR-3^{+}, IL-$5R^{+}$, Gr-1^{mid-hi}) were GFP-positive (Figure 3.8 A.). When all GFP-positive cells were analyzed, ≥ 95% of the cells were positive for CCR-3^{+}, IL-$5R^{+}$ and Gr-1^{mid-hi} (Figure 3.8 B). The Cre-reporter demonstrated a robust and highly efficient Cre-mediated activity when crossed to *eCre*-expressing mice.

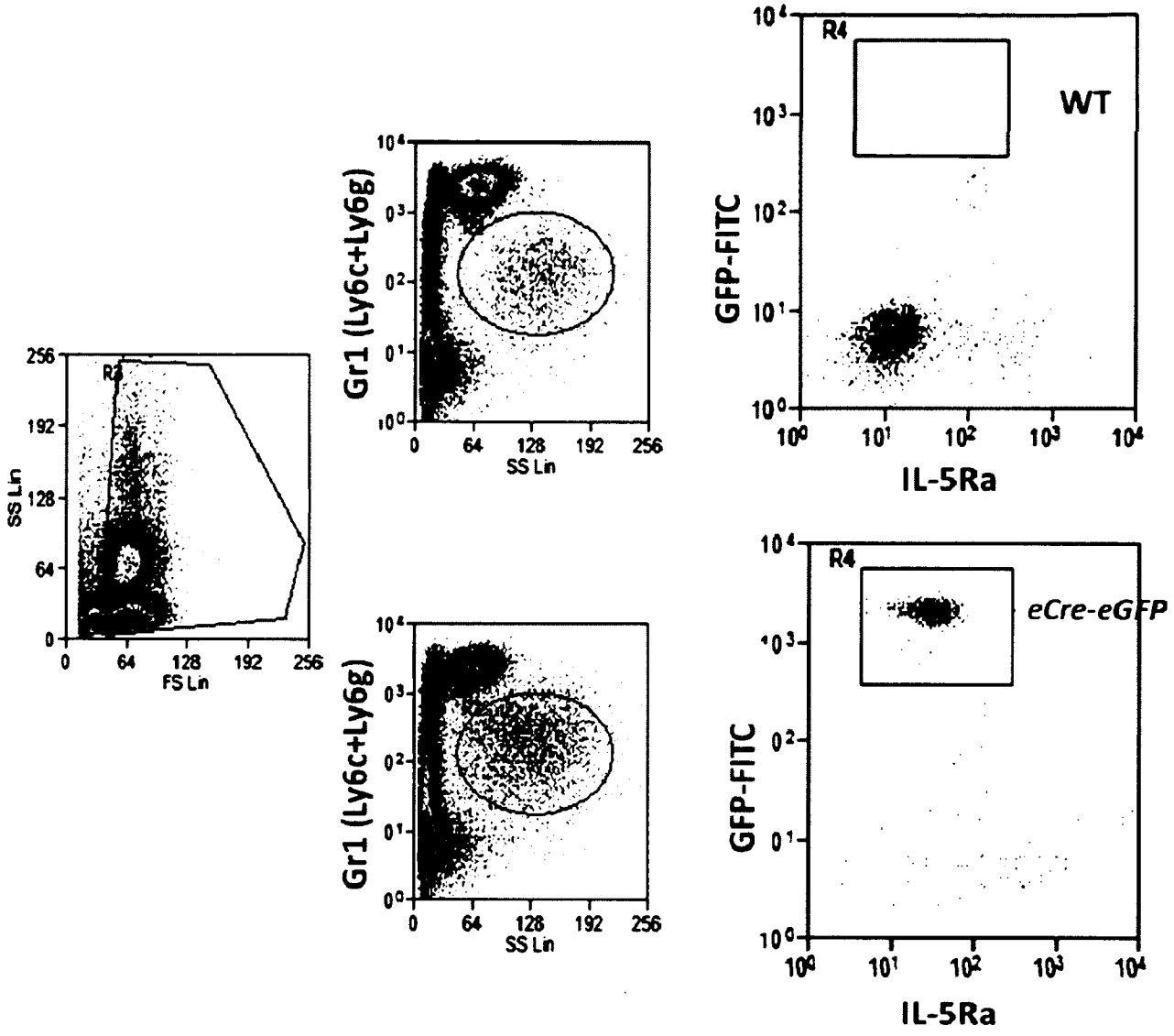


Figure 3.8A. Eosinophil-specific *Cre*-mediated gene arrangement confirmed using enhanced green fluorescent protein (*e-GFP*)-expressing *Cre*-reporter system. Isolated WBC were stained with 1 µg/ml of rat anti-mouse IL-5 receptor α/CD125 (PE channel); rat anti-mouse Ly-6G/Gr-1, clone RB6-8C5 (PE-Cy7 channel); mouse CCR3 fluorescein mAb, clone 83101 (APC channel). Channel FL1 (GFP-FITC) was left open to detect GFP+ cells. Flow cytometry identification of eosinophils (SSC^{hi}, $CCR\text{-}3^{+}$, $IL\text{-}5R^{+}$, $Gr\text{-}1^{mid\text{-}hi}$) from WBC isolated from *eGFP-eCre* and wild-type mice indicated >95% of eosinophils were GFP positive. Generated by Mr. Alfred Doyle and Dr. Jacobsen.

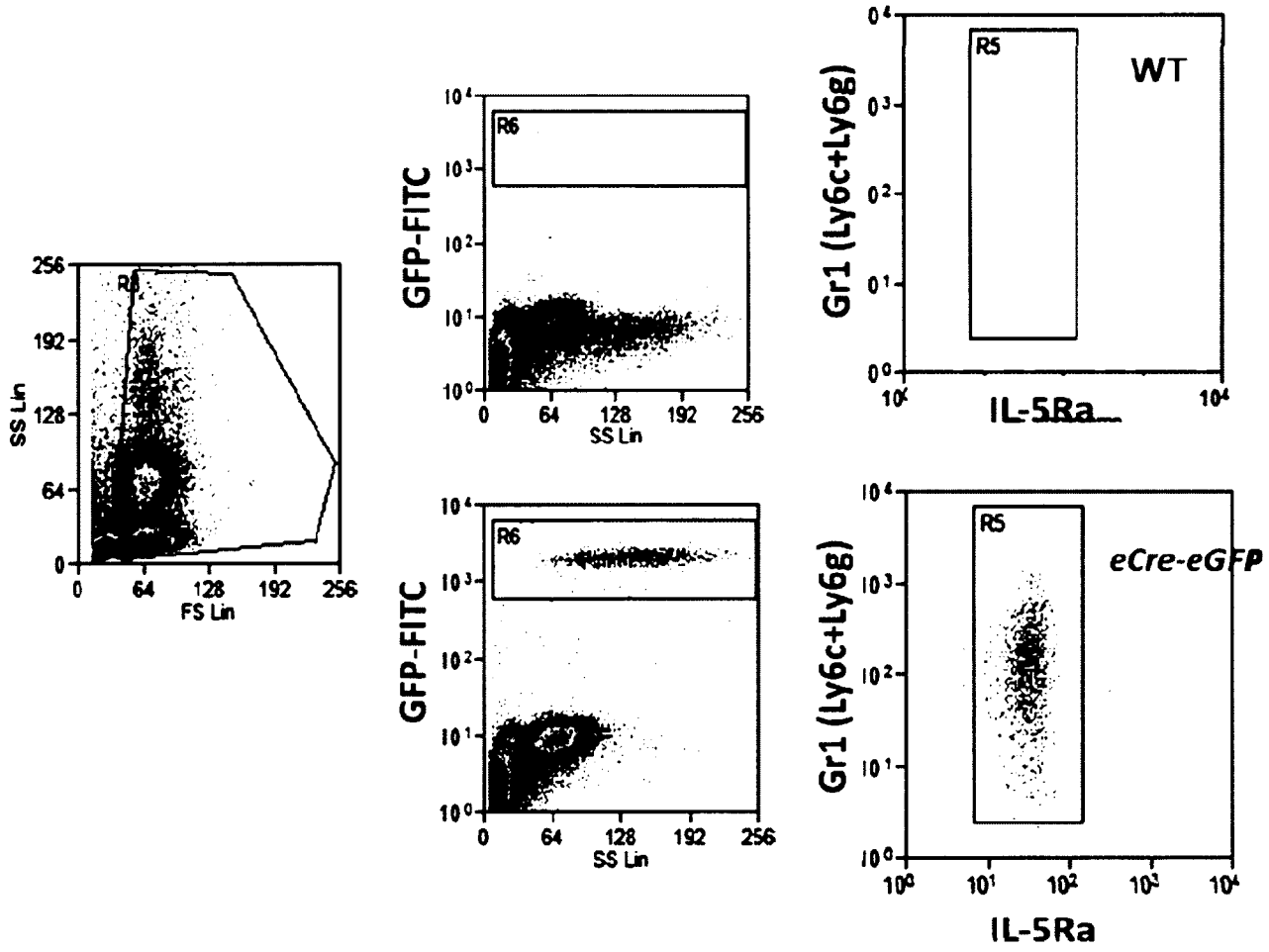


Figure 3.8B. Flow cytometry identification of eosinophils (SSC^{hi}, $CCR\text{-}3^{+}$, $IL\text{-}5R^{+}$, $Gr\text{-}1^{mid\text{-}hi}$) from WBC isolated from *eGFP-eCre* and wild-type mice indicated >95% GFP positive cells were high in side scatter pattern and positive for IL-5R and Gr1 (Ly6c+Ly6g). Generated by Mr. Alfred Doyle and Dr. Jacobsen.

3.3 Summary and Discussion

In this study, the genetic ablation of VAMP-7 was achieved using two different transgenic mouse models based on the *Cre-Lox P* binary system. The cross between *Zp3-Cre* and the floxed *VAMP-7* mice generated ubiquitous VAMP-7 knockout mice. In order to generate mouse eosinophils deficient in VAMP-7 gene expression, *eCre* mice were crossed to floxed *VAMP-7* mice to generate *eCre-V7* mice. In *eCre* mice, the expression of the Cre recombinase is driven by the eosinophil-specific EPX promoter. Homozygous *eCre* mice had less than half of the wild-type EPX protein level in their circulation. Heterozygous *eCre* mice had comparable EPX protein levels to wild-type mice, which suggest that at least one intact copy of the EPX gene is necessary for normal gene function.

Ubiquitous gene ablation occurs in all cells of a mouse, including zygote stage embryo, which could lead to complex phenotypes, including but not limited to embryonic lethality (10). A ubiquitous knockout mouse model is useful, but does not offer information regarding tissue specificity. In contrast, e*Cre-Lox P* binary system offers a means to generate eosinophil gene targeting with specific phenotypes. In this study, both *eCre* and floxed *VAMP-7* mice were knock-in animals generated using gene targeting in ES cells.

The generation of each of the parental mouse strains in the binary system took advantage of the plasticity and accessibility of ES cells (14, 15). ES cells have only one X chromosome, which reduces the complexity of deleting X-linked genes, such as VAMP-7. Homologous recombination-based genetic manipulations were carried out in isolated ES cells and re-introduced back into early embryos. Genetically altered ES cells contribute to the development of mice and allow artificial alteration to be inherited by successive generations of mice (10). This mouse model-based gene-specific targeting allowed the generation of conditional knockout mice carrying null alleles with Mendelian-inheritable traits. It was demonstrated in this study that *Cre-Lox P*-mediated gene targeting was successful in deleting

the VAMP-7 gene from an entire mouse (*Zp3Cre-V7* mice) or from the eosinophil population (*eCre-V7* mice) only.

Tissue specificity in the *Cre-Lox P* system is provided by the promoter chosen to regulate expression of Cre recombinase. Among all eosinophil proteins, EPX appears to be eosinophil-specific and is used in various applications to identify the presence and activation of eosinophils (12, 16, 17). Activation and function of the *EPX* gene are critical in the commitment of unselected bone marrow progenitors to the eosinophil lineage. The expression of the *EPX* gene is transient, and *EPX* reaches its highest level of expression during early eosinophilopoiesis (18). *eCre* mice were designed to express the *Cre* recombinase with the necessary molecular machinery associated with the endogenous *EPX* gene, which offers the tissue specificity advantage over the *Zp3Cre-V7* ubiquitous knockout.

The isolation/purification of eosinophils is important in characterization of both the parental mice expressing *Cre* recombinase and the progeny carrying *Cre-*recombined alleles. Two different approaches were used to generate a sufficient amount of eosinophils for VAMP-7 deletion analysis in this project. Generally, eosinophils are generated in the bone marrow, although extramedullary sites of eosinophil generation have been observed (19). Pluripotent $CD34^+$ hematopoietic stem cells are induced by cytokines to commit to the eosinophil lineage (20, 21) . The *ex vivo* culture used in this study with SCF, FLT ligand, and IL-5 stimulation yielded high percentages of phenotypically mature eosinophils by gross histological examination, although some neutrophils were found in culture samples as well. Unselected bone marrow cells stimulated with a set of cytokines that can induce development of eosinophils or mixed neutrophil-eosinophil colonies could contribute to the impurity of *ex vivo*-derived cell populations.

The presence of the *Cre*-recombined VAMP-7 null allele on day 0 of bone marrow harvesting indicated deletion of the VAMP-7 gene in eosinophilic metamyelocytes in the bone

marrow of *eCre-V7* mice. It was demonstrated for the first time using traditional PCR that eosinophil-specific VAMP-7 targeting was successful using the *eCre-Lox P* binary system in bone marrow-derived eosinophils. Nevertheless, traditional PCR is associated with low resolution, short dynamic range, and low sensitivity. Moreover, ethidium bromide-based staining of gels for PCR evaluation is not quantitative. In order to quantify the percentage of *ex vivo*-derived cells containing eosinophil-specific VAMP-7 deletion, quantitative PCR which measures PCR amplification as it occurs was subsequently conducted. When eosinophils were induced to mature in an *ex vivo* developmental environment using unselected bone marrow progenitors, >77% of cultured cells were deficient in VAMP-7 as determined by qPCR.

The advent of flow cytometry has provided a means to identify and characterize various cell types including eosinophils by their physical nature (i.e. cytoplasmic granularity) and cell surface markers. In accordance with standards set in the current literature, mouse peripheral blood eosinophils were defined as being side-scatter^{hi}, IL-5R^{+}, CCR3^{+}, Siglec-F^{+}, F4/80^{+}, CD11b^{+}, and Gr1$^{low\text{-}med}$, and lacking lineage markers such as CD4, CD8, and B220 (22). It is important to note that the cell surface markers selected are not eosinophil-specific on their own. Siglec-F is found on the surface of alveolar macrophages in mice (23). Gr1 has a high expression level on the surface of neutrophils compared to eosinophils and has been used for splenic myeloid cell identification (24). Nevertheless, a specific combination of cell surface markers can provide an optimized selection of eosinophils by cell sorting using flow cytometry.

In this project, eosinophils were defined as being CCR3^{+}, Siglec-F^{+} and Gr1$^{low\text{-}med}$, CD4^{-}, and B220^{-} based on availability of appropriate quality antibodies. The levels of *Cre*-recombined VAMP-7 null allele quantified by qPCR indicated that ~86% of peripheral blood eosinophils isolated from *eCre-V7* mice were VAMP-7-deficient. qPCR analysis using DNA isolated from separated eosinophil and lymphocyte populations demonstrated that the specificity of *eCre*-mediated recombination events to delete the VAMP-7 gene was restricted to eosinophils.

Using binary *Cre-Lox P* systems, there is a risk of observing Cre mosaicism in offspring. Cre mosaicism occurs when a tissue-specific Cre transgene fails to completely delete floxed alleles. This may occur in two different ways: first, Cre may not be expressed in every eosinophil. Second, expressed Cre recombinase may fail to recombine floxed alleles in every eosinophil. To address the Cre mosaicism in *eCre-V7* model, a Cre reporter line was used in this project (gratitude is extended to Mr. Alfred Doyle and Dr. Elisabeth Jacobsen for generating the mice and using flow cytometry for analysis). As a knock-in Cre, *eCre* can be used to track individual cells with eosinophil lineages (i.e., EPX-positive) by turning transgene expression on or off in the reporter system. A poor reporter activity throughout eosinophil populations is an indication that the Cre line will also have a poor interaction with floxed VAMP-7 alleles.

In the *eCre-eGFP* cross, the expression of GFP indicated successful *Cre*-mediated recombination events at the *Rosa26* locus in all cells committed to eosinophil lineage. Among all the GFP positive cells, >95% were positive for IL-5R^{+}, CCR3^{+}, and Gr1$^{low\text{-}med}$. Concluding from the data obtained using the *eCre-eGFP* system, the *eCre-Lox P* binary system provides eosinophil-specific gene targeting with extremely high (~95%) efficiency. In the past decade, the use of a Cre reporter line has been considered the gold standard evaluation method for Cre-mediated recombination efficiency. However, the *eCre-eGFP* is a surrogate evaluation system to analyze the efficiency of VAMP-7 gene deletion. It would be ideal to use flow cytometry to analyze eosinophils isolated from the peripheral blood of *eCre-V7* mice for their intracellular VAMP-7 expression using a VAMP-7 specific antibody. However, a suitable candidate antibody with high VAMP-7 specificity has not yet been found for single cell based analysis by flow cytometry.

In summary, eosinophil-specific VAMP-7 deletion was successful in *eCre-V7* mice with high specificity and efficiency. Progeny of all crosses were viable and fertile. Differences in eosinophil purity and the sensitivity of the various detection methods may have contributed to differences in detection of VAMP-7 deletion efficiency obtained using these three approaches. Levels of *Cre*-recombined VAMP-7 null allele increased as the purity of eosinophils increased from *ex vivo* cultures to flow cytometry-sorted blood eosinophils. Using a combination of the high-throughput *Cre* reporter system and flow cytometry, *Cre*-mediated recombination events were analyzed at a single cell level, which offers a much more robust and sensitive analysis compared to PCR-based population detection. *eCre-V7* eosinophils were subsequently isolated and stimulated *in vitro* to evaluate the involvement of VAMP-7-mediated events in degranulation. Because of the high efficiency of VAMP-7 removal in blood eosinophils (86%-95%), all *in vitro* experiments thereafter used peripheral blood eosinophils isolated from *eCre-V7-IL5* male mice.

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Chapter Four: Defining the Role of VAMP-7 in Mouse Eosinophil Degranulation

4.1 Introduction

Eosinophil granule proteins have been shown to have immunoregulatory capabilities as they are associated with mast cell degranulation (1), platelet activation (2), and increased reactive oxygen species (ROS) production in the defence against helminth infection (3). Recent evidence has also linked eosinophil granule proteins (i.e., EPX) to post-translational tyrosine modification via nitration (4-6). The inflammatory effector function of eosinophils is therefore modulated, in part, by degranulation (7). Under light or electron microscopy, populations (300-400/cell) of membrane-bound secretory granules occupy the cytoplasm of eosinophils (8). The most distinct granules in mouse and human eosinophils are the crystalloid granules containing preformed cationic proteins, such as MBP, EPX, ECP (human), EDN (human), and Ears (mouse), and cytokines, such as IL-2, -3, -4, -5, -6, -10, -12, -13, and IFNγ (9). In response to various stimulants (e.g., immunoglobulin receptor cross-linking, lipid mediators, chemokines, and IFNγ), eosinophils are activated and recruited to inflammatory foci where they may modulate the micro-milieu via releasing granule-derived proteins and cytokines.

Human eosinophils, when activated, lose some of the electron density in their granules under transmission electron microscopy. Eosinophils isolated from BAL samples of *IL-5/hE2* mice are also associated with hollowed granules and emptied vesicles within intact cells. Degranulation is achieved through the event of exocytosis, which involves fusion between granule membranes and the plasma membrane. The fusion event is a SNARE-dependent process, which occurs in many cell types including eosinophils (10).

It has been found that stimulation-mediated exocytosis of crystalloid granules from human eosinophils requires binding of v-SNARE proteins, such as VAMP-7, to t-SNAREs anchored in the plasma membrane (10, 11). VAMP-7 has been detected in the same fraction as eosinophil crystalloid granules and small secretory vesicles where EPX is stored and transported (12).

VAMP-7 neutralizing antibody treatment induced a dose-dependent decrease in the release of EPX and EDN from permeabilized human eosinophils stimulated *in vitro* (10). However, no studies were found that have shown a role for VAMP-7-mediated events in physiologically relevant activation of eosinophils. To gain insight into VAMP-7-mediated events in eosinophil degranulation, the combination of physiologically relevant *in vitro* and *ex vivo* secretagogues were used to induce activation and consequent degranulation in intact mouse eosinophils deficient in VAMP-7.

VAMP-7-deficient eosinophils were isolated from *eCre-V7-IL-5* mice, and stimulated *in vitro* with established secretagogues, calcium ionomycin and platelet activation factor (PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) to induce degranulation. Recent evidence has shown both human and mouse eosinophils respond to PAF *in vitro* (13). PAF is a proinflammatory lipid mediator produced by basophils, monocytes/macrophages, neutrophils, and eosinophils [1]. Human eosinophils respond to PAF stimulation by releasing cationic granule proteins (14). PAF promotes dose-dependent degranulation responses from cultured eosinophils derived from wild-type mouse progenitors and peripheral blood eosinophils isolated from IL-5 transgenic mice via PAF receptor-independent mechanism(s) (13).

Ionomycin is a potent calcium ionophore, which acts as a motile Ca^{2+} carrier and enhances Ca^{2+} influx by direct stimulation of the entry of stored cations across biological membranes (15, 16). Stimulation of eosinophils with calcium ionomycin by-passes receptor activation pathways, which involve ligand and receptor interaction, and directly activates Ca^{2+}/calmodulin-dependent kinases and phosphatases to stimulate degranulation (17, 18). Ionomycin was used in VAMP-7-deficient eosinophils as a positive control to elicit eosinophil degranulation, and to compare responses with those of the physiological agonist PAF.

Among all the granule proteins stored in eosinophil crystalloid granules, EPX appears eosinophil-specific, and it is secreted as the result of eosinophil activation. Therefore, EPX released was first evaluated in VAMP-7-deficient eosinophils. The release of two other types of cationic proteins (MBP and Ears) from eosinophils stimulated *in vitro* was also evaluated.

In human eosinophils, immunoregulatory cytokines such as IL-4 have been identified to reside in granules containing cationic proteins (12, 19). Immunogold-stained IL-4 has been observed to congregate within granules as well as budding and travelling vesicles after stimulation. The release of immunoregulatory cytokines such as IL-4 and IL-13 from VAMP-7-deficient eosinophils was evaluated to better understand the relationship between the release of signal peptide dependent secretory proteins and VAMP-7-mediated exocytotic events.

In this study, the artificial nature of *in vitro* receptor-mediated stimulation with PAF and ionomycin was considered, and a more physiologically relevant and eosinophil-centric model was used to evaluate the release of EPX in VAMP-7-deficient eosinophils. Eosinophils isolated from *eCre-V7-IL-5* peripheral blood were stimulated in an *ex vivo* airway activation model using *IL-5/hE2/EPX*$^{-/-}$ mice as recipients. Constitutive expression of the eosinophilopoietin, IL-5, and chemokine, eotaxin, in the *IL-5/hE2/EPX*$^{-/-}$ mice provided an inductive environment for intratracheally installed eosinophils to become activated and undergo degranulation. Because the IL-5/hE2 inductive environment is on an EPX$^{-/-}$ background, this transgenic mouse model allows the use of EPX as a measurement to detect the changes in EPX release from the installed eosinophils.

4.2 Results

EPX release is significantly reduced in VAMP-7-deficient eosinophils

In order to evaluate changes in degranulation from eosinophils lacking VAMP-7, VAMP-7-deficient (VAMP-7 -) and wild-type (VAMP-7 +) eosinophils were isolated from the peripheral blood of *eCre-V7-IL-5* and *IL-5Tg* mice respectively. Isolated eosinophils were stimulated with PAF or ionomycin. The amount of EPX released from eosinophils was assayed by EPX ELISA using cell-free supernatant isolated from the *in vitro* culture. It was demonstrated that both VAMP-7 + and VAMP-7- eosinophils released EPX in response to PAF and ionomycin stimulation. As indicated in Figure 4.1, purified VAMP-7 - eosinophils demonstrate a significant reduction ~ 35% in EPX release compared to VAMP-7 + eosinophils in response to PAF stimulation *in vitro*. Mouse eosinophils deficient in VAMP-7 had a significant reduction ~ 70% in EPX release in response to ionomycin when compared to VAMP-7 + eosinophils.

PAF is a G-protein-coupled receptor ligand, and can elicit cellular activity through PAF receptor (PAFR)-dependent and independent fashion (13). PAF activation is associated with signalling pathways such as increasing intracellular calcium concentration and degranulation. Both VAMP-7 + and VAMP-7-deficient eosinophils responded to combined stimulation from PAF and calcium ionomycin much more robustly than to PAF alone. VAMP-7-deficient eosinophils demonstrated a ~72% decrease in the release of EPX compared to VAMP-7 + eosinophils in response to PAF and ionomycin combined stimulation. As indicated by trypan blue stain, >90% of eosinophils were viable after 30 min of stimulation by PAF and ionomycin at 37°C.

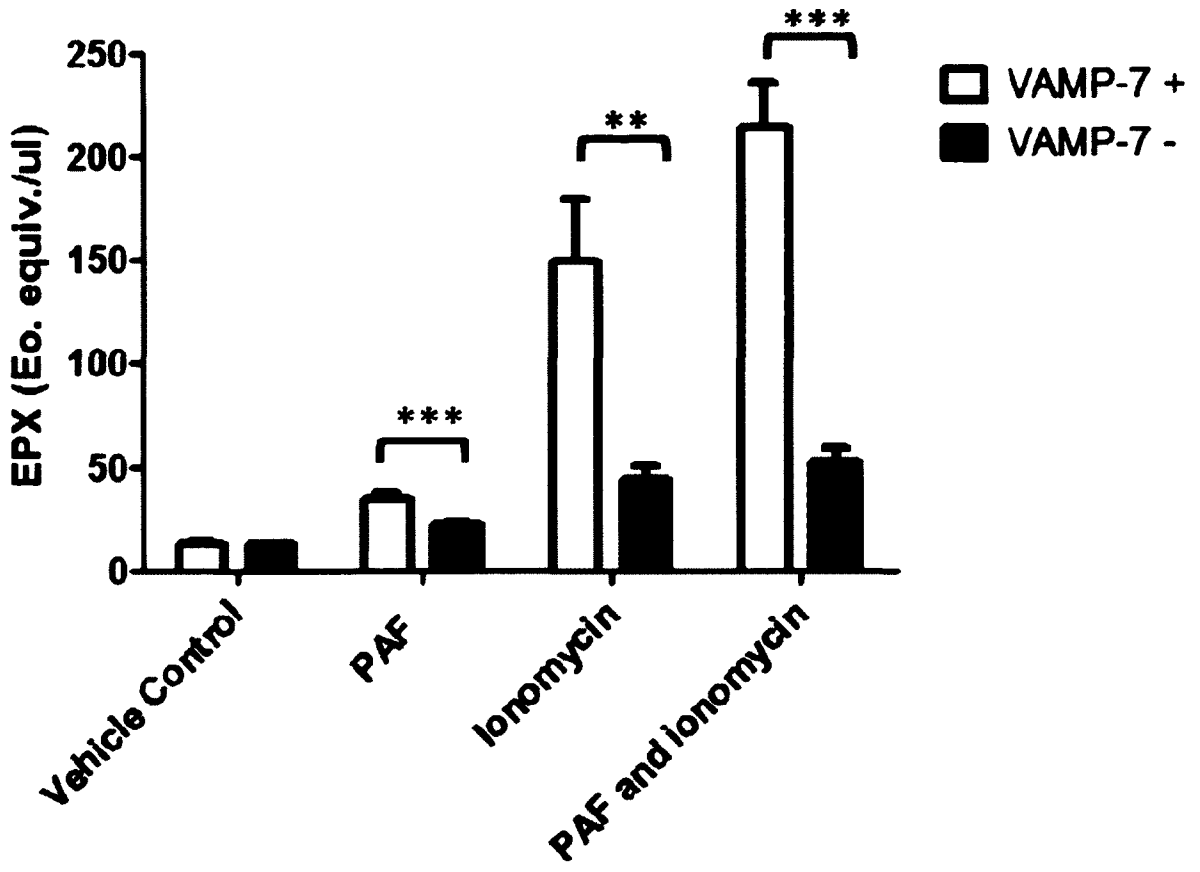

Figure 4.1. EPX release was significantly reduced in VAMP-7-deficient eosinophils. Eosinophils were obtained at > 98% purity from the peripheral blood of 6 to 8 week old *eCre* - VAMP-7 mice on the IL-5 (NJ. 1638) background. Purified eosinophils were resuspended in phenol red-free RPMI 1640. Aliquots of 2 x 10^5 cells were incubated with PAF (200 ng/ml) or ionomycin (50 ng/ml) or DMSO as vehicle control for 30 min. The level of EPX released into supernatants was assayed using EPX ELISA, (n=6). Error bars represent ± SEM, *** indicates a p=0.0008, ** indicates a p= 0.0047, and *** indicates a $p < 0.0001$ as determined by an unpaired t-test.

Release of MBP and Ears is significantly decreased in VAMP-7-deficient eosinophils

MBP is the most abundant granule protein in both mouse and human eosinophils. MBP-1 is the predominant form of the protein expressed in mouse eosinophils. Preformed and stored MBP-1 creates the electron-dense cores observed in eosinophil crystalloid granules. Ears (Ear-1, -2, -6/7, -5/11), the second most abundant granule protein group in mouse eosinophils, are a family of preformed ribonucleases that are stored in the electron-translucent matrix of the crystalloid granules (20, 21). Unlike EPX which appears to be specific to eosinophils, both MBP and Ears are expressed by cell types other than eosinophils in humans (20, 22, 23). In addition, the release of MBP from human eosinophils has been linked to cell activation and disease pathology (7, 24, 25).

Cell-free supernatants collected from *in vitro* stimulation of VAMP-7+ and VAMP-7-deficient eosinophils were evaluated for the presence of soluble MBP and Ears using a protein-specific immunoblot assay with a rat-anti mouse MBP monoclonal antibody or a rat-anti mouse Ears antibody. As indicated in Figure 4.2 A (MBP) and Figure 4.2 B (Ears), results collected from the single-dimensional ELISAs indicated that VAMP-7-deficient and VAMP-7+ eosinophils responded to PAF and ionomycin stimulation by releasing soluble MBP and Ears. The amount of soluble MBP and Ears was reduced drastically in supernatants collected from VAMP-7-deficient eosinophils compared to VAMP-7+ eosinophils.

Immunoregulatory cytokine (IL-4 and IL-13) release is unaffected in VAMP-7-deficient eosinophils

Human eosinophils release their granule contents (i.e. EPX) via more than one form of regulated exocytosis. Classical exocytosis, where a single granule can fuse with the plasma membrane to release granule proteins has been shown *in vitro*. Cytokine release from human eosinophils has been shown to occur via piecemeal degranulation, which is a variation of regulated exocytosis.

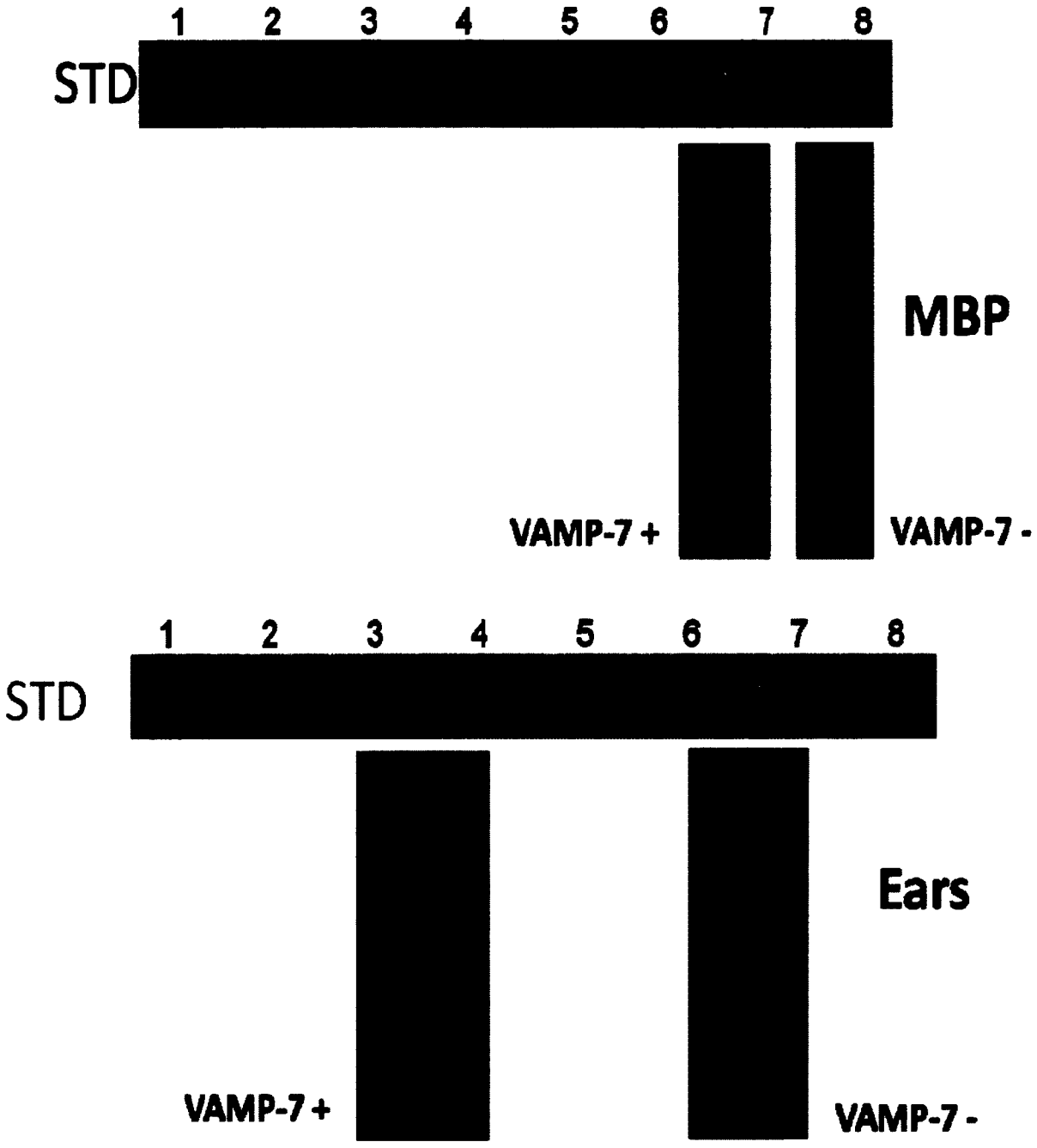

Figure 4.2. MBP and Ears release was reduced in VAMP-7-deficient eosinophils. MBP/Ears standards numbered 1-8 are eosinophil cell lysates blotted as a serial dilution before reaction with biotinylated rat-anti mouse MBP or biotinylated rat-anti mouse Ears corresponding to 22,531,7510, 2503, 834, 278, 93, 31, 0 eosinophils/well equivalents, respectively. Each dot represents a sample from one isolation and stimulation.

The relationship between release of immunomodulatory cytokines (IL-4 and IL-13) and VAMP-7-mediated activities in mouse eosinophil degranulation was evaluated. As indicated in Figure 4.3 A and B, levels of IL-13 and IL-4 released in cell-free supernatants from PAF and ionomycin stimulation were comparable between VAMP-7-deficient and VAMP-7+ eosinophils, and no significant difference was observed.

EPX levels are significantly reduced from VAMP-7-defecient eosinophils in response to *ex vivo* airway stimulation

VAMP-7-deficient eosinophils had significantly reduced levels of granule proteins released in response to *in vitro* stimulation with PAF and ionomycin. The ability of VAMP-7-deficient eosinophils to release EPX in the lung environment of *EPX*$^{-/-}$ mice crossed with those who are expressing *IL-5/hE2* was evaluated. *IL-5/hE2* mice were observed to have a severe asthmatic pathology as a result of overwhelming eosinophil recruitment and activation in the lungs (26). Because recipient mice were EPX$^{-/-}$, any EPX signal detected from the BAL of the recipient mice would be solely from intratracheally installed eosinophils isolated from VAMP-7 + or VAMP-7 - mice peripheral blood.

Eosinophils were isolated and intratracheally installed into the recipient *IL-5/hE2/EPX*$^{-/-}$ mice airways. As indicated in Figure 4.4, both VAMP-7-deficient and VAMP-7 + eosinophils responded to environmental stimulation in the airways of *IL-5/hE2/EPX*$^{-/-}$ mice. The release of EPX from VAMP-7-deficient eosinophils was significantly reduced ~ 76.5% compared to VAMP-7+ eosinophils in response to *ex vivo* airway stimulation.

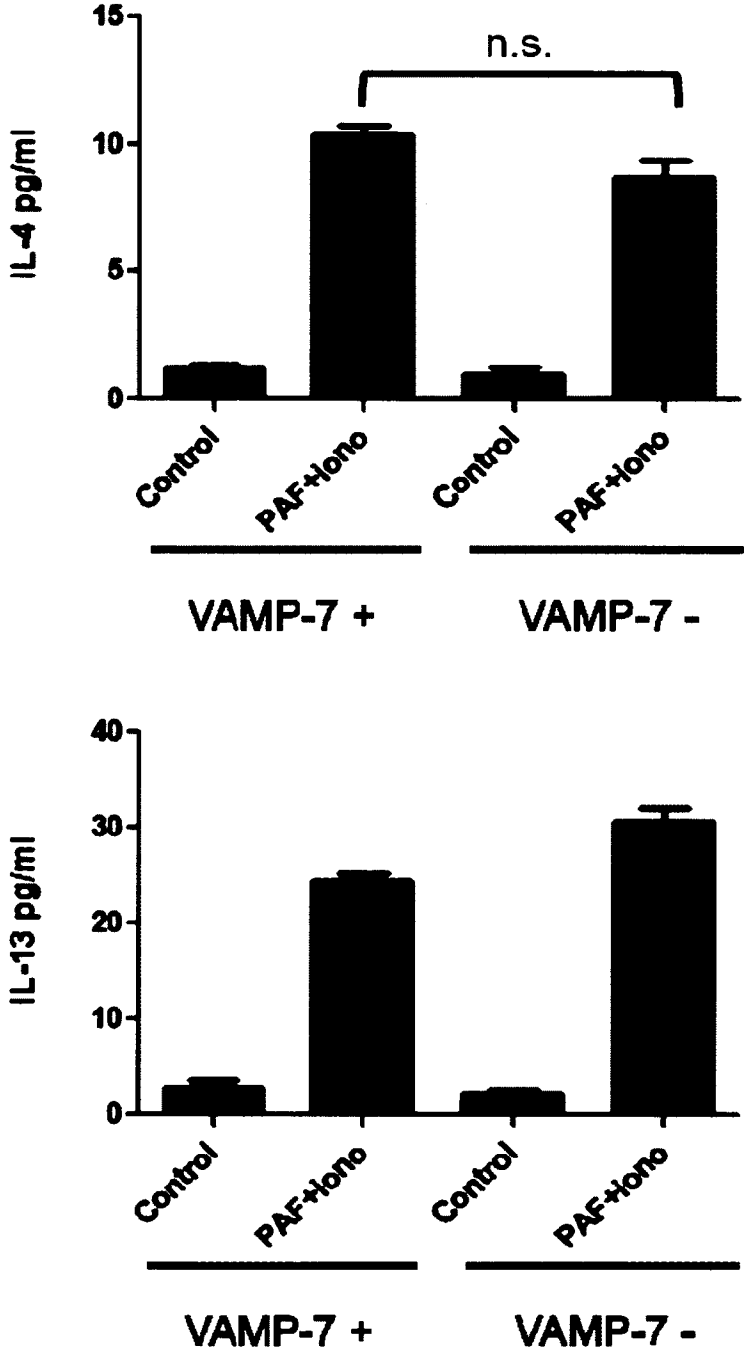

Figure 4.3. Release of IL-4 and IL-13 were unaffected in eosinophils deficient in VAMP-7 gene expression. The levels of IL-4 (A, *n*=3) and IL-13 (B, *n*=3) released by VAMP-7 deficient eosinophils were comparable to that of the wild-type eosinophils. Error bars represent ± SEM.

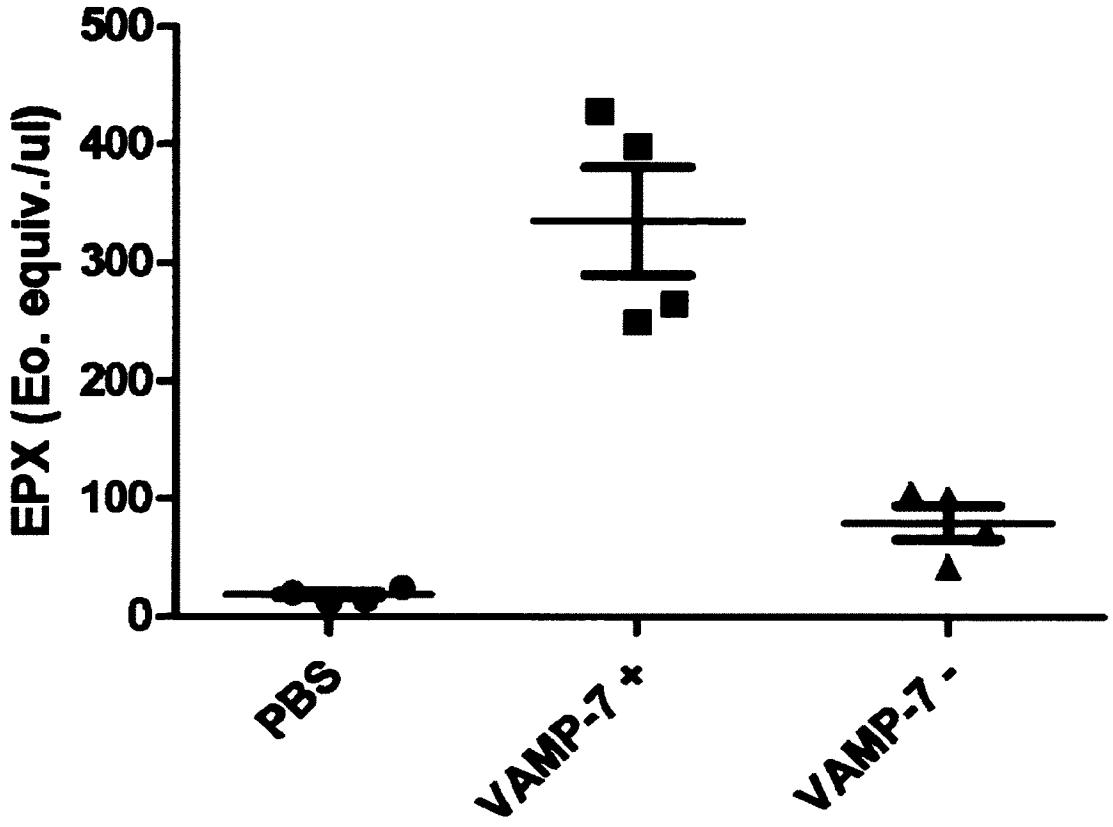

Figure 4.4. EPX levels were significantly reduced in VAMP-7-defecient eosinophils in response to *ex vivo* airways activation. Eosinophils were obtained at > 98% purity. Purified peripheral blood eosinophils were installed into the trachea of *IL-5/hE2/EPX-/-* recipient mice. BAL samples were collected from recipient mice 24 h later. The levels of EPX released into cell-free BAL supernatants was assayed using ELISA with biotinylated rat anti-EPX monoclonal antibodies, (n=4). Error bars represent ± SEM, ** indicates a p=0.0018 as determined by unpaired t-test.

4.3 Summary and Discussion

Exocytosis is a critical event in the activation of eosinophils. SNARE family proteins VAMP-2, -7, -8, syntaxin-4, and SNAP-23 are expressed in human eosinophils (10, 27, 28). VAMP-7 and -8 were found in subcellular fractions containing crystalloid granules and other membrane-bound compartments in human eosinophils (10). A dose-dependent reduction in EPX and EDN was observed in human eosinophils treated with neutralizing antibodies against VAMP-7 introduced intracellularly via streptolysin-O (SLO) permeabilization of cell membranes (10). Antibodies are unable to traverse through the plasma membrane, and it is necessary to manipulate cells to allow antibodies to enter them. Therefore *in vitro* cell membrane permeabilization with SLO was required to introduce anti-VAMP-7 antibodies and inhibit VAMP-7 function. This is an artificial system that required stimulation of degranulation using extracellular GTPγS and Ca^{2+}, and did not indicate a role for VAMP-7 in exocytosis induced by receptor stimulation. In this study, gene targeting-based transgenic mouse models were applied to generate intact mouse eosinophils lacking VAMP-7 for physiologically relevant receptor-mediated degranulation responses. The use of such transgenic mouse models has provided a unique advancement for the study of SNARE functions in eosinophils.

For the first time, it was demonstrated that receptor-dependent release of eosinophil granule proteins such as EPX, MBP, and Ears was significantly reduced in mouse eosinophils deficient in VAMP-7. The reduction in EPX released from VAMP-7-deficient eosinophils was confirmed in the *ex vivo* airway stimulation model. In purified eosinophil preparations stimulated *in vitro*, limited cytolysis was observed. EPX released by cytolysis could explain incomplete inhibition of granule protein release from eosinophils stimulated *in vitro*, since cytolysis is not a direct result of receptor stimulation and intracellular signalling through VAMP-7.

Neutralizing antibody-mediated inhibition of VAMP-7 function impaired the release of EPX and EDN in a dose-dependent manner in human eosinophils (10). The release of cytokines, however, was not evaluated in the SLO-permeabilized *in vitro* model. In this study, the release of crystalloid granule proteins as well as immunoregulatory cytokines was investigated from intact mouse eosinophils deficient in VAMP-7. Whereas granule protein release was dependent on VAMP-7, cytokine (IL-4 and IL-13) release in response to PAF and ionomycin appeared to be unaffected by deletion of the VAMP-7 gene from mouse eosinophils. The differential secretion of granule proteins and cytokines from VAMP-7-deficient eosinophils may be attributable to the mode of degranulation and/or stimuli used *in vitro.* Vesicular-based PMD and cytolysis are the predominant mechanisms of release of preformed mediators under physiological conditions (9, 29, 30). Classical exocytosis of complete granule release from eosinophils has only been observed in stimulated cell preparations *in vitro* and is absent in biopsy samples collected from allergic patients (8).

Eosinophil-derived cytokines are found in both crystalloid granules and small secretory vesicles, which are differentially released into the extracellular matrix via PMD in response to selective stimuli [25,(31). A "ready-to-release" pool of Th1 (IL-12, IL-27, IFNγ), Th2 (IL-4, IL-13), pro-inflammatory (TNF-α), and regulatory (IL-10) cytokines has been shown to be transported by a tubulovesicular system shuttling vesicles from the crystalloid granule storage compartment for rapid release from human eosinophils (30-60 min) following specific receptor stimulation (12, 32, 33). IFNγ stimulation was associated with the secretion of IL-12 but not IL-4 (34-36), whereas eotaxin, CCL5, or IL-16 stimulation was associated with IL-4 secretion but not IL-12 from human eosinophils (19, 36). In human eosinophils, the IL-4 receptor α chain is anchored to the inner membrane leaflet of small secretory vesicles, and has been observed to colocalize with IL-4 during CCL11-mediated selective mobilization and secretion (19). The differential secretion pattern from stimulated eosinophils suggests a complex mechanism of selective

sorting, transport, or fusion exists in response to activation. Cytokine release is a major means of immune modulation by eosinophils, and thus it is critical to evaluate the release of immunoregulatory cytokines from eosinophils in order to characterize the role that this particular cell population plays in inflammation.

This differential pattern of secretion can be explained, in part, by the transport and fusion machinery that eosinophils employ. Evidence from the Moqbel and Lacy lab, Edmonton, Alberta, Canada has shown differential expression patterns of transport and fusion machinery in crystalloid granules and secretory vesicles (37). Specific and overlapping complements of SNARE proteins have been shown to be expressed on both eosinophil crystalloid granules and secretory vesicles. VAMP-7 and -8 are localized to crystalloid granules and small secretory vesicles of human eosinophils (10, 38), but only VAMP-7 was associated with the release of EPX and EDN (10). VAMP-2 is absent on crystalloid granules, but was colocalized with secretory vesicles containing chemokine CCL5 and was observed to traffic to the plasma membrane in response to IFNγ stimulation (27). In human eosinophils, VAMP-2 has been considered a major participant in the machinery regulating PMD to release eosinophil-derived cytokines, whereas membrane fusion events necessary for complete crystalloid granule exocytosis is mainly VAMP-7-dependent (10, 37). The significant reduction in crystalloid granule protein release and the lack of reduction in cytokine release from VAMP-7-deficient mouse eosinophils suggests that a selective mechanism exists in which eosinophils employ for sorting and mobilization during receptor-mediated exocytosis.

In this study, only robust stimuli (PAF and ionomycin) were used to elicit *in vitro* eosinophil degranulation. PAF has been shown to function in a PAF receptor-independent manner and may act through an alternative receptor-linked pathway (13). Conversely, ionomycin bypasses receptor-mediated binding to initiate an intracellular Ca^{2+}-dependent signalling cascade that results in degranulation. Eosinophils express various surface receptors

(e.g., C5 receptor, PAFR, and chemokine receptor) that can elicit various modes of degranulation in response to ligand-receptor interaction (39, 40). Cytokine and chemokine receptors may play a role in differential secretion in addition to signalling (19, 37). Eotaxin stimulation of eosinophils elicited the movement of secretory vesicles but not crystalloid granules (19) which could lead to a different profile of secreted mediators compared with PAF and/or ionomycin stimulation. In the future, VAMP-7-deficient eosinophils should be subjected to further *in vitro* stimulation with various stimuli (e.g., IFNγ, CCL11, and IL-6) to evaluate the role of VAMP-7 in cytokine secretion.

All four modes of exocytosis can be visually confirmed using transmission election microscopy (TEM). Therefore, in future studies, differentially stimulated VAMP-7-deficient eosinophils should be subject to TEM evaluation to determine specific modes of degranulation associated with each stimulus.

Patch clamp analysis is another way to distinguish PMD and classical exocytosis in VAMP-7-deficient eosinophils. Each mode of exocytosis is associated with a specific pattern of cell capacitance increase observed when eosinophils are patched in the whole cell configuration with GTPγS and Ca^{2+} to stimulate degranulation (41). Electrical capacitance is directly proportional to the area of plasma membrane. As a result of exocytosis, granule membranes are added to the total area of plasma membrane upon fusion. Changes in the plasma membrane area when a secretory granule fused with the plasma membrane can be measured by changes in the electrical capacitance, which is directly proportional to the granule membrane area.

In summary, it has been shown that the SNARE protein VAMP-7 plays a crucial role in receptor-mediated exocytotic release of EPX, MBP, and Ears from intact mouse eosinophils. This has important implications for an *in vivo* role for VAMP-7-mediated eosinophil degranulation and allows testing for the role of eosinophil degranulation in allergic airway inflammation.

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Chapter Five: Defining the Role of VAMP-7-mediated Eosinophil Degranulation in Mouse Models of Allergic Airway Inflammation

5.1 Introduction

The pathogenesis of allergic asthma is associated with recruitment and accumulation of immune cells in the lungs, which is hypothesized to be orchestrated by innate and adaptive immunity (1). Clinically, allergic asthma is characterized by reversible airway hyperresponsiveness (AHR), mucus hyperproduction, airway remodelling, and in most cases airway and tissue eosinophilia accompanied with extensive degranulation. Eosinophil numbers and activity are increased in airway mucosa, lumen, and circulation in patients with allergic asthma as well as in mouse models of the disease (2). Evidence from clinical and animal model-based investigations suggest eosinophils have a critical role in the pathogenesis of allergic asthma (3-5), although the exact mechanism of how eosinophils influence the pathology requires further study.

There is strong evidence indicating that the release of granular proteins is a critical function for eosinophils in orchestrating the micro-milieu during inflammation (6). Eosinophil degranulation has been associated with AHR and remodelling in human asthmatics, guinea pig, and mouse models of the disease (7-9). Human eosinophils undergo gradual depletion of their crystalloid granular content via small secretory vesicles known as piecemeal degranulation during inflammation (10). Piecemeal degranulation has been observed in tissue eosinophils in nasal polyps from human allergic subjects, and the magnitude of eosinophil degranulation is correlated with severity of the disease (11). Mouse models and allergen provocation have been used extensively to study the role of eosinophils in the pathogenesis of allergic asthma.

To study VAMP-7-mediated eosinophil degranulation in the development of allergic pulmonary inflammation, C57BL/6J wild-type (WT) mice with eosinophil-specific VAMP-7 gene deletion (*eCre-V7*) mice were subjected to acute allergen sensitization and challenge using

OVA/alum, which induces disease-like changes in the lungs when animals are sensitized intraperitoneally and challenged using nebulization. Pulmonary inflammation may be evaluated by cellular infiltrate to airways, eosinophil crystalloid granule protein release in airways, and AHR in response to methacholine challenge subsequent to the OVA provocation. Airway cellular infiltrate profiles generated from BAL samples provide a general view of airway inflammation.

In this project, lung sections from *eCre-V7* and WT mice were analyzed for general histopathology. The extent of inflammatory cell infiltration to the lungs, goblet cell metaplasia, mucin accumulation, and collagen deposition were analyzed in *eCre-V7* and WT mice treated with OVA. Sensitized and challenged *eCre-V7* mice were also subjected to AHR measurement using increasing doses of methacholine, which induce airway smooth muscle contraction. Baseline airway resistance as well as airway resistance to methacholine challenge were evaluated in WT mice and *eCre-V7* mice.

It was demonstrated that VAMP-7-mediated eosinophil degranulation contributed to the development of pulmonary inflammation induced by acute OVA provocation and challenge in mouse model of allergic asthma.

5.2 Results

OVA treated *eCre-V7* mice exhibit comparable pulmonary cellular infiltration to wild-type mice

Age and sex-matched mice were subjected to an acute OVA sensitization and challenge protocol to induce pulmonary inflammation. Specifically, *eCre-V7* mice and WT controls (which were floxed *VAMP-7* mice in all experiments) were sensitized on day 0 and 14 with chicken OVA/alum and challenged with aerosolized OVA in PBS on days 24, 25, and 26 (Figure 5.1) The control mice received saline alone. BAL samples were collected on day 28. BAL cellularity was determined by differential count of Diff Quik stained cytospin slides. The magnitude of recruitment of inflammatory cells (e.g., eosinophils, lymphocytes, and neutrophils) in the lung parenchyma was comparable between *eCre-V7* mice and WT wild-type mice in response to the induced allergic inflammation (Figure 5.2). No difference between the total BAL cellularity and eosinophils numbers was observed among experimental groups.

VAMP-7 is not required for the release of IL-13 in an OVA model of allergic airway inflammation

Levels of immunoregulatory cytokines (IL-4 and IL-13) were analyzed in BAL samples to better understand the relationship between VAMP-7-mediated eosinophil events and the secretion of cytokines from the airways. Levels of IL-4 in the BAL of OVA-sensitized and OVA-challenged mice were below the ELISA detection sensitivity. However, IL-13 levels were detected in cell-free BAL fluid samples collected from both WT and *eCre-V7* mice treated with

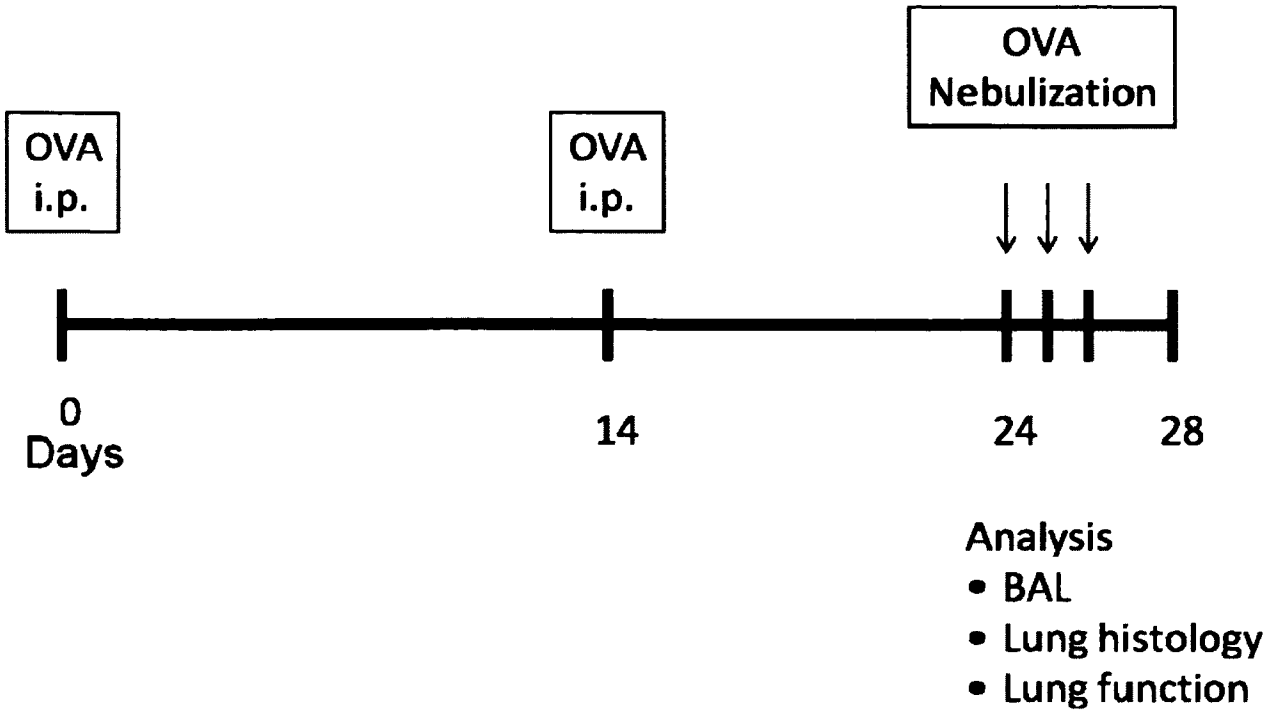

Figure 5.1. OVA sensitization and challenge protocol to induce allergic airway inflammation in the mouse model.

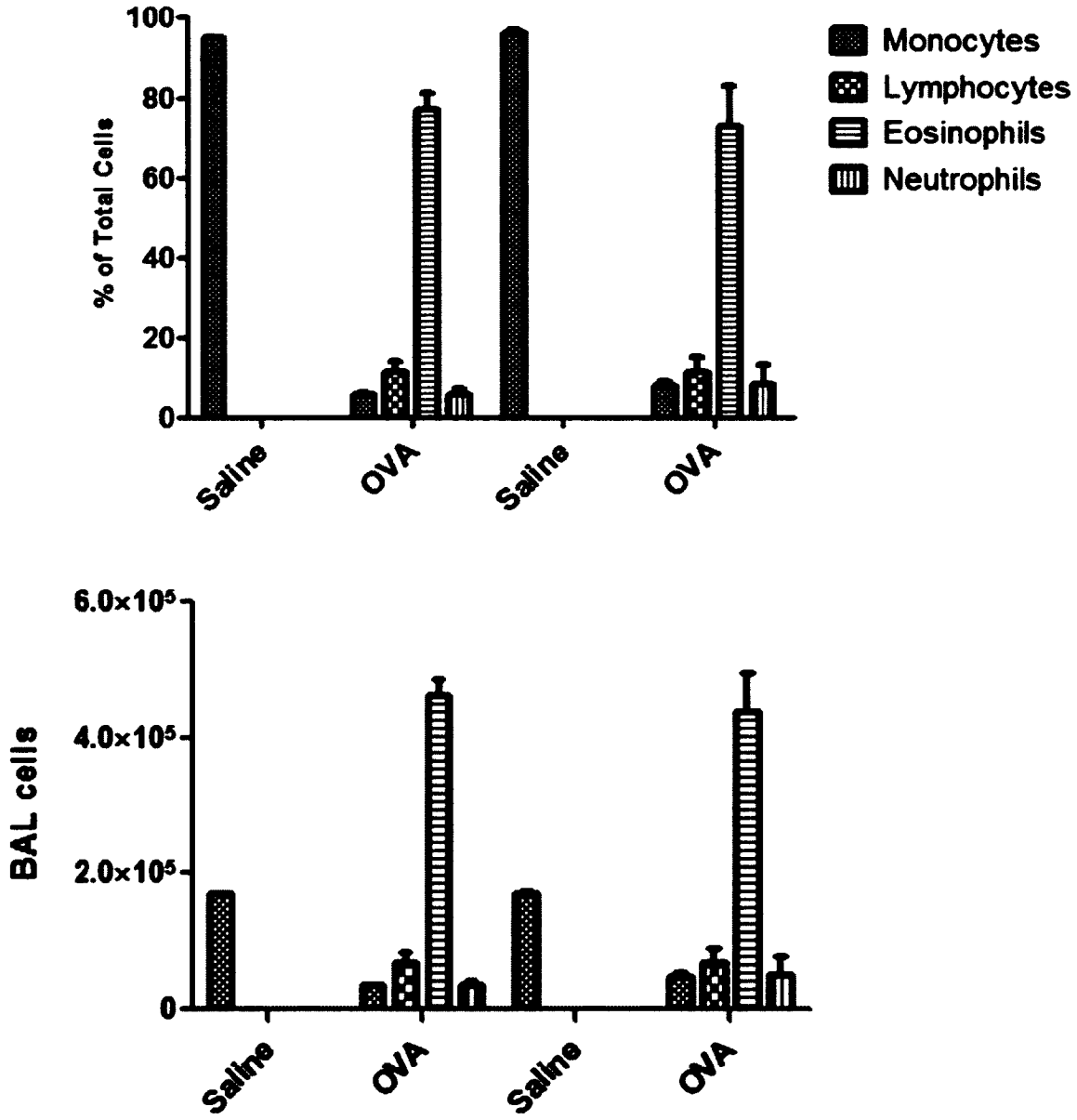

Figure 5.2. Allergen-mediated pulmonary inflammatory cellular infiltration of the lung is comparable between *eCre-V7* and wild-type mice. Wild-type and *eCre-V7* mice were subjected to the acute OVA sensitization/challenge protocol. BAL was collated 24 h after the last OVA challenge. Cells from BAL samples were assessed for total cell number by hemacytometer. Assessment of BAL cellularity demonstrated that the acute OVA protocol elicited inflammatory response in both WT and *eCre-V7* mice. The recruitment of eosinophils, lymphocytes and neutrophils by *eCre-V7* mice to the airways after OVA challenge was comparable to WT mice (n=6). Error bars represent ± SEM.

OVA. As indicated in Figure 5.3, IL-13 release was unaffected in the airways of OVA sensitized and challenged *eCre-V7* mice compared to WT mice.

VAMP-7 contributes to EPX release from mouse eosinophils *in vivo*

WT and *eCre-V7* mice were subjected to acute OVA sensitization/challenge. Levels of EPX in BAL samples were assayed using EPX ELISA. WT control mice treated with saline alone did not show any EPX release in their BAL samples. However, OVA treated *eCre-V7* mice had a significant (~30%) reduction in the amount of EPX in their BAL compared to WT mice treated with OVA (Figure 5.4).

VAMP-7 contributes to the release of MBP-1 and Ears from mouse eosinophils *in vivo*

Single-dimensioned immunoblot assays using biotinylated MBP and Ears antibodies were used to analyze levels of soluble MBP and Ears in BAL samples from *eCre-V7* mice subjected to acute OVA provocation. As indicated in Figure 5.5 A, lower levels of soluble MBP-1 were detected in BAL samples collected from *eCre-V7* mice treated with OVA compared to WT mice. Levels of soluble Ears detected in the BAL of *eCre-V7* mice treated with OVA were lower than that of WT mice (Figure 5.5 B).

Histological evaluation of acute allergen-mediated airway inflammation

Lung sections were obtained 24 h after the last OVA challenge and stained with hematoxylin and eosin. OVA sensitization and challenge increased the number of eosinophils (white arrows) and other cell types infiltrated in mice airways compared to non-sensitized mice. Both *eCre-V7* and WT mice demonstrated similar levels of cellular infiltration in the lungs in response to OVA treatment (Figure 5.6). Acute allergen provocation increased perialveolar and perivascular thicknesses in airways of both *eCre-V7* and WT mice compared to saline-treated control mice. Airway smooth muscle thickening was observed in both strains of mice treated with OVA compared to saline controls. The level of airway smooth muscle thickening was comparable between *eCre-V7* mice and wild-type mice treated with OVA.

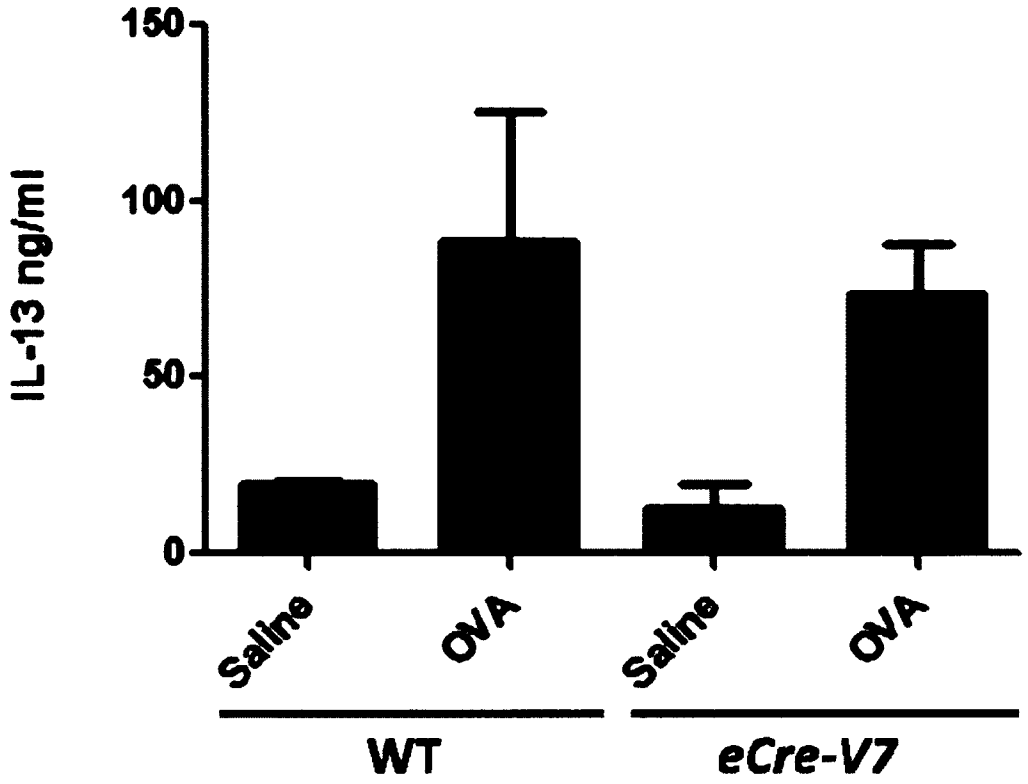

Figure 5.3. VAMP-7-mediated events are not involved in the release of IL-13 in an OVA model of allergic airway inflammation. The Th2 cytokine IL-13 (IL-4 was undetectable) was assessed by ELISA (*n*=3). Error bars represent ± SEM.

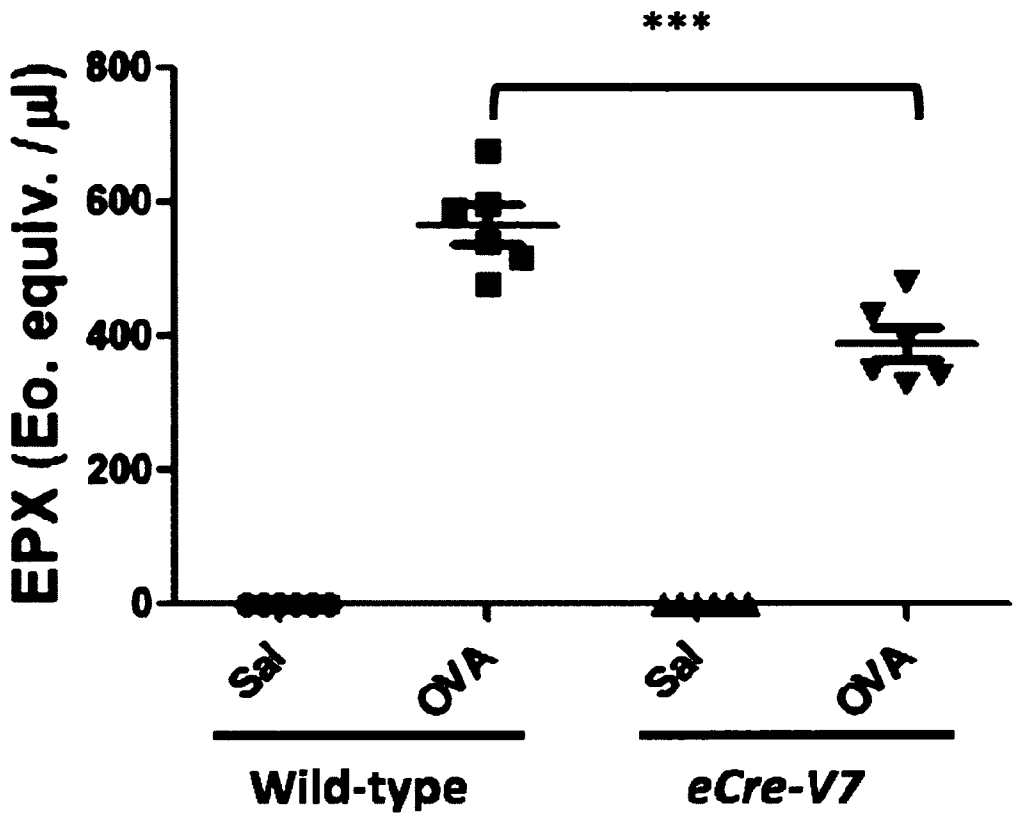

Figure 5.4. VAMP-7-mediated events are necessary for EPX release from mouse eosinophils *in vivo*. Extracellular EPX in the airways of both WT and *eCre-V7* mice provided evidence of eosinophil degranulation. Significant levels of EPX were detected in cell-free BAL isolated from *eCre-V7* and WT mice. Saline control and OVA-sensitized/challenged WT mice are shown for comparison. The highly specific EPX sandwich ELISA demonstrated a significant reduction (~30%) in levels of EPX released into the cell-free BAL of *eCre-V7* mice compared to WT controls (n=6). Error bars represent ± SEM, *** indicates a p=0.0009 as determined by unpaired t test.

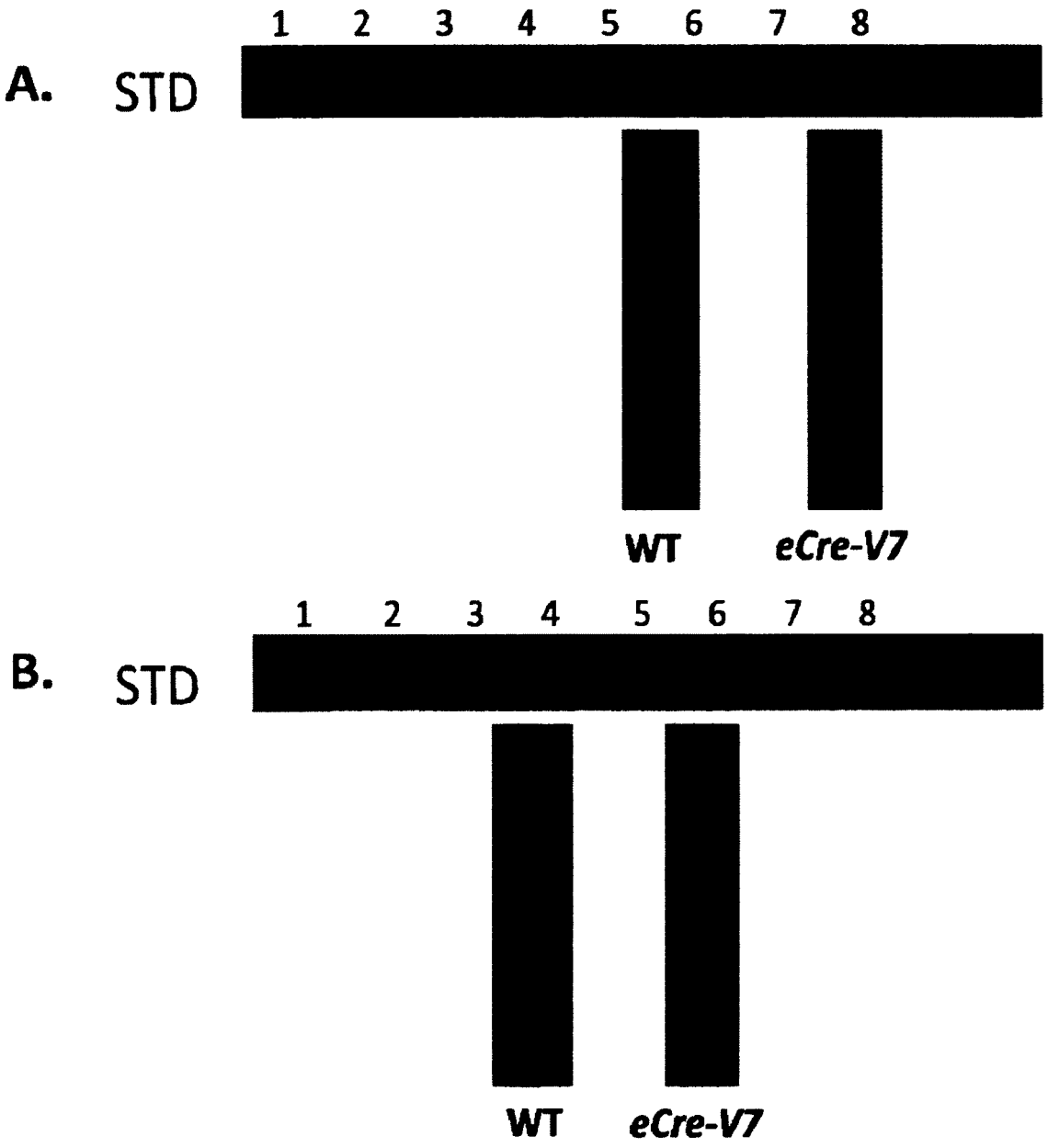

Figure 5.5. VAMP-7-mediated events contribute to MBP-1 and EARs release from mouse eosinophils *in vivo*. Immunoblot assay using a biotinylated rat-anti mouse MBP and Ears antibodies were use to detected the presence of soluble MBP-1 protein (A.) and Ears (B.) in cell-free BAL samples collected from both WT and *eCre-V7* mice subjected to acute allergen protocol. Lower levels of MBP-1 and Ears were detected in *eCre-V7* BAL fluid. Standards numbered 1-8 are eosinophil cell lysates blotted as a serial dilution corresponding to 22, 531, 7510, 2503, 834, 278, 93, 31, 0 eosinophils/well equivalents, respectively.

Assessment of goblet cell metaplasia and airway epithelial cell mucin accumulation by Periodic acid-Schiff (PAS) staining

Lung sections from *eCre-V7* and WT mice were stained with PAS and changes in the airway epithelia were examined. As shown in Figure 5.7, there was a significant increase in the number of PAS-positive, mucin-containing epithelial cells (black arrows) in the airways of OVA challenged WT and *eCre-V7* mice, which were absent in the lungs of control mice treated with saline. The magnitude of mucin accumulation and goblet cell metaplasia appears comparable between *eCre-V7* and WT mice treated with OVA, however, a trend towards a decrease in PAS-positive cells in *eCre-V7* mice compared to WT mice was observed.

MBP immunohistochemistry confirms eosinophil infiltration in the lung

Lung sections were stained using an anti-mouse MBP rat mAb (red) for immunohistochemistry detection of eosinophils. As indicated in Figure 5.8, there was a significant increase in MBP-1 positive cells in the lung parenchyma of OVA treated WT and *eCre-V7* mice compared to saline treated controls. Tissue eosinophilia was evident in the peribronchial and submucosal region of the lung parenchyma of OVA treated *eCre-V7* and WT mice (indicated by the black arrows in Figure 5.8), and absent in the airways of saline treated control mice. In confirmation of BAL sample analysis, the magnitude of eosinophil recruitment in the lungs parenchyma in response to OVA provocation was similar between *eCre-V7* mice and WT mice.

MBP-immunohistochemistry (IHC) staining provides information regarding eosinophil infiltration into tissues as well as their activity, which can be partially reflected by the extent of soluble MBP-1 present in the extracellular matrix as a result of degranulation. Intact eosinophils were observed as punctate structures accumulating in the lung parenchyma using MBP-IHC staining.

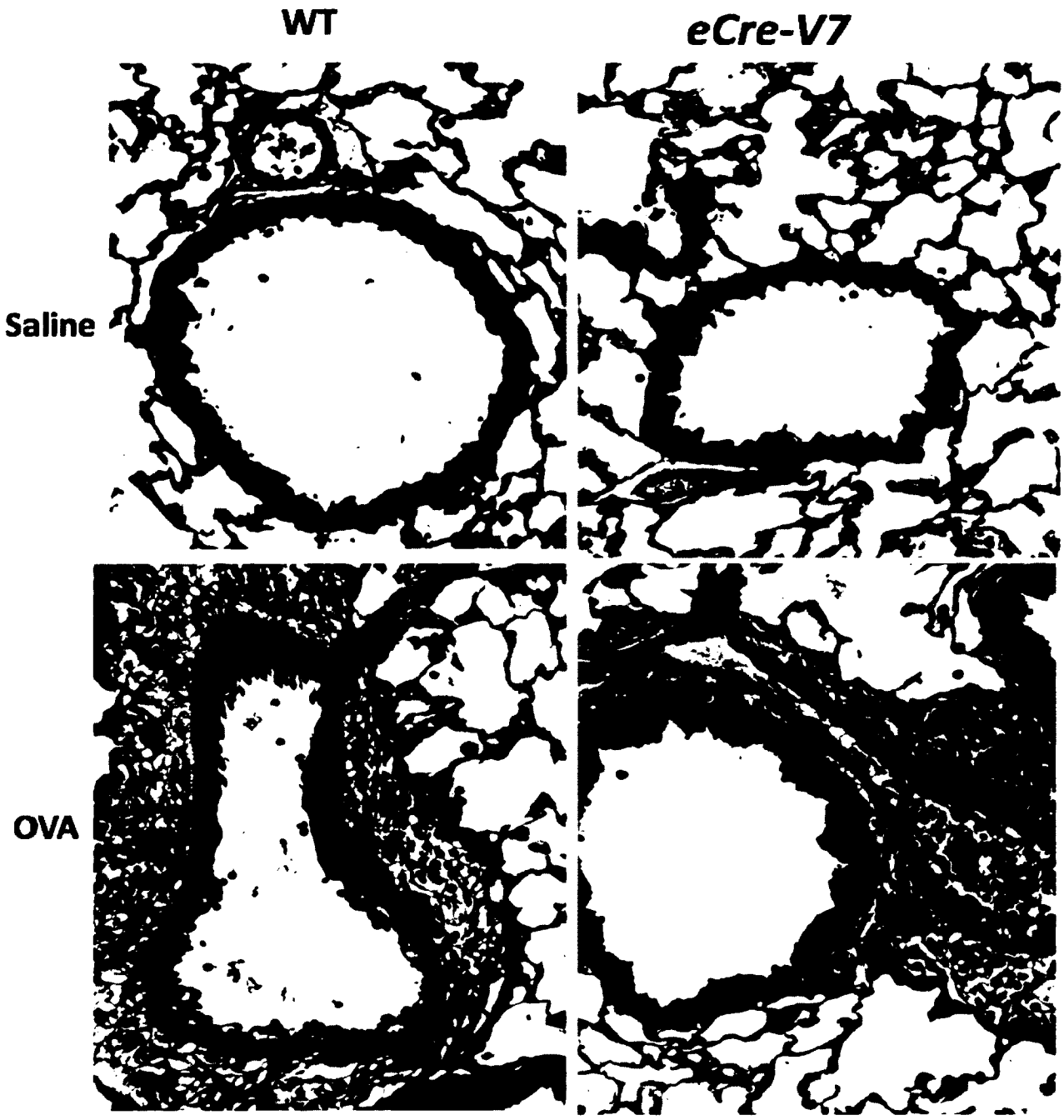

Figure 5.6. H&E histological evaluation of acute allergen-mediated airway inflammation. Representative views of airway sections from wild-type and *eCre-V7* mice subjected to acute OVA sensitization/challenge are shown. Inflammatory cell infiltrate patterns were comparable between *eCre-V7* and wild-type mice. Micrographs were taken using a 20X objective.

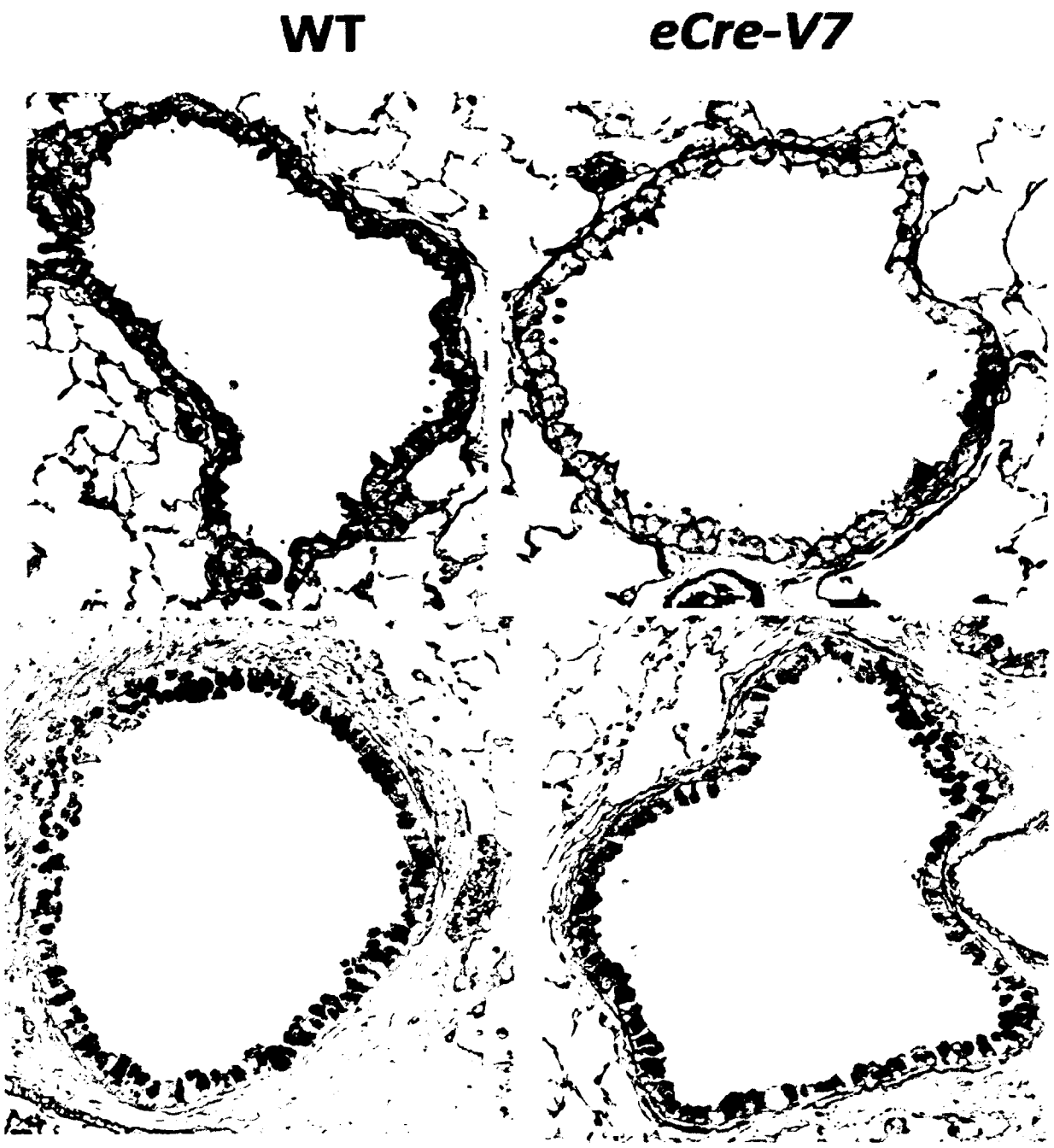

Figure 5.7. Assessments of goblet cell metaplasia and airway epithelial cell mucin accumulation by PAS staining. Representative views of airway sections from allergen-naive wild-type (C57BL/6J) mice, OVA treated *eCre-V7* and wild-type mice stained with PAS. The degree of goblet cell metaplasia and mucin accumulation (purple) was comparable between *eCre-V7* and wild-type mice. Micrographs were taken using a 20X objective.

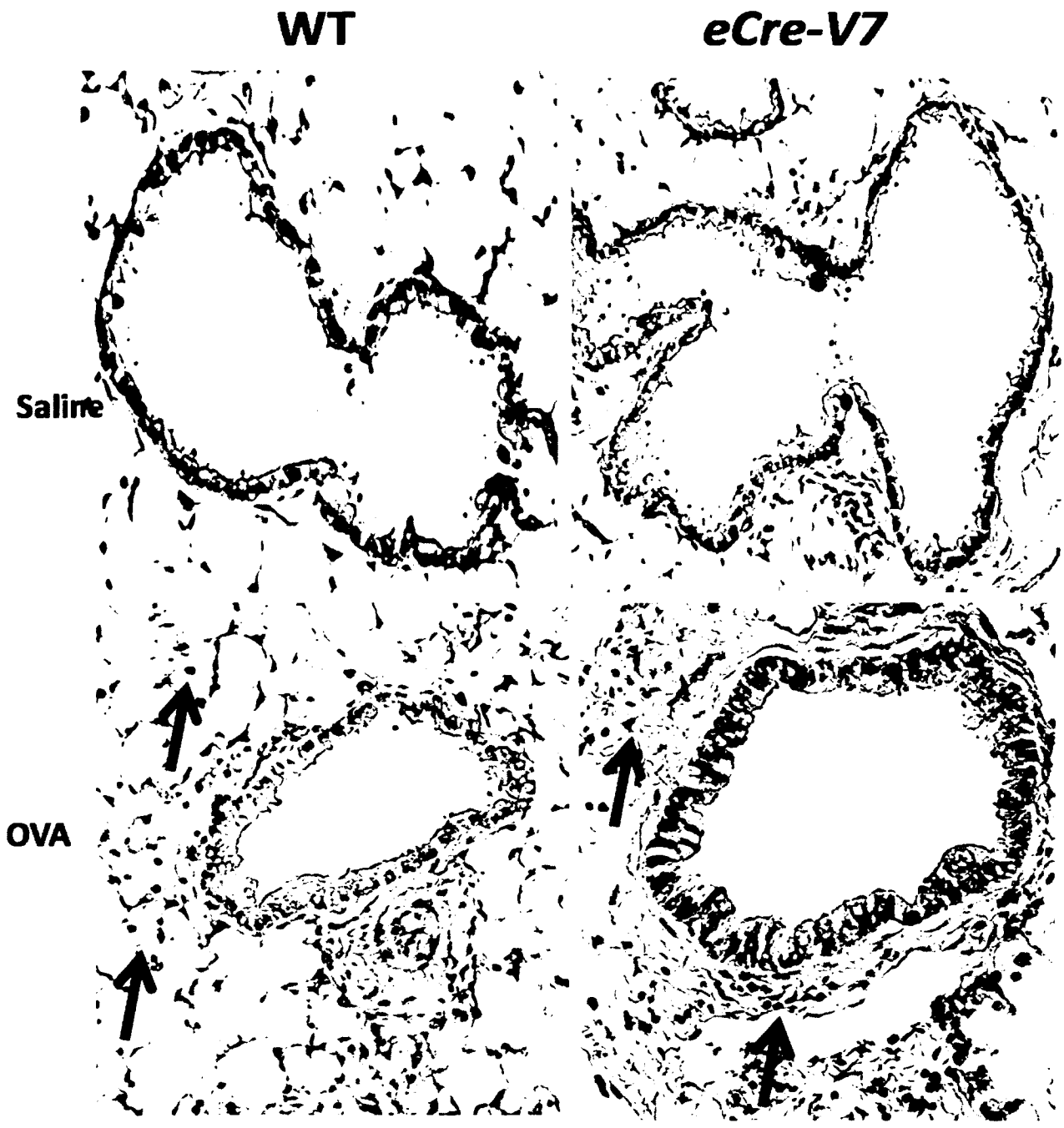

Figure 5.8. Eosinophil infiltration of the lung indicated by immunohistochemistry against MBP. Representative views of lung sections stained with immunohistochemistry using an anti-mouse MBP rat mAb indicated infiltrating eosinophils (red) in allergen-naive wild-type (C57BL/6J) mice, OVA sensitized/challenged wild-type (WT) and *eCre-V7* mice. Micrographs were taken using a 20X objective.

Extracellular MBP as the result of eosinophil activation and degranulation in the lung was seen as a lacy pattern that spread across the tissue accompanied with or without intact eosinophils. Extracellular granules distributed in the tissues may also be detected through MBP-IHC. As indicated in Figure 5.7, eosinophils observed in the MBP-IHC stained lung remained intact and showed little evidence of degranulation in the airways of *eCre-V7* and WT mice treated with OVA.

Masson's trichrome evaluation of collagen deposition and remodelling

Lung sections were stained with Masson's trichrome to evaluate changes in the magnitude of collagen deposition (indicated by the blue stain) in airways as a result of allergen-induced inflammation. OVA sensitization/challenge increased the amount of collagen deposited in peribronchial regions of both WT and *eCre-V7* mice. There appears to be a trend towards a decrease in collagen deposition in the lung parenchyma of OVA treated *eCre-V7* mice compared to WT mice (Figure 5.9) as the collagen staining was less intense in the peribronchial regions of the airway. As observed at a higher magnification (Figure 5.10) the deposition of collagen (indicated by the black arrows) in the airways of OVA treated *eCre-V7* mice appeared to be less compared to WT mice.

***eCre-V7* mice have similar baseline airway resistances to wild type controls**

Baseline airway resistance was measured using Flexivent. As indicated in Figure 5.11, the baseline airway resistance of *eCre-V7* mice in the absence of methacholine was not different from that of the allergen-naïve WT animals.

Eosinophil VAMP-7 may be required for development of airway hyperresponsiveness following OVA challenge of sensitized mice

From the results obtained in BAL samples, VAMP-7-deficient mice have reduced release of granule proteins (i.e., EPX, MBP, and Ears) in their airways following OVA sensitization and challenge. Consequently, it was determined that the influence of reduced eosinophil granule

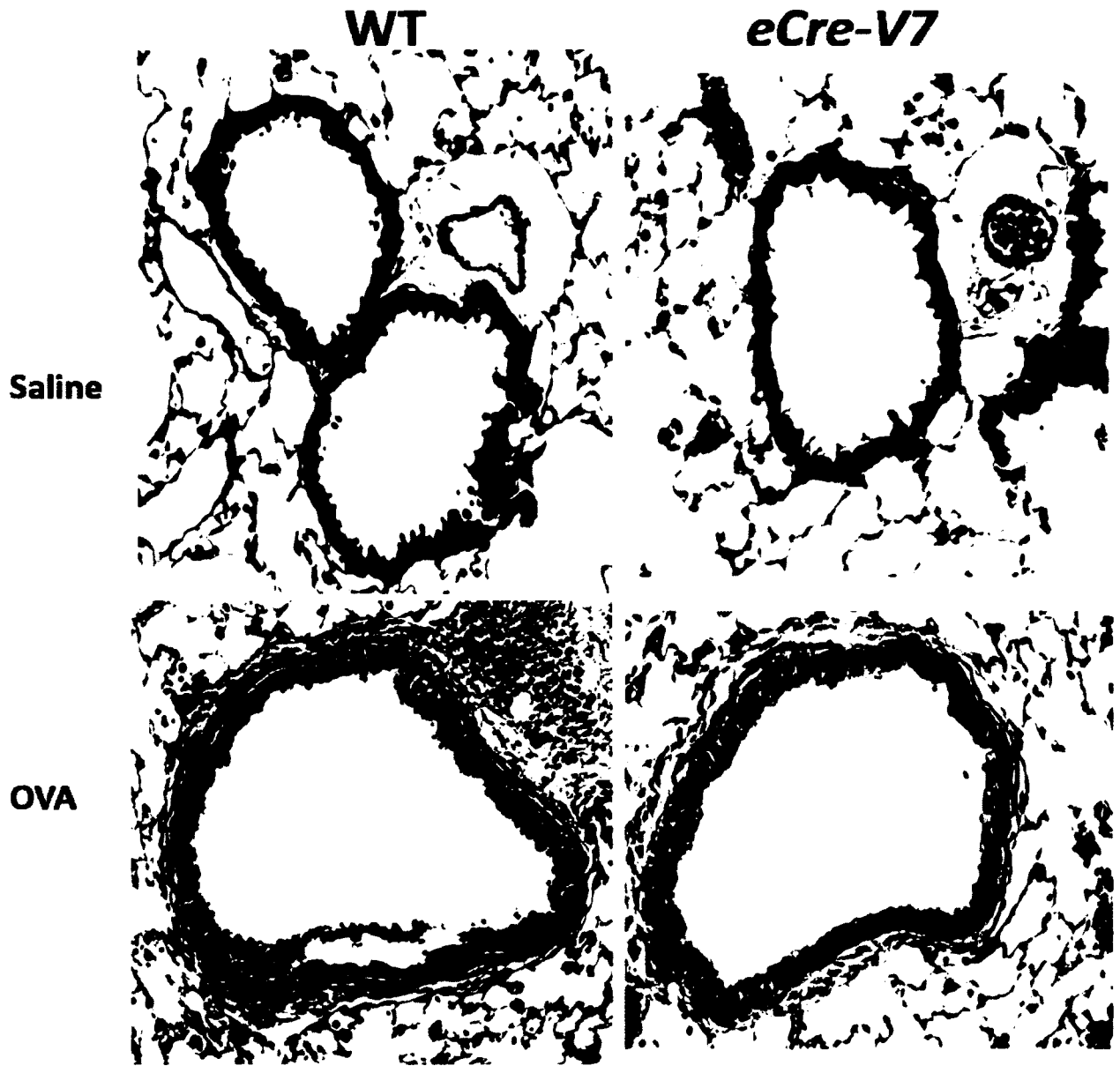

Figure 5.9. Masson's trichrome evaluation of collagen deposition and remodelling. Representative lung sections from allergen-naive wild-type mice, OVA treated wild-type and *eCre-V7* mice were stained with Masson's trichrome. A trend of reduced collagen deposition (stained blue) was observed in the airway of *eCre-V7* mice treated with OVA. Micrographs were taken using a 20X objective.

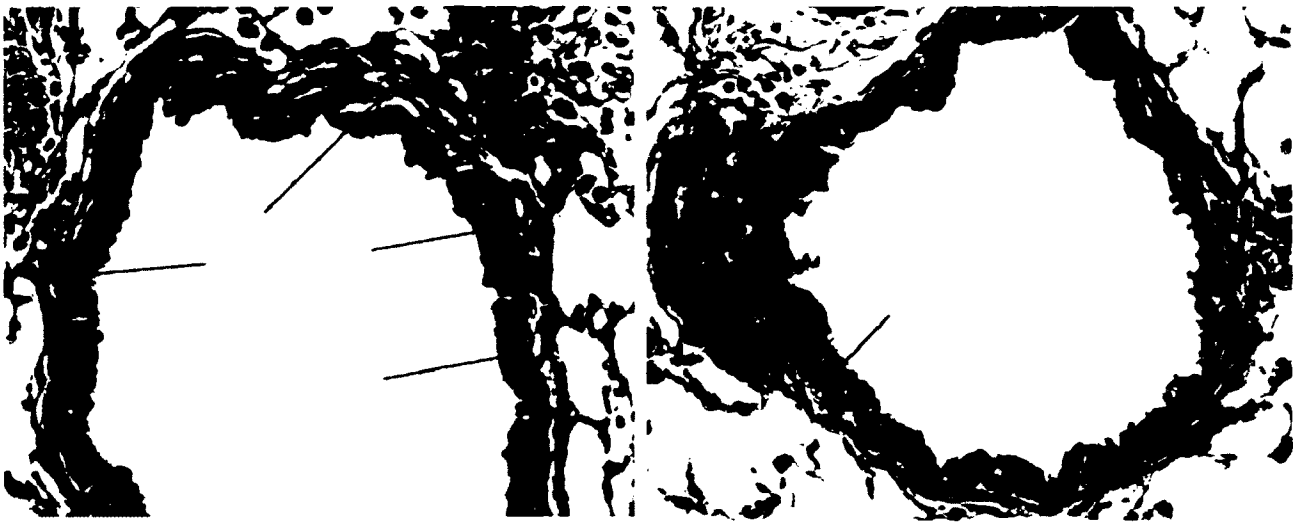

Figure 5.10. Masson's trichrome evaluation of collagen deposition and remodelling. Airways of mice treated with OVA showed less collagen staining in *eCre-V7* mice compared to WT mice. Micrographs were taken using a 40X objective.

protein release in AHR was evaluated following aerosolized methacholine challenge. As indicated in Figure 5.12, airway resistance elicited by each dose of methacholine appeared to be lower in OVA treated *eCre-V7* mice compared to OVA treated WT mice; however, the difference was not statistically significant. The AHR data gathered in this project came from 3 *eCre-V7* mice, thus the number of experimental mice are needed in the future.

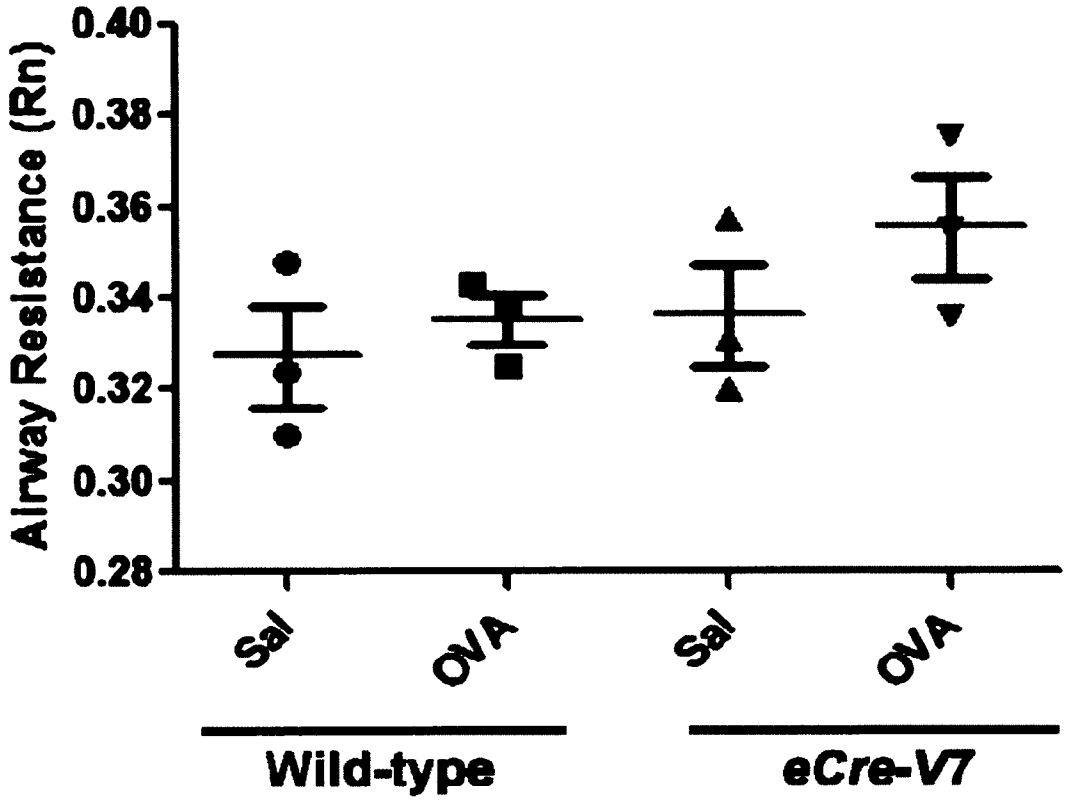

Figure 5.11. *eCre-V7* mice have comparable baseline airway resistances as wild-type controls. *Cre-V7* mice (8 weeks old) were subjected to acute OVA sensitization/challenge. Saline control and OVA-sensitized/challenged wild-type mice are shown for comparison. Sensitized and challenged mice were subject to one dose of aerosolized saline. Airway resistance was assessed using Flexivent. Baseline airway resistance of *eCre-V7* mice was similar to that of wild-type controls (n=3). Error bars represent ± SEM.

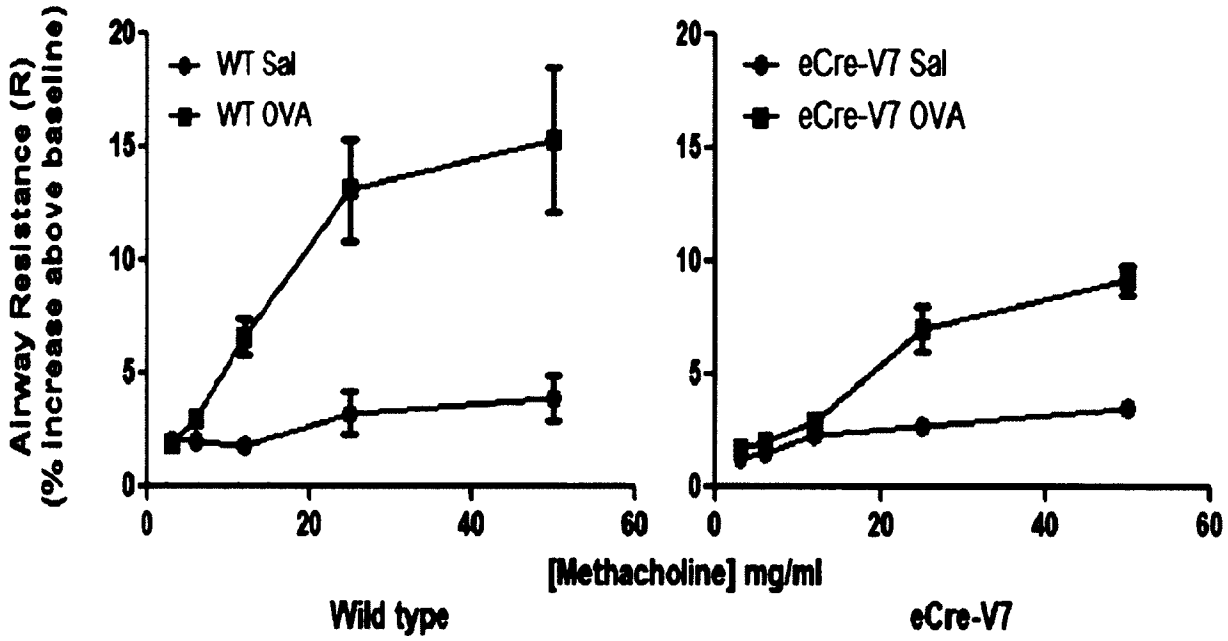

Figure 5.12. VAMP-7-mediated events in eosinophils appear to be required for the development of airway hyperresponsiveness (AHR) following OVA challenge of sensitized mice. *eCre-V7* mice (8 week old) were subjected to acute OVA sensitization/challenge. Saline control and OVA-sensitized/challenged WT mice are shown for comparison. Sensitized and challenged *eCre-V7* and wild-type mice were subjected to increasing doses of aerosolized methacholine (0, 3, 6, 12, and 15 mg/ml) Airway resistance (R) was assessed using an invasive ventilator-based technique (Flexivent). *n*=3, Error bars represent ± SEM.

5.3 Summary and Discussion

This is the first demonstration that eosinophil degranulation may have a role in the development of allergic airway inflammation. Previously there has been circumstantial evidence linking eosinophil degranulation with the development of pulmonary pathology and AHR in allergic asthma. The use of a transgenic-based mouse model in this study not only generated intact VAMP-7-deficient eosinophils for degranulation studies *in vitro*, but also provided a means to study eosinophil degranulation in the context of allergic pulmonary inflammation using mouse models of disease. *In vivo* models in the mouse provide a more systematic evaluation regarding the development of pathology as tissue samples and lung function can be measured after allergen provocation.

It was demonstrated that *eCre-V7* mice had similar BAL cellularity profiles in response to OVA treatment compared to WT mice. Although IL-13 levels remained unchanged, levels of eosinophil secondary granule proteins (i.e., EPX, MBP, and Ears) were significantly reduced in the airways of OVA sensitized/challenged *eCre-V7* mice. The lack of change in magnitude of cytokines released in the BAL of *eCre-V7* and WT mice confirmed similar observations *in vitro*, which demonstrated that eosinophils from *eCre-V7/IL-5* mice produced IL-13 in response to stimulation. Although human and mouse eosinophils store and release IL-13, Th2 cells produce the majority of this cytokine in this model. Therefore IL-13 is likely to derive from a mixture of cells, and most of the IL-13 produced will be released by Th2 cells.

The magnitude of reduction in EPX release in the *in vivo* OVA model (~30%), although significant, was low when compared to the magnitude of reduction in the release of the same proteins from VAMP-7-deficient eosinophils stimulated *in vitro* (~70%). This difference suggests that the contribution of VAMP-7-mediated events in the release of EPX *in vivo* is significant. Nevertheless, other VAMP-7-independent pathways exist in eosinophils in response to stimulation. For example, VAMP-2-mediated PMD may also play a significant role in the release

of crystalloid granule proteins as well as immunoregulatory cytokines in addition to VAMP-7-mediated events. Moreover, eosinophils may release granule proteins and stored mediators by cytolysis.

General histopathology (i.e., hematoxylin and eosin) revealed comparable changes in the magnitude of inflammatory cell infiltration in OVA-treated WT and *eCre-V7* mice. Nevertheless, the degree of goblet cell metaplasia and collagen deposition (i.e., PAS and Masson's trichrome) appeared lower in the lung parenchyma of *eCre-V7* mice compared to WT mice treated with OVA. Immunohistochemical examination of lung parenchyma revealed similar levels of tissue eosinophilia in OVA-treated *eCre-V7* and WT mice, with little evidence of tissue degranulation, which also confirmed the observations seen in other studies using this model of allergy induction (12).

AHR evaluation revealed that OVA-treated *eCre-V7* mice had comparable baseline airway resistances to WT mice. There was a trend toward reduced AHR in response to methacholine challenges in *eCre-V7* mice treated with OVA. This trend did not reach significance as the number of animals examined was too small for statistical analysis. Further experiments are needed to determine significance.

Extensive eosinophilia and degranulation in the airway and lung parenchyma are considered cardinal symptoms of human allergic asthma. Heretofore, the lack of evidence for degranulation from mouse eosinophils has been a prominent difference between human disease and mouse models using OVA. It has been shown that in the OVA immunization model, mouse eosinophils do not have extensive degranulation and peroxidase-mediated oxidation of airway proteins (12). The lack of extensive eosinophil degranulation within the lung parenchyma was also observed in our study as indicated by the MBP-IHC staining of lung tissue from mice treated with OVA. Various models exist to elicit allergic airway inflammation in mouse models and they range from allergen provocation, passive transfer models (i.e. antibody and or cell

types), and transgenic mice. Recent evidence has shown that a combination of house dust mite and OVA can elicit more inflammatory cell infiltration into the airways, increase AHR, and increase trends of airway remodelling when compared to mice treated with a single allergen (13). Multiple allergens consisting of cockroach, dust mite, ragweed, and fungi antigens have been shown to elicit enhanced allergic responses with special emphasis in eosinophilic inflammation in chronic models of asthma in mice (14). In order to study eosinophil-specific VAMP-7 deletion in the pathophysiology of allergic asthma, *eCre-V7* mice may be subject to various disease models using different combinations of allergens and provocation protocols, which are robust enough to elicit eosinophil infiltration and degranulation in the lung parenchyma beyond what was observed with the single allergen OVA model.

The development and outcome of inflammation is also strain-dependent in mice. C57BL/6 and BALB/c mice are most commonly used in modeled studies and genetic manipulations associated with asthma research (15). All mice used in this project were on the C57BL/6 background. However, AHR and all measurable Th2 responses, including eosinophilia, in C57BL/6 mice are present to a lesser degree than BALB/c mice (16). It would be interesting to generate *eCre-V7* mice on a BALB/c background to analyze airway Th2 inflammation in these mice, as eosinophils have been shown to be necessary for recruitment of effector T cells in mouse models (17).

One way to overcome limitations posed by the relatively inert nature of mouse eosinophils and the lack of extensive degranulation in the OVA provocation model is the use of *IL-5/hE2* double transgenic mice. These transgenic mice overexpress the eosinophil maturation and survival cytokine IL-5 from their T cells (18), along with the eosinophil-specific chemokine, eotaxin-2 (hE2) (19), from the lung epithelium, to promote eosinophil recruitment to the lungs without allergen provocation (20). Clinical and histological pathology of severe allergic asthma in humans is closely mimicked in the *IL-5/hE2* double transgenic mouse model, as they

spontaneously present symptoms such as AHR, mucus hyper-secretion, and airway remodelling in the absence of any allergen sensitization and challenge (20). Eosinophils isolated from the BAL of *IL-5/hE2* mice have been observed to undergo activation and extensive piecemeal degranulation which occurs in parallel with detection of EPX in BAL (20). Thus, *IL-5/hE2* mice serve as an excellent model of severe asthma.

In future studies, *eCre-V7* mice may be bred with *IL-5/hE2* mice to generate a colony of transgenic mice that are *IL-5/hE2/VAMP-7-*. The difference in pulmonary function and histopathology of this line of mice and *IL-5/hE2/VAMP-7+* mice will allow the evaluation of the VAMP-7-mediated pathophysiology in this model of severe asthma. The OVA provocation model of genetically altered mice used in this project elicits a general airway inflammation in response to a single allergen. In contrast, the *IL-5/hE2/VAMP-7-* model may provide more information on the development of pathology specifically caused by eosinophil degranulation using an eosinophil-mediated model of severe airway inflammation.

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Chapter Six: Discussion and Future Directions

6.1 Summary of the project

The observations of this study are in agreement with previous *in vitro* models and demonstrated a critical role of SNARE isoform (VAMP-7) in releasing crystalloid granule proteins from eosinophils. Using *eCre-LoxP* binary system, we achieved high efficiency in *Cre*-mediated activity that resulted in VAMP-7 gene deletion in eosinophils. VAMP-7-deficient eosinophils demonstrated reduced granule protein release *in vitro.* This reduction in secretion is reflected in three different types of eosinophil granule proteins (i.e., EPX, MBP, and Ears). The significant reduction in secreted EPX from VAMP-7-deficient eosinophils was confirmed following adoptive airway transfer into airways of IL-5/hE2/EPX $^{-/-}$ mice. Using the mouse model of allergic airway inflammation, I have demonstrated the contribution of VAMP-7-mediated degranulation from mouse eosinophils *in vivo.* Preliminary findings suggest that VAMP-7 to degranulation from airway eosinophils and allergic airway responses are important in establishing pathology and airway hyperresponsiveness.

6.2 SNARE mediated exocytosis

More than one eosinophil effector function may be associated to the release of granule contents. Exocytosis is a central process for all receptor-mediated granule release from eosinophils, and thus contributes significantly to eosinophil activity in maintaining homeostasis and mediating disease pathology. VAMP-mediated events in degranulation have been observed in many immune cells (1-8). However, the role of VAMP-7 in receptor-mediated secretion from eosinophils isolated from mice congenitally devoid of the gene expression with cell specificity is only achieved in this model.

In this study, VAMP-7 deletion in eosinophils is not associated with changes in cytokine release *in vitro,* which confirms a pattern of differential secretion in releasing cytokines and granular proteins from eosinophils upon stimulation. Differential secretion has been observed in cells other than eosinophils such as activated T helper cells (13), suggesting a commonality in exocytotic mechanisms employed by immune cells. Unlike piecemeal degranulation in eosinophils, T cell mediators are not stored, but newly synthesized and differentially sorted. Specific complements of fusion proteins are differentially expressed in vesicles containing IL-2^{+}/IFNγ^{+} or TNF (13). Selective packaging together with differential expression of SNARE isomers on granules can explain, in part, the differential release of receptor stimulated immune cells.

Exocytosis is a synergistic process that involves numerous upstream proteins in addition to the SNARE core complex. Rab 27a protein, a member of the Rab family of small guanosine triphosphatases, has elevated expression in asthma patients compared to healthy individuals (14) and is critical in releasing EPX from mouse eosinophils. Rab27a has been associated with the exocytosis of secretory lysosomes in many cell types (15-18). The reduction in EPX released from Rab27a knockout mice is also associated with reduction in airway hyperresponsiveness to cholinergic agonist challenge (unpublished data). The magnitude of changes in AHR observed in the eosinophil-specific VAMP-7 knockout mouse model is parallel to the trend observed in Rab27a knockout mouse model of allergic airway inflammation. Even though the Rab27a knockout model is not specific to eosinophils, *in vivo* evidence generated using IL-5/hE2/Rab27a$^{-/-}$ and ubiquitous Rab27a$^{-/-}$ knockout mice suggests the importance of the protein in receptor-mediated exocytosis from eosinophils and in mediating allergic inflammatory response in mouse airways.

In this study, one of the key molecular elements involved in eosinophil exocytosis were dissected. The involvement of VAMP-7 in eosinophil-specific degranulation has provided a major piece of puzzle regarding the role of the cells in development of pathology in allergic airway inflammation. However, since VAMP-7 gene deletion did not fully inhibit exocytosis, further refinement of the model is needed to reveal the alternative route for receptor-mediated exocytosis.

Specifically, intact extracellular granules have been observed in tissue biopsies from many diseases (19-24). Eosinophil cytoplasmic granules, predominant in number and striking in appearance, have shown to function as secretion-competent entities that are responsive to receptor stimulation in absence of intact cells (25, 26). The expression of extracellular ligand-binding receptor domains on granule membranes also suggests a role of cytokines in receptor-mediated secretion. The exact mechanism of secretion from cell-free granules from eosinophils remains unclear. Are SNAREs involved in granule release of mediators in absence of interaction with the plasma membrane? If not, what alternative means is critical in extracellular granule release of eosinophil-associated proteins and cytokines in the event of cytolysis?

6.3 Eosinophils and allergic airway inflammation

From a clinical perspective, eosinophils are present in a sub-group of asthmatic patients. Anti-eosinophil treatment (i.e., mepolizumab) in asthmatic patients with hyper-eosinophilia is associated with improvement in clinical variables such as decreased exacerbation rate. Along with evidence provided by animal models of allergic airway inflammation, it has been suggested that eosinophils play an active role in mediating airway inflammation (27-30).

Airway remodelling is intimately associated with the chronicity and increased severity of asthma. Permanent tissue structure changes such as cellular trans-differentiation, extracellular matrix breakdown, excessive proliferation and fibrosis are considered as signs of remodelling as

a result of inflammation (31). The remodelling process is orchestrated by many immune and non-immune cells including eosinophils (32, 33). Anti-eosinophil treatment has been associated with evidence of attenuated tissue structural changes (34). The aforementioned tissue transformations are initiated by the release of enzymes, growth factors, and cytokines. Eosinophils manufacture and secrete cytokines (e.g., TGF-β, bFGF, and MMPs) (35-37) and cationic proteins involved in mesenchymal transition and airway epithelial changes, which is considered to be a major driving force for remodelling (38, 39).

The histopathology evidence collected in this study has provided preliminary data suggesting SNARE-mediated eosinophil degranulation appears to influence development of airway immune response to allergen. The disease model chosen in this study elicited eosinophil infiltration to the inflammatory foci with little or no evidence of eosinophil degranulation in the lung parenchyma as observed with immunohistochemistry staining patters. However, BAL EPX levels were reduced in *eCre-V7* mice. In order to study the effect of VAMP-mediated degranulation events in the remodelling and repair process, an eosinophil-centric model (e.g., IL-5/hE2 double transgenic model) should be considered in future studies.

6.4 Transgenic mouse models and their role in airway inflammation research

Even as the journey in establishing a causative link between eosinophils and allergic asthma is long and tedious in human studies, mouse models of the disease have provided invaluable information. Physiological and genetic similarities between mice and human have been capitalized in using mice as models of human disease. Two eosinophil-deficient transgenic mouse strains (PHIL (29) and ΔdbI-GATA (40)) have been used in modeled studies of allergic pulmonary inflammation. Changes observed in airway function and histopathology using both PHIL and ΔdbI-GATA mice suggest an active role of eosinophils in immune-regulation in addition to their destructive end-stage effector functions.

PHIL mice were generated using eosinophil peroxidase promoter to drive the expression of diphtheria toxin A chain to eliminate eosinophils during development (29). ΔdblGATA mice lack eosinophil lineage committed cells by a deletion of eosinophilopoietic transcription factor GATA -1 binding sites from its auto-regulatory region (40). PHIL and ΔdblGATA are both considered as eosinophil-deficient mouse models; however, they do differ in development of pathology. Even though the two eosinophil-deficient models are different, several experimental parameters, such as the protectiveness against AHR, airway and tissue eosinophilia, mucus production and Th2 cytokines production when exposed to OVA, have altered in the absence to eosinophils (29, 30) suggesting an active role for this enigmatic cell type.

In the initial stage of trying to understand a human disease, the focus on pathologies and causal origins are direct and simple with single gene or cell type depletion (e.g., PHIL and ΔdblGATA). Nevertheless, as our knowledge base expand with research, methods such as a cell type depletion or a single gene deletion, albeit critical, are limited in their sensitivity and specificity as disease pathologies are invariably rooted in elaborate cell-to-cell communication and complex pattern of genetic modulation. With the link established in the mouse model of allergic airway inflammation using either hyper-eosinophilic or eosinophil-less models, we have gained insight into the role eosinophils play in the establishment of pathophysiology. Using eosinophil specific gene targeting strategy (e.g., *eCre-LoxP*), a more refined approach can be taken to study eosinophil-specific influences in health and disease. Molecular mechanisms and signalling pathways that are critical in eosinophil system biology (e.g., development, priming, activation, and degranulation) can be evaluated in intact cells and genetically modified mice in hypothesis-driven research. However, the availability of the floxed animal is a major limiting factor.

In general, to generate transgenic Cre expressing mice, Cre recombinase is expressed by a transgene that randomly inserted into the mice genome after introduction of target vector into the zygotes' pronuclei. This method of generating Cre-expressing transgenic mice is associated with ectopic activities such as Cre-phenotype (41), varying magnitude of mosaicism in the founders (42), Cre-toxicity (43-45), high cost and low efficiency as the percentage of founders are low (1-4% transgenic offspring) (46), and decreased specificity (47-50).Eosinophil-specific (*eCre*) mice were generated by the next generation of Cre-expressing system using homologous recombination in ES cells, which is designed to erase major evidence of genetic manipulation in the construction of transgenic animals. Instead of random integration into the genome, the transgene vector replaces a portion of the wild-type allele in the knock-in Cre, which helps to reduce the incidence of unexpected and ectopic activities of Cre recombinase since they are being translated *in situ*.

Using mouse models, we have gained insights in some of the cardinal features of Th2 allergic pulmonary inflammation in mice. IL-4, IL-13, and IL-5 production, eosinophil infiltration, and IgE can be reproduced in OVA sensitized and challenged mice. In combination with transgenic engineering, the central role of T cells, eosinophils, Th2 cytokine in airway inflammation and pathophysiology have been studied extensively in knockout or transgenic strains. Potential targets (i.e., anti-IL-4, IL-5, Il-13) (51-53) are chosen for therapeutic potential because of their critical role in the development of inflammation, and the inhibition of which have led to changes in inflammatory profile in mice.

The application of mouse models to study human diseases may be extremely informative in providing causative links between disease development and research targets. For the mouse model to reach its full potential in understanding and treating asthma, observational studies in the human population are critical as they provide correlation and context to confirm animal studies. Acute inflammatory responses to allergen, in addition to

chronic underlying pulmonary inflammation, have been thought to induce asthma symptoms (54, 55). Clinical observations of asthmatics have provided pathological and physiological measurements to establish experimental parameters for empirical research.

Mouse models may also be applied to provide information on inflammatory parameters and airway physiology in model-based research. The physiological and genetic differences between mice and humans place a limit on how far we may extrapolate the significance of the findings in mouse models of asthma, but the information we gain in the area of allergic inflammation has helped us understand the disease better, and more potential agents can be selected for therapeutic agents that promote symptom control in patients that have a suboptimal response to steroid alone.

6.5 Future Directions

VAMP-7-mediated events are not limited to granule and plasma membrane fusion. VAMP-7 is important in heterotypic fusion of late endosomes and homotypic fusion of lysosomes (9). It would be interesting to investigate if compound exocytosis is augmented in *eCre-V7* mice in response to helminth infection. VAMP- 7 is involved in the fusion of transport vesicles to target membrane in mast cells (10) and early endosomes to the lysosome in alveolar macrophages (11). VAMP-7 has been associated with calcium-regulated lysosomal exocytosis in the human parotid epithelial cell line (12). The role of VAMP-7-mediated events in exocytosis and its consequence in mouse models of disease may now be evaluated in other cell types given the availability of the cell-specific *Cre* expressing mice.

A tissue-specific *Cre-Lox P* binary system was used to target the VAMP-7 gene in this project to evaluate eosinophil-specific release of granule proteins and its implication in mouse models of allergic airway inflammation. A similar construct may be used to study the role of VAMP-2 in differential secretion of cytokines from eosinophils, and to determine its implications

in mediating disease pathology. In future, it may be possible to generate a VAMP-7 and VAMP-2 double knockout model to investigate the combined role of PMD and classical exocytosis of eosinophils in disease pathology.

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Appendix

Introduction

In order to inhibit VAMP-7 function, we first tried to overexpress VAMP-7 coiled-coil (CC) domain (Figure 7.1) (1) that compete with vesicle associated VAMP-7 for binding to t-SNAREs. Preliminary findings in murine MC9 cells suggested that the expression of VAMP-7 CC domain is associated with inhibition of calcium ionophore-induced degranulation (unpublished data generated in the Moqbel and Lacy lab).

Methods and Results

Tet-Op system

To determine the role VAMP-7 play *in vivo*, we first attempted to generate a transgenic mouse model with tetracycline dependent over-expression of VAMP-7 CC domain. In this mouse model, as indicated in Figure 7.2, the "Tet-off" system, a tetracycline-controlled transcriptional silencer (tTS), is used in combination with a "Tet-on" system (rtTA) to control gene expression (2). In the absence of tetracycline (Dox), tTA binds the responsive elements in tetracycline-resistance operon of *E. coli* transposon Tn10 (*tetO*). In the presence of tetracycline, tetracycline activates the minimum promoter from human cytomegalovirus resulting in the transcription and expression of VAMP-7 CC domain when given tetracycline in drinking water. In the presence of tetracycline/Dox, tTA dissociates from *tetO* and the transcription of the VAMP-7 gene is terminated.

Cloning

To generate a transgene construct containing *tetO* and VAMP-7 CC sequences, the VAMP-7 CC domain sequence was excised from its primary construct, which was in a pGEX vector and did not have compatible restriction enzyme digest sites for insertion into the *tetO*

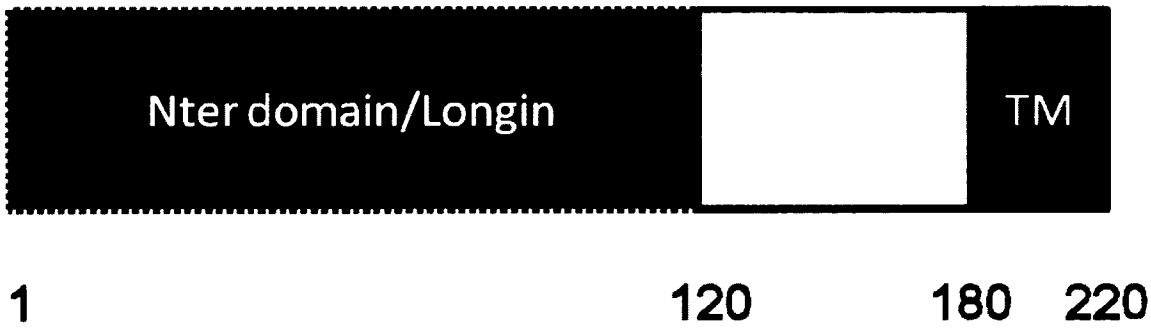

Figure 7.1. Scheme of VAMP-7 protein structure. VAMP-7 is composed of three domains: N terminal domain/Longin, which exerts intermolecular inhibition of SNARE complex formation; CC domain, which allows binding to t-SNAREs; and the transmembrane domain, which anchors the protein on to the lipid membranes.

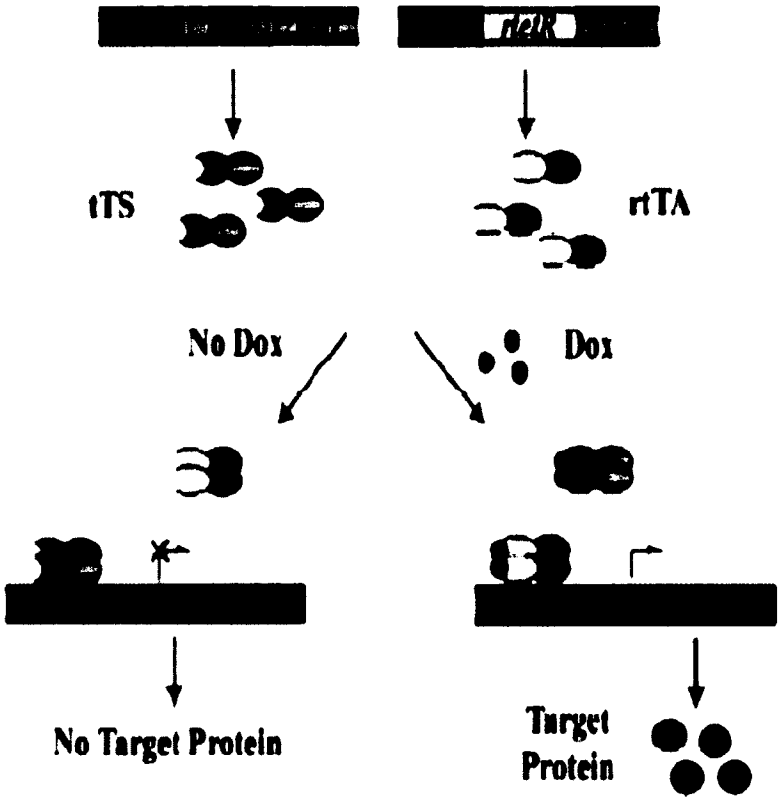

Figure 7.2. Tetracycline-controlled transcriptional silencer and rtTA system. From Zhu, Z., *et al.*, 2002 (1).

construct (Figure 7.3). The primary constructed was linearized using BamH1 and Not1 restriction digestion (Figure 7.4) and the VAMP-7 CC sequence was removed and introduced into a secondary vector (pBluescript SK+) (Figure 7.5) using heat shock in *E. coli.* Transformed *E. coli.* were grown on ampicillin-containing agar for 12 h at 37°C. Ampicillin-resistant colonies were picked from the agar and grown overnight in ampicillin-supplemented medium. DNA was extracted for *E. coli* for analytical digest (Figure 7.6). The secondary construct was subsequently linearized with Sal1, and the VAMP-7 CC domain sequence was removed from the secondary construct and inserted into the final construct with the *tetO* sequence (Figure 7.7 and Figure 7.8). The final construct was linearized with BssHII and micro-injected into the pro-nucleus of the zygote.

Micro-injection of embryos

Female mice were treated by pregnant serum gonadotrophin and human chorionic gonadotrophin injection prior to copulation with male breeder mice in preparation for embryo isolation.

Embryos were collected from the oviduct of these female mice and plated to 2-pronuclei state. The linearized *tetO*-VAMP-7 CC sequence, rTS (the silencer) and rtTA (activator) sequences were all introduced into the pro-nucleus of the zygote using a micro-injection technique.

Injected embryos were planted into the ampula of age-matched pseudo-pregnant mice via surgery. Recipient mice were allowed to recover. The first generation of the transgenic mice was obtained 19 to 21 days after implantation surgery. A total of 29 mice were born in the first generation in this project. Embryo injection was done by Mr. Alfred Doyle, Lee Labs, Mayo Clinic, Arizona.

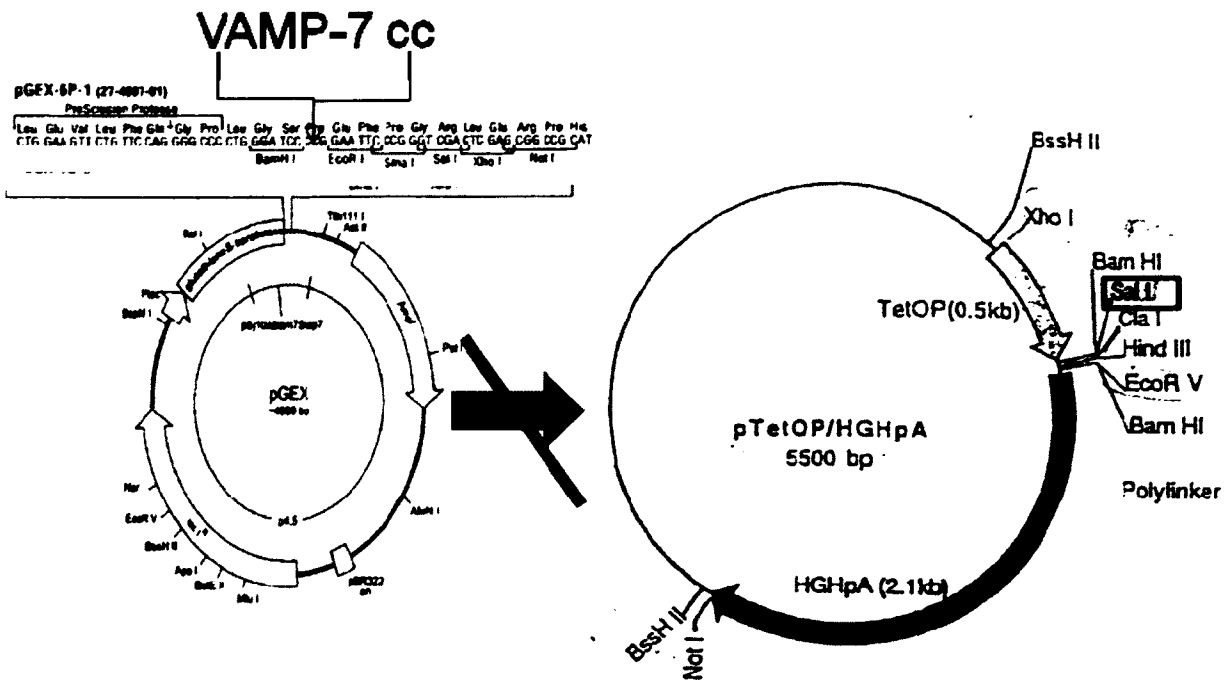

Figure 7.3. Primary VAMP-7 CC domain construct. VAMP-7 CC in the pGEX backbone do not have compatible sites for restriction digest necessary for the insertion of VAMP-7CC into the pTetOP vector.

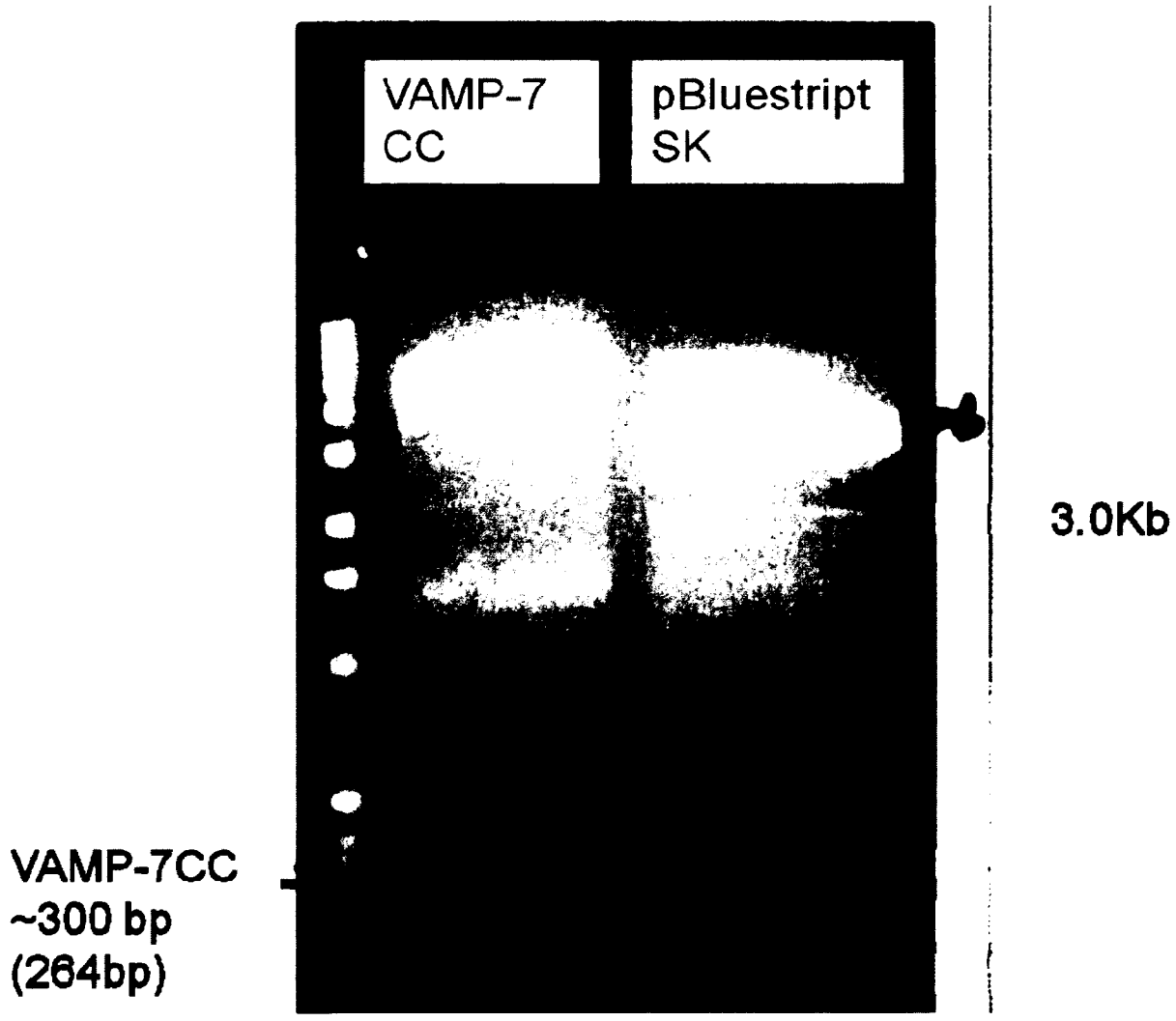

Figure 7.4. Not1 and BamH1 restriction digests to remove the VAMP-7CC sequence out of the primary construct.

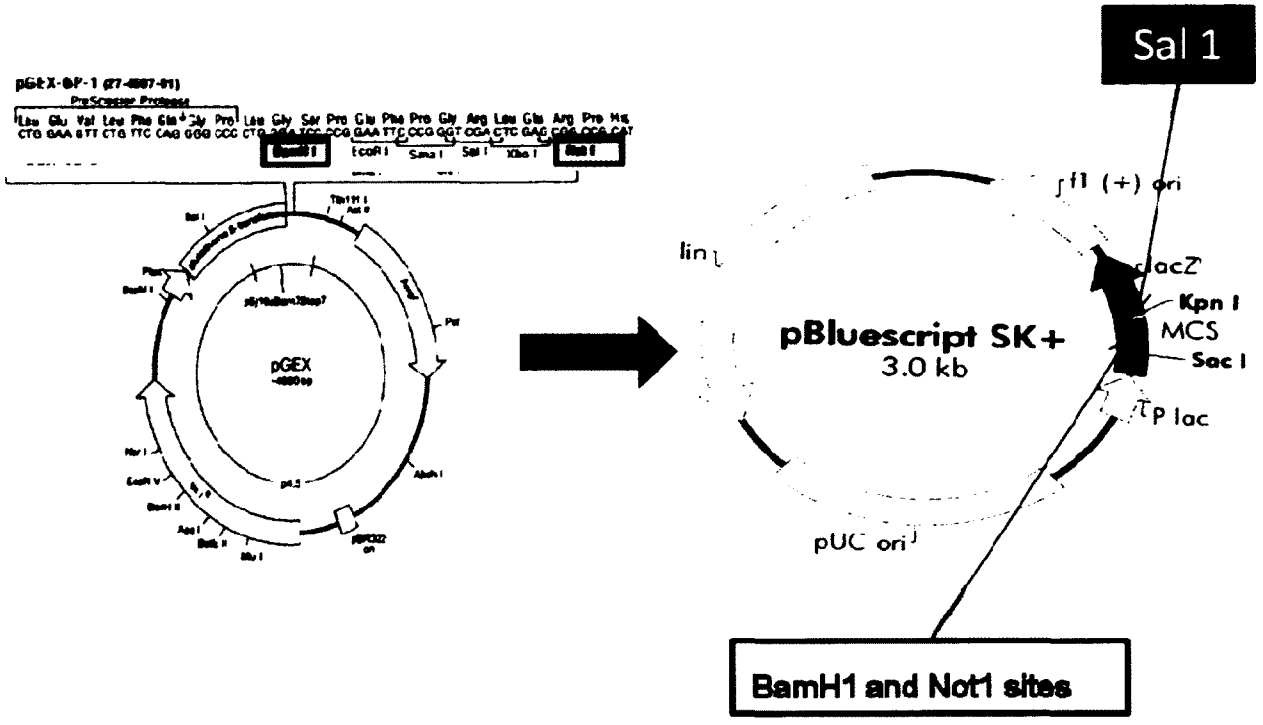

Figure 7.5. VAMP-7 CC removed with BamH1 and Not1 from the primary construct is inserted into the pBluscript SK+ vector.

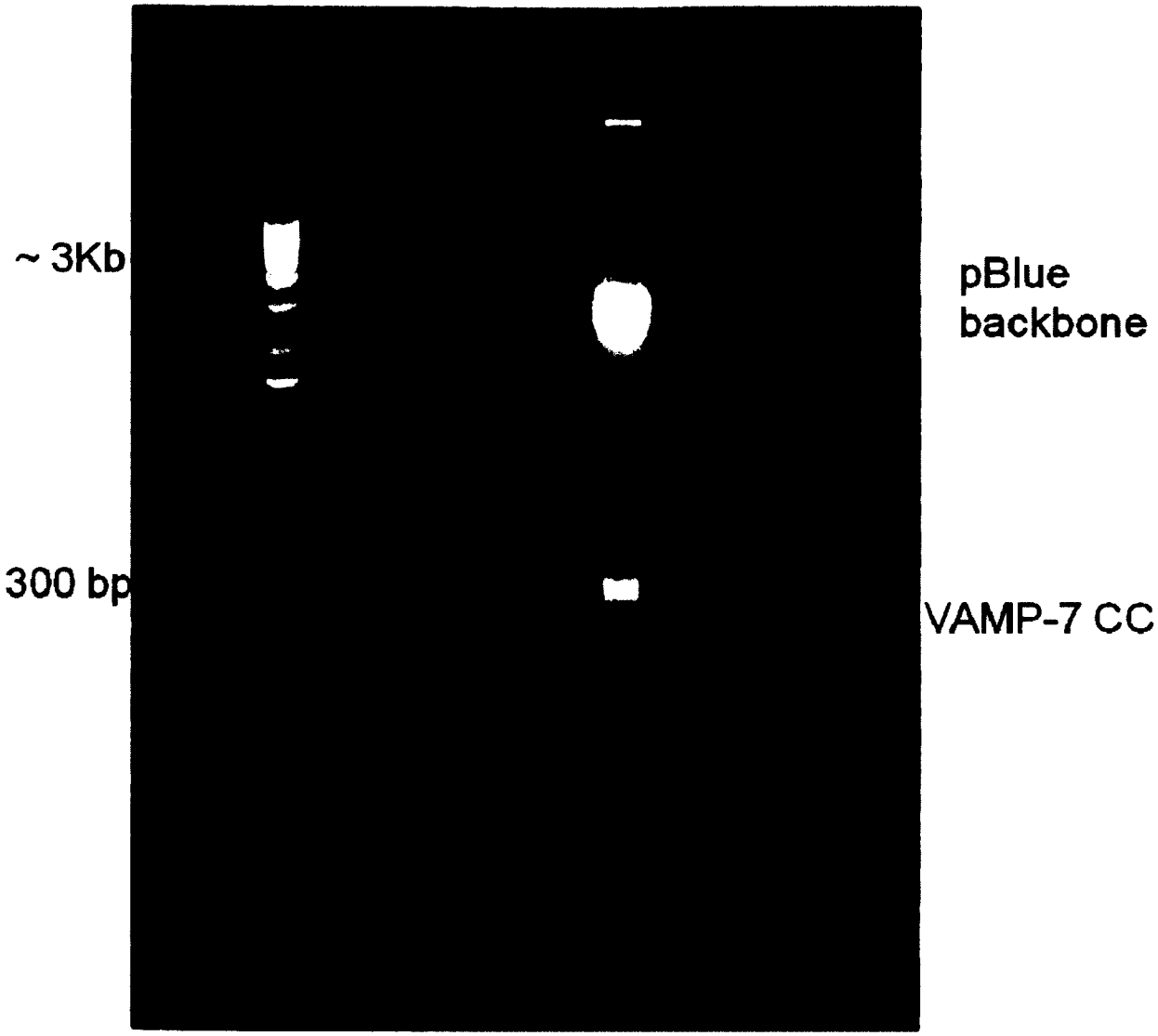

Figure 7.6. Analytical digest of VAMP-7 CC in the pBluescript SK+ backbone.

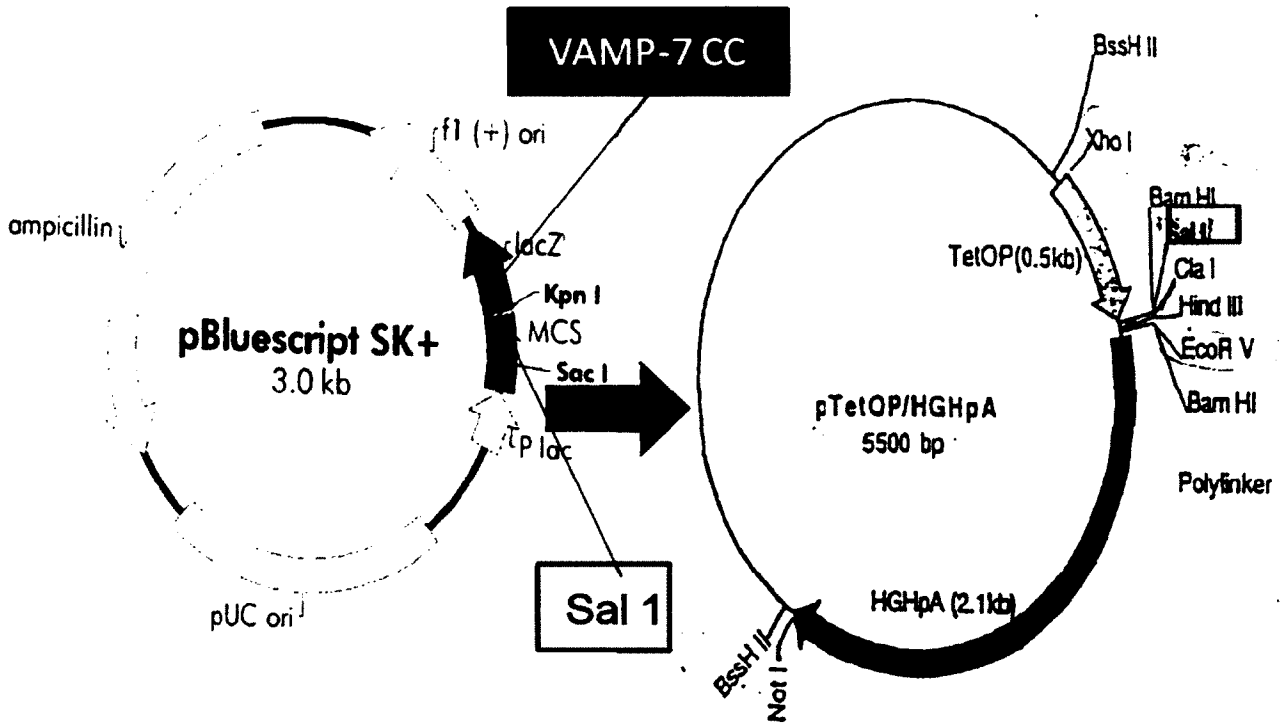

Figure 7.7. Restriction digestion and ligation to generate Tet-op/VAMP-7 CC construct. VAMP-7CC sequence was removed from the secondary construct using Sal1 restriction digest, and ligated in the t*etO* construct.

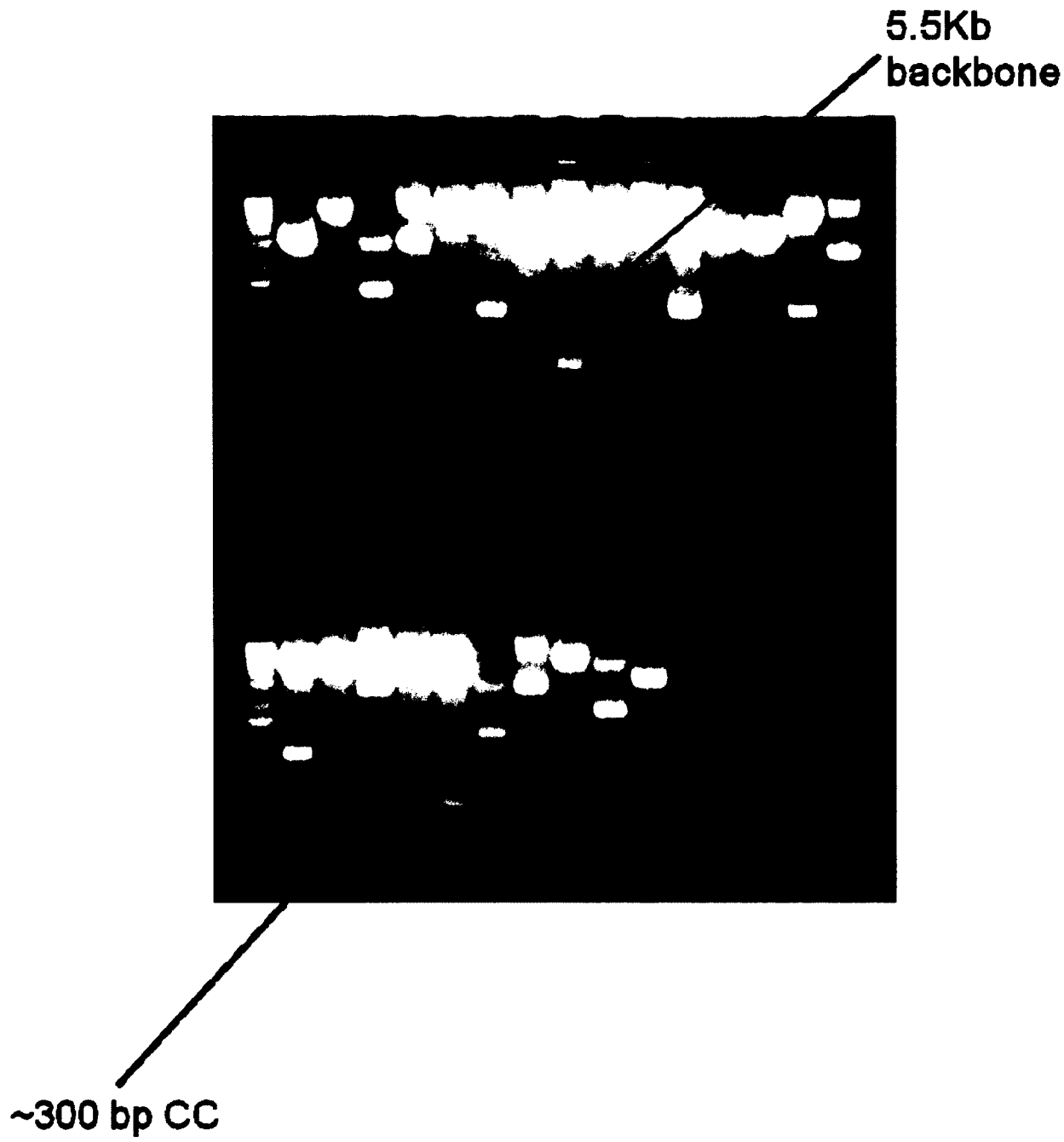

Figure 7.8. PCR identification of positive clones with VAMP-7 CC sequence Tet-op/VAMP-7 CC construct.

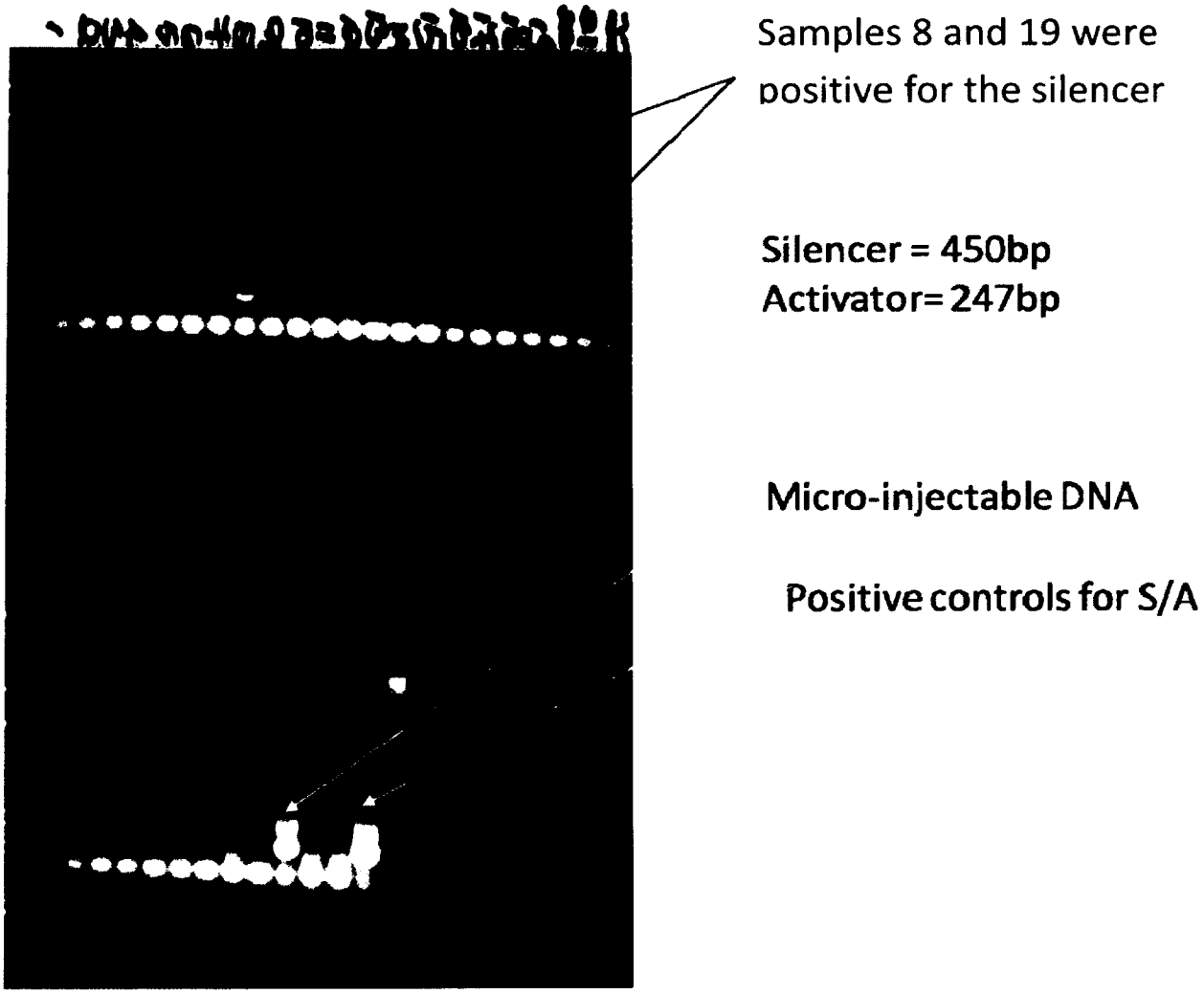

Figure 7.9. PCR identification of transgenic mice positive for the expression of the silencer and the activator transgene. Silencer Primer:(forward) gagttggcagcagtttgt(reverse) gagcacagccagatcttcaa; Activator Primer:(forward) gagcaaagtcataaacggcgc(reverse) cgcgatgtgagaggagagca.

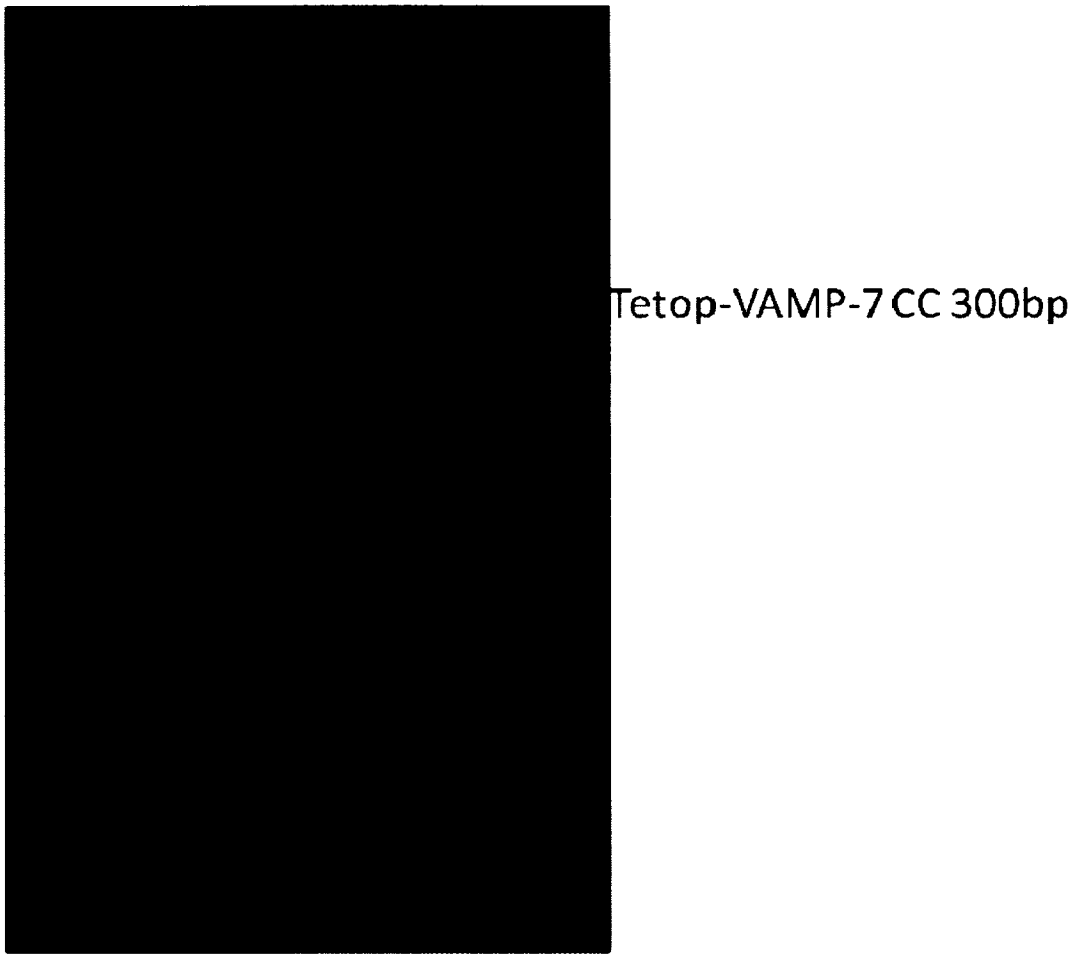

Figure 7.10. PCR detection for the VAMP-7 CC in the two mice that were positive for both the silencer and activator transgene. Tetop-VAMP-7 forward primer:agctcgtttagtgaaccgtc ; and HghRs reverse primer: agggaatggttgggaaggca.

PCR genotyping of triple transgenic mice

As indicated in Figure 7.9, 2 out of 29 mice in the first generation were positive for both rTs silencer and the rtTA activator transgene. The presence of the *tetO*-VAMP-7CC transgene was subsequently evaluated in these 2 mice. As indicated in Figure 1.10, neither of the 2 mice (samples 8 and 19) expressed the tetO-VAMP-7CC transgene.

References

1. Galli, T, A Zahraoui, VV Vaidyanathan, G Raposo, JM Tian, M Karin, H Niemann, and D Louvard. 1998. A novel tetanus neurotoxin-insensitive vesicle-associated membrane protein in SNARE complexes of the apical plasma membrane of epithelial cells. *Mol Biol Cell* 9: 1437-1448.

2. Zhu, Z, T Zheng, CG Lee, RJ Homer, and JA Elias. 2002. Tetracycline-controlled transcriptional regulation systems: advances and application in transgenic animal modeling. *Semin Cell Dev Biol* 13: 121-128.