The Role of Proteinase Activated Receptor -2 (PAR-2) Activation in the Airways

by

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Abstract

Allergens possessing serine proteinase activity like house dust mite (HDM) and cockroach can activate proteinase-activated receptor-2 (PAR-2), a pro-inflammatory G-coupled receptor.

Par-2 $^{-/-}$ mice develop attenuated airway inflammation compared to WT mice. PAR-2 blockage in the airways abrogated the ability of $CD4^+$ T cells to transfer the disease to naïve mice, decreased lung Th2 cytokines, and decreased *ex vivo* splenocyte proliferation in response to HDM. As a result, activation of PAR-2 is critical for allergic sensitization and allergic airway inflammation. Inhibiting PAR-2 activation in the airways is interesting, although cells mediating PAR-2 activation in the airways and the role of PAR-2 activation on the formation of $CD4^+$ T memory cells are not fully understood.

Using mouse models, we demonstrated that PAR-2 activation in the airways induce the release of inflammatory mediators essential for Th2 skewing of the immune response. In addition, the loss of PAR-2 expression on airway structural cells, possibly airway epithelium attenuates allergic airway inflammation. Moreover, we have evidence indicating that the loss of PAR-2 expression abrogated the ability of $CD4^+$ T cells to transfer the disease to naïve mice. Furthermore, we propose that the loss of PAR-2 expression attenuates the formation of $CD4^+$ T memory cells in the lung and spleens of mice treated with house dust mite (HDM).

We hypothesize that the loss of PAR-2 expression on structural cells, such as airway epithelium, suppresses the production of inflammatory mediators necessary for Th2 polarization of the immune response, resulting in a decrease in $CD4^+$ T memory cells in the lung and spleen.

Our study is highlighting the critical role of PAR-2 activation on airway structural cells and its role on memory cells formation. PAR-2 antagonists, or the neutralisation of mediators released

after PAR-2 activation, could be a very appealing therapeutic approach for preventing asthmatic airway inflammation. The capacity to apply targeted therapeutic techniques will be enhanced if the involvement of airway epithelial cells can be determined.

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Preface

This is an original work by Yahya Fiteih. I was responsible for performing all the experiments, data collection and analysis. All animal procedures were approved by the University of Alberta Animal Care and Use Committee under animal use protocol #353(Vliagoftis; approved in 2003 and renewed in 2020).

Dedication

I dedicate my MSc to my beloved wife, Enji Elmenofy and my lovely kids Ali and Hoda.

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I am sincerely thankful to my MSc supervisor Dr. Harissios Vliagoftis for his support and encouragement.

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▪	AHR	Airway hyperresponsiveness
▪	BM	Bone marrow
▪	BMDCs	Bone marrow derived dendritic cells
▪	CCL	CC chemokine ligand
▪	CD	Cluster of differentiation
▪	DCs	Dendritic cells
▪	ERK1/2	Extracellular signal-regulated kinase ½
▪	GATA3	GATA binding protein 3
▪	G-CSF	Granulocyte colony-stimulating factor
▪	GDP	Guanosine diphosphate
▪	GM-CSF	Granulocyte-macrophage colony-stimulating factor
▪	GPCRs	G-protein-coupled receptors
▪	GTP	Guanosine triphosphate
▪	HAT	Human airway trypsin like protease
▪	HDM	House dust mite
▪	I.N	Intranasal
▪	I.P	Intraperitoneal
▪	I.T	Intratracheal
▪	IFN	Interferon
▪	IL-	Interleukin
▪	IP-10	Interferon gamma-induced protein 10
▪	KC	Keratinocyte Chemoattractant
▪	KO	Knock out
▪	LIF	Leukemia inhibitory factor
▪	LIX	LPS-induced CXC chemokine

- LN Lymph node
- LPS Lipopolysaccharide
- LPS Lipopolysaccharide
- MCP-1 Monocyte chemoattractant protein 1
- M-CSF Macrophage colony-stimulating factor
- MIG Monokine induced by gamma interferon
- MIP Macrophage Inflammatory Proteins
- MMP-1 Matrix metalloproteinase -1
- Ova Ovalbumin
- PAMPs Molecular patterns associated with contaminating microbial pathogens
- PAR-1 - Proteinase-activated receptor-1
- PAR-2 Proteinase-activated receptor-2
- PAR-2 AP PAR-2 activating peptide
- PAR-2 CP PAR-2 control peptide
- PARs Proteinase-activated receptors
- PI3K Phosphoinositide 3-kinase
- PRR Pattern recognition receptors
- RANTES Regulated on Activation, Normal T Expressed and Secreted
- STAT6 Signal transducer and activator of transcription 6
- Tcm Central memory T cells
- Tem Effector memory T cells
- Th2 Type 2 helper T
- TNF Tumor necrosis factor
- Trm Tissue resident memory T cells
- TSLP Thymic stromal lymphopoietin
- VEGF Vascular endothelial growth factor

Chapter 1: Introduction

The prevalence of allergic diseases especially asthma is increasing and the mechanism(s) behind asthma is not fully clarified. Many common allergens, such as HDM, cockroach and fungal allergens, are proteinases. Their proteinase activity is important for the development of allergic sensitization. It has been demonstrated that these allergens can possibly induce their detrimental effect through activating proteinase activated receptor -2 (PAR-2) (1) (2) (3) (4). Our lab is specializing in studying the role of PAR-2 in allergic sensitization and allergic airway inflammation development.

1.1 Serine proteinases

In mammals, serine proteinases are the most frequent form of proteinase. They acquire their name from the presence of a catalytically essential serine residue in their active sites. Serine proteinases are important participants in extracellular proteolysis, and they are most active when the pH is neutral. This class of proteinases includes digestive enzymes (trypsin and chymotrypsin), leukocyte proteinases, and other proteins involved in fibrinolysis, coagulation, and complement activation (5).

Serine proteinases are multiple groups of enzymes that are characterized by the presence of three critical amino acids: histidine, aspartic acid, and serine in the catalytic site. Serine proteinases are involved in many biological processes. The family name descends from the nucleophilic Ser in the enzyme active site, which attacks the carbonyl group of the substrate peptide bond to form an acyl-enzyme intermediate(5) (6). Nucleophilicity of the catalytic Ser is mainly dependent on the

catalytic activity of Asp, His, and Ser residues, which is known as the charge relay system(7). The polypeptide substrate binds to the outside of the serine proteinase protein where the peptide bond is embedded into the dynamic site of the catalyst, with the carbonyl carbon close to the nucleophilic serine. The serine - OH strike the carbonyl carbon, and the nitrogen of the histidine acknowledges the hydrogen from the - OH of the serine and a couple of electrons from double bond of the carbonyl oxygen moves to the oxygen. The covalent electrons making this bond move to strike the hydrogen of the histidine, breaking the association. The electrons that recently moved from the carbonyl oxygen double bond move over from the negative oxygen to reproduce the bond, producing an acyl-enzyme intermediate (5) (6).

1.2 Serine proteinases and asthma

Proteinases such as neutrophil elastase, cathepsin G, mast cell tryptase, and mast cell chymases are secreted by inflammatory cells in the airways. An increasing body of evidence suggests that proteinase and anti-proteinase abnormalities may play a role in asthma etiology.

Many serine proteinases are involved in asthma such as neutrophil elastase (involved in asthma exacerbation episodes)(8)(9), mast cell tryptase (increased in the airways of asthmatic patients) (10)and human airway trypsin -like proteinase (HAT) (associated with chronic bronchitis and asthma) (11) and they can activate PAR-2 (3)(12).

House dust mite (HDM) (13) and cockroach (14) are the most common allergens related to asthma in vast parts of the world. However, many allergens such as fungus, HDM and cockroach have serine proteinase activity which has been demonstrated to play a role in allergic sensitization and asthma development (15)(12).

Such allergens lose their potency when their proteinase activity is neutralized (16). Theories have been published regarding the mechanism behind proteinases and PAR-2 and allergic airway inflammation development, including activation of innate immunity and breaking epithelium integrity (17)(18)(3) (19)

1.3 Proteinase activated receptors

G protein-coupled receptors (GPCRs) are the biggest class of signaling receptors in the human genome, regulating a wide range of physiological responses, and being targeted by several medications (20) (21).

The family of proteinase-activated receptors (PARs) is comprised of four members: PAR-1, PAR-2, PAR-3, and PAR-4 (22) (23). PARs are G protein-coupled receptors (GPCRs) that are activated by proteolysis and are expressed mainly in immune, vascular, and epithelial cells, astrocytes, and neurons (24) (25). PARs play a major role in thrombosis, hemostasis, inflammation, and cancer (26) (27).

PARs are activated by an irreversible proteolytic mechanism. Proteinases bind and cleave the extracellular N terminal domain at certain sites to reveal a new N-terminus which act as a tethered ligand that binds to the receptor and stimulate intracellular signaling(21) (28) (29). Thrombin, matrix metalloproteinases, neutrophil elastase, and activated protein C can cleave PAR-1 (30) .Trypsin, neutrophil elastase, neutrophil proteinase 3 (PR3), and cathepsin G can all cleave PAR-2 (30). In comparison to other PARs, there are fewer proteinases that can cleave PAR-3. Apart from thrombin, anticoagulant activated protein C (APC) is the only proteinase known to have proteolytic activity against PAR-3 (30) . Unlike other PARs that are preferentially cleaved by trypsin or thrombin, PAR-4 has a similar susceptibility to both enzymes (30).

Synthetic peptides mimicking the first 6 amino acids can activate PARs without the need for the receptor to be cleaved (27) (21) (31) (32), except for PAR-3 which does not respond to synthetic peptides. PAR-3 appears to be a co-factor for the activation of other PARs, such as PAR-4 and PAR-1, rather than signaling on its own (33).

PAR-1 and PAR-2 genes are localized to chromosome 5q13, separated only by 90 kb of DNA (34) (35) (36). The PAR-1 gene is flanked upstream by the PAR-3 gene and downstream by the PAR-2 gene (36) (34). PAR-4 gene is localized to chromosome 19p12; however, it shares similar structure with the other PAR genes(37) (34).

Human PAR-1 is a 425 amino acids protein, whereas PAR-2 is a protein of 397 amino acid residues that is 35% similar to the sequence of human PAR-1(28) (35) (36). Human PAR-3 is a protein of 374 amino acid residues that is 27% and 28% homologous with the sequences of human PAR-1 and PAR-2, respectively(36) (38). PAR-4 consists of 385 amino acid residues with about 33% overall identity with the sequences of PAR-1, PAR-2 and PAR-3 (37) (36) .

1.4 PARs signaling and function

Once PARs are activated, conformational changes are induced within the receptor. Such changes expose the cytoplasmic component essential for its interaction with alpha subunit of heterotrimeric G proteins (39) (40). Activated PARs act like guanine nucleotide exchange factors and facilitate the exchange of GDP for GTP. This exchange enables the ‘release’ of the Gα subunit from its tight binding to the Gβγ dimer subunit with subsequent induction of various signaling responses(39) (40). Studies showed that activated PAR-1 and PAR-2 couples to various heterotrimeric G proteins subtypes including Gi, Gq and G12/13, while PAR-4 couples to Gq and G12/13 (24) (25) . Studies suggested that PAR-3 cannot signal autonomously (33) however, other

studies demonstrated that thrombin activated PAR3, induce Rho- and Ca2+-dependent release of ATP from A549, lung epithelial cells not expressing PAR-1 or PAR-4 (41).

In addition, activated PARs interact with other adaptor proteins that induce signal transduction independent to G protein signaling. The best studied G-protein independent effectors for GPCRs are the adaptor proteins β –arrestins(42) (43) (44). Studies showed that, β-arrestins control the degree and the duration of GPCR-mediated G protein signaling as well as signaling to non-G protein effectors by acting like a scaffold to enhance different signaling complexes with specific GPCRs (44) (45). C-Src will be recruited to activated GPCRs by β- arrestins to enhance the activation of ERK1, 2 (21).

Studies suggested that different agonists, proteinases, and synthetic peptide ligands induce different signaling responses through the activation of the same PAR. The observation that different ligands can induce distinct signaling responses when acting at the same receptor is best characterized for GPCRs, a process termed 'functional selectivity' or 'biased agonism'(46) (47). So, in our lab we used different allergens (HDM and cockroach) and PAR-2 synthetic activating peptide (PAR-2 AP). This helped us to explore PAR-2 responses with different stimulants.

Classically, activated GPCRs are desensitized and uncoupled from G- protein signalling by phosphorylation and β- arrestins coupling. Phosphorylation of GPCR occurs mainly on serine and threonine residues in the cytoplasmic tail and the third intracellular loop and rarely on the tyrosine residues(21). G protein coupled receptor kinases induce phosphorylation of activated GPCRs, increasing the receptor attraction towards β- arrestins, resulting in inhibition of receptor-G protein coupling and signaling induction (48) (49). The 2nd signaling kinases include protein kinase A and protein kinase C, where phosphorylation and desensitization occur regardless to the activation status of the receptor. This process does not include β arrestin recruitment to the

receptor(50). Further studying PAR family showed that proteolytic cleavage leads to activation of the same receptor only, however, evidence showed that it can indirectly affect other PARs (crosstalk between different PARs). It has been demonstrated that PAR-3 localizes thrombin to PAR-4 (receptor with low affinity for thrombin), facilitating and enhancing the activation of PAR-4 to initiate platelet activation(33). Moreover, PAR-3 dimerizes with PAR-1 and subsequently augments thrombin signaling in endothelial cells, suggesting that PAR-3 acts like a modulator for PAR-1 signaling(33) (51). In addition, the tethered ligand domain of PAR-1 can interacts with un-cleaved PAR-2 to induce signal transduction(52). Moreover, it has been shown that during the progression of sepsis, activated PAR-1 can control the inflammatory process by switching the damaging signaling to a protective signaling via PAR-2 transactivation(53).

Our lab is dedicated to studying PAR-2 and its role in allergic airway inflammation so in this study, we will focus on PAR-2 as a member of GPCRs.

PAR-2 plays a major role in many physiological processes associated with respiratory and gastrointestinal functions, tissue metabolism, immunity, and neuronal signaling(26). The N-terminus of PAR-2 can be cleaved by many proteinases such as trypsin, elastase, human airway trypsin like proteinase (HAT), matrix metalloproteinase -1 (MMP-1) and factor Xa/VIIa. PAR-2 is cleaved at the canonical site ($R^{36}S^{37}$) by serine proteinase mentioned above (54) (55), (54) (56) (57) (58).

The activation of G protein after PAR-2 cleavage includes PLC-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) and activation of the Ca2+/inositol 1,4,5-trisphosphate (IP3)/PKC signaling pathway (35). Downstream of this pathway, phosphorylation of IKKα and IKKβ induce activation and nuclear translocation of NFκB (59) (60) (61) (62). Moreover, activation of PAR-2 also induces the formation of arachidonic acid and the release of

its metabolites including prostaglandin E2 (PGE2) via a signaling pathway including phospholipase A2 and cyclooxygenases 1 and 2 (63) (64).

The increase in intracellular Ca2+ concentration induces Ca2+-dependent Ca2+/calmodulin-dependent protein kinase beta (CamKKβ) that activates 5′AMP-activated protein kinase (AMPK), a critical regulator of metabolism. However, this pathway is inhibited by high levels of β-arrestin 2, suggesting that G protein-dependent signaling is suppressed by recruitment of β-arrestin to PAR-2 (65). Also, PAR-2 G protein signaling activates PI3 kinase by Cdc42-dependent activation of p110 involving phosphorylation of p85 by PYK2(66) (67). However, the catalytic activity of the p110 subunit of PI3 kinase is down regulated by direct interactions with β-arrestin.

Essentially, association of β-arrestin with PAR-2 activates ERK1, 2 signaling. This is crucial for the regulation of cell migration, and it impacts G protein-coupled receptor signaling pathways negatively (42) (65) (68). PAR-2 is the only member of proteinase activated receptor family that bind and co-internalize with β- arrestins. This enhances ERK 1, 2 signaling from endocytic vesicles to induce changes in actin cytoskeleton and cell migration (42) (69) (70). PAR-2 signaling is mainly terminated by lysosomal degradation of the receptor. Lysosomal recruitment of the receptor involves ubiquitination of the carboxyl-terminus of PAR-2 by E3 ligase c-abl, a src-dependent process that occurs after receptor activation (71) .

It became possible to test the impact of selectively activating PARs 1, 2, and 3 in a variety of situations. By the development of PAR-selective synthetic activating peptides, PARs can be activated by synthetic soluble ligands corresponding to cleaved N-terminal sequences, or it can be transactivated by cleavage-generated N-terminal portions of homo- or heterodimer partners, independent of proteolytic cleavage. Synthetic PAR-2 activating peptides (PAR-2 AP) like the

canonical TL sequences (SLIGRL, rodent; SLIGKV, human), and ending with a C-terminal amide have been beneficial in studying PAR-2 function. The C-terminal amide is essential for agonist strength compared to the N-terminal amine. The rodent hexapeptide (SLIGRL-NH2) was stronger than the human analog (SLIGKV-NH2) in vitro studies (26) (72). Using PAR-2 synthetic peptides in studies is beneficial especially by evading the PAR-2-independent effects of proteolytic enzymes.

PAR-2 plays significant role in regulating inflammatory(73),(74)(75)(76), gastrointestinal, (77) (78), cardiovascular(79), respiratory (80) (81) (82), and metabolic diseases(83,84) and cancers(85,86). However, in specific settings and disease states, PAR-2 activation applies defensive and protective impacts.

Activation of PAR-2 has appeared to induce histamine-induced bronchoconstriction(87), ischemia/reperfusion injury(88), colitis(89), and showed to be significant in recovery from injury(90) (91). Moreover, evidence suggests that PAR-2 plays a role in protecting against airway inflammation (92) and bronchoconstriction (93). However, its ability to induce smooth muscle contraction (94) and the fact that PAR-2 is up-regulated in the airways of asthmatic individuals (95) point to a different role. Therefore, PAR-2 activation may be beneficial or harmful depending on the type of tissue, PAR-2 activators, disease status and the presence of other environmental provocation.

1.5 Asthma

Asthma is a chronic inflammatory illness of the airways that affects people of all ages. Associated with recurring episodes of wheezing, dyspnea, chest tightness, and/or coughing that can vary in duration and intensity. The recurrent symptoms are usually accompanied by widespread but variable airway obstruction. This is usually reversible either spontaneously or with

appropriate asthma treatment, such as a fast-acting bronchodilator (96) (97). Airways morphology in asthma is not only highlighted by intense unfavorable inflammation, yet additionally by primary air way changes including subepithelial fibrosis or smooth muscle hypertrophy as well as hyperplasia (98). Hallmark of asthma is the presence of bronchial hyperresponsiveness (BHR). This is reflected by an expansion both in sensitivity and (99)reactivity of the airway(100).

Asthma is characterized by increased amounts of Th2 cells in the airways such as interleukin (IL)-4, IL-5, IL-9, and IL-13 which enhance eosinophilic inflammation and the production of immunoglobulin E (IgE). Also, asthma is characterized by the release of inflammatory mediators like histamine and cysteinyl leukotrienes. Such mediators, produce bronchospasm (airway smooth muscle contraction), edema, and increased mucus secretion, all of which contribute to asthma symptoms (96,97,101).

Non-atopic or "intrinsic" asthma and atopic or "extrinsic" asthma were long regarded to be two distinct phenotypes of asthma. Early-onset atopic asthma is most common throughout childhood and young adulthood, following which older age groups are more likely to develop non-atopic asthma (102–104).

In this study we will focus on atopic or allergic asthma, characterized by airway inflammation because of an improper adaptive immunological response to an inhaled allergen (105).

Allergic sensitization is a result of a complicated interaction between the allergen, the host, and environmental factors. Even though we know a lot about the effector mechanisms that enhance airway hyperresponsiveness, eosinophilia, IgE, and mucus formation, the mechanisms that allow for early allergen sensitization are still poorly understood. Allergic sensitization necessitates the

activation of antigen-presenting cells, such as dendritic cells (DCs) (106) ,which induce $CD4^+$ T cells (T-helper, Th) activation. DCs also influence which subset of Th cells will dominate, which is determined by the spectrum of co-stimulatory signals coming from DCs. Importantly, the pulmonary epithelium can influence dendritic cell activity and accordingly, $CD4^+$ T cell activity. This is crucial for qualitatively influencing the sort of allergic reaction manifested in the future(105).

Airway epithelial cells is the initial line of defense against allergens. Increased permeability of the epithelium makes it more vulnerable to external triggers by facilitating the interaction between allergens and the immune system(107).

Aeroallergens are a complex mix of various components, including proteins with different structures and activities, which can interact with epithelial cells through a variety of mechanisms (108) . The airway epithelium not only acts as a passive barrier that prevents allergens from penetrating the mucosal surface, but also plays an active role in allergen recognition and initiating innate immune responses. It has been demonstrated that certain proteinase containing allergens break down the airway epithelial barrier by acting on tight junctions (109). In addition, the detection of this proteinase activity by airway epithelial cells can also induce the release of various inflammatory cytokines. For example, it has been shown that Alternaria alternata can trigger the proteinase-dependent PAR-2 mediated release of IL-6, CXCL8 and GM-CSF (109). Cockroach proteinase has also similar effect(109) (111,112).

In addition, allergens can also activate airway epithelial cells through a proteinase-independent mechanism. It has been reported that, in bronchial epithelial cell lines, HDM triggers the proteinase-independent release of CCL20 through the interaction of HDM-derived β-glucan with CLR dectin-1 (108). Also, the non-proteolytic HDM allergen Derp2 has been shown to induce

airway epithelial release of CCL20, IL-6, CXCL8, GM-CSF and MCP-1 by activating the NF-κB and MAPK pathways(109).

Our lab demonstrated that PAR-2 activation in airway epithelium promotes the release of GM-CSF and eotaxin (113), inflammatory mediators associated with allergic airway inflammation. Interestingly, Thymic stromal lymphopoietin (TSLP) [inflammatory mediator crucial for allergic sensitization] (114,115) and GM-CSF can be released from airway epithelium induced by TNF stimulation. This can be produced by PAR-2 activation in DCs and alveolar macrophage (116) (117) (118).

Dendritic cells (DCs) are the most potent antigen presenting cells (APC) linking innate and adaptive immunity (119). Mucosal DCs form a dense network associated with the airway epithelium (120) (121), and can project lengthy extensions into the airways (122). DCs are crucial airway antigen presenting cells. They can capture and present inhaled allergens to $CD4^+$ T cells when migrating to regional lymph nodes. Some of these subsets of DCs induce Th2 immunity to allergens (CD11b conventional DCs) while others such as plasmacytoid and CD103 conventional DCs, induce immune tolerance to inhaled antigens (123) (124).

DCs regulate immune function to a wide range of antigens inhaled into the lungs, including allergens and viruses. DCs deletion during the sensitization phase of the allergic response has been shown to suppress common hallmarks of asthma, such as eosinophilic influx, Th2 cytokine production, and airway hyperresponsiveness (AHR)(107) (125).

The direct contribution of PAR-2 in DC antigen processing and presentation has not been fully described however, the involvement of proteinases in DC antigen processing and presentation has been demonstrated. It has been shown that primary DCs culture with serine proteinases is

essential for antigen processing, however the direct contribution of PAR-2 was not validated (126). Another study indicated that serine proteinases are essential for DCs maturation (127), and allergens possessing serine proteinases upregulate PAR-2 expressions on myeloid DCs in the lungs (128).

To date, not much detailed information is known regarding the role of PAR-2 expression on DCs. It has been demonstrated that the lack of PAR-2 expression on sensitizing DCs markedly affects Th2 cytokine production in a murine model of asthma (128). Furthermore, it has been demonstrated that bone marrow derived dendritic cells (BMDCs) lacking PAR-2, have decreased cytokine production and costimulatory molecules upon stimulation with an allergen with serine proteinase activity (128). Furthermore, serine proteinases and/or PAR-2 AP treated DCs up-regulate the expression of costimulatory molecules, which is induced through PAR-2 activation(129). These findings highlighted the significant role for PAR-2 expression on DCs in developing Th2 immune responses(107).

Inhibiting DCs activity may be advantageous in the treatment of asthma, but it is not a safe method as it may hinder host-immune responses to infections. As a result, determining a specific role for different DC subsets in specific diseases and attempting to target only these subsets may be a preferable option. In conclusion, allergic asthma occurs as a result of a complicated interaction between allergen and susceptible host in a specific environment (107).

Inflammatory mediators contributing to allergic asthma will be discussed in the next section; however, we will focus on mediators associated with PAR-2 activation.

Alarmins, which include thymic stromal lymphopoietin (TSLP), IL-25, and IL-33, are epithelial-derived mediators. These are cytokine mediators that activate Th2 signalling pathways

in response to infection and allergen-induced inflammation (104). **TSLP** is a pluripotent cytokine that was known to enhance B and T cells survival and maturation (130), however, it has been also demonstrated that TSLP play a major role in the function of other immune cells that are related to asthma such as DCs, $CD4^+$ T cells and eosinophils (131) (132). TSLP is mainly produced by epithelial cells, but recently it has been shown to be produced from DCs and mast cells. Also, TSLP is upregulated in asthmatic patients suggesting that TSLP might be a significant disease biomarker and could be a potential target for asthma treatment (133). Allergens with proteinase activity induce the release of TSLP from airway epithelial cells by activating PAR-2 (134). TSLP induce the maturation of DCs with upregulation of costimulatory molecules specially OX-40 ligand. This ligand augments DCs interaction with naïve $CD4^+$ T cells and induce their differentiation to proinflammatory Th2 cells expressing IL-4, IL-13, and IL-5 (135) (114). Interestingly, allergic airway inflammation cannot be induced in mice lacking TSLP receptor, and TSLP neutralization inhibited chronic house dust mite- induced asthma in a murine model of allergic asthma (136) (115). Altogether, this suggests an indispensable role for TSLP in allergic sensitization.

Studies demonstrated that PAR-2 activation on airway epithelial cells induce the release of IL-6 (137) (138) .**IL-6** is a pleotropic cytokine secreted by inflammatory cells and lung epithelial cells (139)(140). Studies suggested that IL-6 plays an essential role in deciding the type of adaptive immune response. It has been demonstrated that IL-6 induce Th2 differentiation of $CD4^+$ T cells while suppressing Th1 differentiation. IL-6 enhance the differentiation of Th2 cells by activating endogenous IL-4 production by $CD4^+$ T cells (141) (142). In addition, IL-6 has a regulatory role on $CD4^+$ T cells through supporting its activation with anti-apoptotic signals (143) (142) (144) .

Tumour necrosis factor (TNF) is a pro-inflammatory cytokine that plays a role in asthma pathogenesis. Upon allergen exposure, TNF is produced by innate immune cells, such as macrophages, DCs, and lung epithelial cells (145) (146). TNF inhibits airway tolerance to inhaled allergens by up-regulation of DC maturation, and by promoting Th2 response via up-regulation of Th2-polarizing cytokine production (147) (148).

Interestingly, TNF can induce the release of TSLP form airway epithelial cells (130). This suggests that TNF induced by PAR-2 activation on antigen presenting cells, such as DCs (128)and alveolar macrophages (149) could indirectly shift the immune response towards Th2 by the production of TSLP from airway epithelium.

GM-CSF (granulocyte-macrophage colony stimulating factor) can be produced by many cells, including fibroblasts, endothelial cells, T cells, macrophages, and epithelial cells (150). GM-CSF induce DC development and maturation (151) (152), resulting in increased expression of DC costimulatory molecules and increased priming of T-lymphocyte responses. In turn this leads to skewing of lymphocytes responses toward Th2 pathways (116). Our laboratory was one of the first to show that PAR-2-mediated activation of airway epithelial cells leads to the release of GM-CSF (113).

Eotaxin is generated in the lungs of asthmatic patients. It works as a chemoattractant for eosinophils by binding to the CC chemokine receptor (CCR3) on the surface of leukocytes (153) (154). Eotaxin is produced by lung structural cells and is secreted at higher amounts after exposure to allergens. It has been demonstrated that in vitro activation of PAR-2 induces the release of eotaxin from airway epithelial cells (113).

1.6 Mouse models of asthma

Animal models can provide useful insight into the pathophysiology and therapy of asthma. The mouse is increasingly being employed in these models, owing to its ability to apply a wide range of immunological techniques in vivo, including gene deletion technology. Mice appear to be especially beneficial in several aspects, including clarifying elements that influence the response to inhaled allergens, the importance of immunoregulatory systems in preventing Th2 cell development, T cell trafficking, and the contribution of innate immunity (155). In the previous two decades, mice, particularly BALB/c mice, have been the most extensively studied species for asthma. (156) .

Murine models, like other animal species, have their own set of limitations. Asthma does not develop naturally in mice; the morphological and critical features of asthma are not completely mirrored in the presently murine models of asthma and the pattern of the inflammation is not the same as that of human asthma. In mouse models, the inflammatory changes are not only in or around the airways but also, incorporate lung parenchymal and mainly perivascular changes. Restriction of now accessible knockout models is that most of these models is comprised of deletion or blocking the gene of interest which inhibits proper assessment of the gene in a particular period of the sickness cycle. Conditional deletion will help scientists to control the time of turning the gene of interest off (99)(157,158) allowing better assessment of the role of the gene function during specific periods. Moreover, conditional deletion will likewise empower specialists to analyze the gene function explicitly during the 1ry or 2ry antigen exposures(99).

Traditionally, murine models of asthma have two phases: sensitization and challenge. Traditional methods of sensitization include intraperitoneal (I.P), epicutaneous, and subcutaneous (SC) injections. Given that allergen inhalation causes human asthma, intranasal (I.N) and

intratracheal instillation of allergens are becoming more popular in an attempt to replicate the same pattern of allergen exposure in humans. Although the SC and IP routes have been evaluated in terms of Ova sensitization, with mixed results, there has been no published data on this comparison in animal models of asthma produced by aeroallergens (156).

Scientists have created more experimental animal models to study the pathophysiology of allergic lung inflammation and to characterize the role of specific cells in asthma development. These include adoptive transfer models, Ab-mediated depletion of leukocyte subsets or mice lacking $CD4^+$ or $CD8^+$ T cells(159) (160) (161) (162). Regarding adoptive transfer models that have been utilized to study asthma, T cells(159) (163), alveolar macrophages (164) and DCs (165) adoptive transfer models are the most common models.

In a mouse model of asthma, it has been shown that the adoptive transfer of Th1 cells suppressed lung eosinophilia and airway responsiveness while increasing non-eosinophilic inflammation (166). Moreover, it has been indicated that $CD4^+$ T cells, but not $CD8^+$ T cells, are essential for allergic lung inflammation in rodents, according to adoptive transfer models (159). Adoptive transfer systems in the rat and mouse demonstrated that Ag specific $CD4^+$ T cells may transfer the ability to respond to an Ag aerosol challenge to naïve animals (3) (159). Indicating the major role of $CD4^+$ T cells in asthma.

In a mouse model of asthma, alveolar macrophages (AM) depletion following sensitization increased the number of eosinophils and lymphocytes, as well as the amounts of IL-4, IL-5, and GM-CSF in BAL fluid. Furthermore, after AM depletion, elevated numbers of eosinophils and lymphocytes, were significantly reduced by the adoptive transfer of unsensitized AMs (164). The findings, together with a prior report that unsensitized AMs can guard against the development of

airway hyperresponsiveness (167), implying that AMs may play a key role in asthma development possibly by modulating allergic airway inflammation.

In the absence of antigen challenge, adoptive transfer of dendritic cells from allergic mice generates specific immunoglobulin E antibodies in naive recipients. Indicating that DCs play a critical role in host sensitization (168) .On the other hand, it has been demonstrated that in cases of chronic inflammation, autoimmunity, and allergies, DCs could be used to suppress aberrant immune responses (169–171). Furthermore, in experimental asthma, a growing body of evidence suggests that transferring immunoregulatory DCs inhibits aberrant Th2 allergic responses and avoids, or even reverses, established allergic airway inflammation (171–174). This suggests that DCs plays a major role in asthma, and they are considered as critical asthma modulators.

Another crucial consideration in building a suitable animal model is the causal allergen. Ovalbumin (Ova) was the most common allergen used in research however, recently scientists switched to more physiological aeroallergens, such as house dust mites which are common allergens that cause human asthma. Ova is the main protein found in egg white, making up approximately 55% of the total protein and can be mass-produced in enormous quantities, making it more affordable. It has been utilized in asthma research and has been shown to induce allergic lung inflammation (156)(175). Nonetheless, I.N administration of Ova by itself doesn’t cause airway irritation in humans, and its use suitability as an asthma allergen without an adjuvant has been doubted. Mice exposed to I.N HDM develop allergen sensitization (176), whereas mice exposed to I.N Ova develop immune tolerance (177).

In animal models, HDM has been used to efficiently cause asthma. This allergen is ideal because it has intrinsic enzymatic activity, immunogenicity, and direct activation of innate immune cells via the Dectin-2 receptor, which promotes allergic inflammation (156) (178) (179). Blatella

germanica extracts are the most utilized cockroach extracts in animal models. Previous research has found that the protein Blag 2 is a powerful allergen in cockroach and that it can be identified in 60–80% of individuals allergic to domestic cockroaches (156) (180)

The nature of the acute inflammatory model can be influenced by the selection of mouse strain, allergen, and sensitization and challenge procedures (181) (182) (183). The most used mouse line for antigen challenge models is BALB/c because they produce well-developed immune responses based on T helper 2 (Th2) (181) (184). However, other strains (C57BL / 6 and A / J) have been used successfully in allergen challenge studies (181) (183). Phenotypic differences between BALB / c and C57BL/6 mice have been identified in asthma models. Airway reactivity to methacholine is higher in BALB/c mice than in C57BL/6 mice. Furthermore, as compared to C57BL/6 mice, BALB/c animals have a larger number of mast cells in lung tissue. On the other hand, C57BL/6 mice have higher eosinophil and neutrophil counts in bronchoalveolar lavage fluid (BALF), as well as peribronchial eosinophilia (185).

Following Ova sensitization and challenge, variations in inflammatory responses between C57BL/6 and BALB/c mice were studied. C57BL/6 mice had higher levels of serum immunoglobulin (Ig)E and IgG1 than BALB/c mice. C57BL/6 mice, on the other hand, had lower IgG2a levels than BALB/c mice (186). However, the numbers of eosinophil entering the lungs in C57BL/6 mice was considerably higher than in BALB/c animals. The levels of Th2 cytokines IL-4 and IL-5, were also higher in challenged C57BL/6 lung tissues than in BALB/c lung tissues (186). Interestingly, they also demonstrated that C57BL/6 mice produce Th2 responses in the lungs, whereas BALB/c mice produce T helper 1 (Th1) responses (186). C57BL/6 WT and PAR-2 KO mice have been utilized extensively in asthma research with consistent and trustworthy results (187) (188) (19). In our experiments, we used WT on the C57BL/6 background as our *Par-*

2 $^{-/-}$ mouse purchased from the UC DAVIS KOMP (Knockout mouse project) repository is also on the C57BL/6 background. According to the information presented above, C57BL/6 is a reliable strain for researching asthma, particularly studying the role of Th2 cells.

Acute sensitization regimens generally require multiple systemic administration of allergens with/without the presence of adjuvants. Adjuvants such as Aluminum hydroxide and potassium aluminum sulphate are known to increase allergen immunogenicity, and hence increasing the chances of sensitization(156). Ova and HDM alone have been used via the airways for sensitization, where success was limited with Ova inducing only immune tolerance (177).

Acute mouse models of asthma simulate many main features of clinical asthma, for example, elevated IgE levels, airway inflammation, goblet cell hyperplasia, and epithelial hypertrophy. On the other hand, the pattern and localization of pulmonary inflammation in acute models, differ from that seen in asthmatic people (189) (181).

Despite these obvious shortcomings, acute allergen exposure models have been successfully used to study asthma, especially the relationship between cells and inflammatory mediators, and how they coordinate the inflammatory process in the lungs. Also, acute mouse model of asthma helped to validate the hypothesis that asthma is a Th2-mediated disease. It also helped in investigating the role of T cells in allergies and the role of eosinophils and their participation in the occurrence of AHR (181).

The chronic allergen challenge model appears to be able to reproduce some of the characteristics of chronic asthma. It may, as well solve key problems related to asthma pathogenesis and potential new therapies. When inferring findings from animal models to human diseases, both acute and chronic allergen exposure models have certain limitations. Mouse

models must be effective and reflect clinical asthma as closely as possible. To improve its practicality, research is being carried out to improve the existing models (181) (190) (191) (192). The combination of 's improved animal model (which can better reflect asthma) and related human systems will deepen our understanding of this disease and help identify and evaluate new therapeutic targets.

1.7 PAR-2 and asthma

PAR-2 is expressed on inflammatory cells such as neutrophils, DCs, macrophages (193) (194), as well as on structural cells such as epithelial cells, endothelial cells, and bronchial smooth muscle cells (195) (94) (196). The role of PAR-2 in asthma was a matter of debate. Studies suggested that PAR-2 activation play a role in the protection against bronchoconstriction and airway inflammation (92)(93). However, the fact that PAR-2 knockout (KO) mice develop attenuated airway inflammation (12) (3)and that PAR-2 is upregulated in asthmatic patients (95) supports the theory that PAR-2 activation plays a crucial role in the development of allergic airway inflammation.

PAR-2 activation has been explored widely by PAR-2 KO models, showing that PAR-2 activation have a crucial role in different diseases, including asthma (197) (198) , joint pain (199), cardiovascular illnesses (200) , malignant growths (201), and other inflammatory infections like inflammatory bowel disease (IBD)(202) and pancreatitis(203). As a result, strong and specific PAR-2 inhibitors could be promising treatments for these or related conditions (26).

PAR-2 KO mice developed attenuated allergic airway inflammation compared to WT mice (188) (3) (19). On the other hand, mice overexpressing PAR-2 develop increased allergic airway inflammation compared to WT mice (204). This suggests that PAR-2 activation is a critical step in the development of allergic airway inflammation.

Our lab demonstrated that PAR-2 activation mediates allergic sensitization to innocuous antigens presented simultaneously to the nasal mucosa (205). Furthermore, in WT mice, inhibiting PAR-2 activation prior to HDM sensitization leads to lower levels of Th2 cytokines in the lungs, as well as lower splenocyte proliferation in response to HDM, and it abrogated the ability of $CD4^+$ T cells to transfer the disease to naïve mice (3). As a result, these findings suggest that PAR-2 activation is a common mechanism mediating allergic sensitization to allergens.

Interestingly, it has been demonstrated that blocking PAR-2 activation in the airways during the challenge phase (using antibodies against PAR-2) attenuated allergic airway inflammation even if inflammation is already developed (206). This suggests that PAR-2 activation is not only essential for allergic sensitization but also the development of allergic airway inflammation.

1.8 T cells and asthma

Mature thymus T cells express TCR (T cell receptor) and can express CD8 glycoproteins on their surface. They are called $CD8^+$ T cells (cytotoxic) or CD4 glycoproteins, then are called $CD4^+$ T cells (helper T cells). $CD4^+$ cells differentiate into different subgroups: Th (T helper) 1, Th2, Th9, Th17, Th22, Treg (regulatory T cells) and Tfh (follicular helper T cells) (207) .

Th subgroups were differentiated from naive $CD4^+$ T cells induced by specific cytokines (207). Th1 is induced by IL-12, and interferon gamma (IFNγ), a pro-inflammatory cytokine with multiple functions such as activation of antigen presenting cells. Th2 cells is induced by IL-4, Treg by IL-2 and transforming growth factor beta (TGF- β)(208) (209) , and Th17 by IL-6 and TGF-β (210).

Each Th subgroup produces cytokines with pro- or anti-inflammatory, survival, or protective properties. Th1 produces IFNγ and TNF, while Th2 produces IL-4 (a crucial survival factor for B

cells), IL-5 and IL-13. Th9 generates IL-9 and Treg secretes IL-10 (a cytokine with immunosuppressive function) and TGF (211) . Finally, Th17 secrets IL-17, IL-21 and IL-22 (208)

Once T cells become activated by antigens presented on APCs, they develop into effector cells. Effector cells are short-lived cells, but memory cells are a subset of effector cells with the ability for long-term survival. Memory cells can be found in secondary lymphoid organs (central memory cells, Tcm) or in infected tissues (effector memory cells, Tem and tissue resident memory cells, Trm) (212) (208). Memory T cells expand rapidly after re-exposure to antigen during the second immune reaction, resulting in a more powerful and quicker immune response (208).

Moving forward to understand T-cell functions and the impact of innate cytokines on T-cell reactions at the epithelial level will help us better understanding asthma pathophysiology(213).

1.9 CD4 T cells and asthma

There is significant experimental evidence from human and animal studies suggesting that Th2 cells and their cytokines mediate allergic airway inflammation(214). Human studies have shown that Th2 cytokines levels are high in asthmatic patients and increase after challenge with allergens (215–217). Consistent with these findings, additional studies have shown increased Th2 cytokine production on CD4 $^{+}$ T cells in the lungs of asthma patients (214) (218,219). In addition, bronchial biopsies from asthma patients (214) (220,221), found high levels of transcription factors STAT6 and GATA3. Where, GATA3 and STAT6 are transcription factors that are important for both Th2 cell differentiation and TH2 cytokine secretion (222–225) and high levels of these transcription factors indicate a growing population of Th2 cells(214).

One of the important Th2 cytokines, IL-13 is associated with asthma. IL-13 was found to be most increased in the sputum of mild asthma patients (214) (226,227), and IL-13 mRNA expression was enhanced in bronchial biopsy specimens of mild asthma patients(228) (229). Saha et al, further supported the link between IL-13 production and asthma, which provides further evidence that not only increased IL-13 expression in sputum and bronchial biopsy specimens from acute asthma patients but also, its expression is related to the degree of eosinophilic inflammation (214). Therefore, these studies support the hypothesis that Th2 cells play an important role in the pathogenesis of asthma.

Murine studies also support the theory that Th2 cells play an important role in allergic airway inflammation. Studies have shown that Th2-type cytokine IL-13 is critical for AHR in murine models of allergic-induced eosinophil recruitment and allergic airway inflammation, similar to findings in human studies. Furthermore, depletion of Th2 cells from mouse models prevents the development of airway hyperresponsiveness (AHR) and eosinophilic inflammation(214) . Finally, transfer of antigen specific Th2 cells into naive mice and aerosol challenge with antigens later leads to eosinophilic airway inflammation, mucosal hypersecretion, and airway hyperresponsiveness(230).

Asthma is more than just an adaptive immune system disease, and interaction between the innate and adaptive immune systems is crucial for the initiation and propagation of allergic immune responses. Indeed, the typical clinical variability of asthma may represent the complex interactions that occur in the allergic lung between different populations of stromal cells, epithelial cells, and leukocytes, as well as the contribution of T cell subsets (231,232) (233). A better understanding of the contribution of different $CD4^+$ T cell subsets in the pathogenesis of asthma may lead to new

therapeutic targets. New studies focusing on studying memory cells and their role in asthma are an exciting extension of asthma research.

1.10 Memory T cells

The immune system's ability to maintain memory of previous antigen encounters is fundamental to long-term immunity, and health. Antigen-specific T cells are activated, multiply, and develop into effector cells after being exposed to the antigen, such as during an infection or after vaccination. The increased number of antigen-specific cells with effector capabilities can help remove the infection, but the vast majority of these cells will eventually die. Memory cells are the cells that have survived (234) (235) . When exposed to the same disease or antigen again, these memory cells can provide protection or an increased response. The improved reaction is the result of two main alterations that occurred after the initial exposure. First, while the majority of activated cells die after the initial response, the remaining cells are found in higher numbers than the original naive T cell. Because of the larger number of antigen specific cells, any re-infection is more likely to be detected promptly, allowing the immune response to begin before the pathogen spread. The ability of memory cells to create effector responses more quickly than initial responding cells is the second difference between naive and memory cells (234) (236,237). T cells can respond in a variety of ways depending on the type of infection and the signals transmitted from antigen presentation cells like DCs

Based on cell surface molecules, memory cells can be distinguished from naïve cells, and these changes are also used to define memory cell subgroups (235). The overexpression of the hyaluronate receptor, CD44, is one of the changes in cell surface molecules that occurs after T cell activation. This may make it possible for activated and memory T cells to infiltrate inflamed peripheral locations where an infection may be present (238). The activation of T cells leads to the

downregulation of CD62L and CCR7. These molecules are needed to enter the lymph nodes and the T cell area of the lymph nodes, respectively. Although the changes in CD45 expression and CD44 up-regulation may be permanent, some memory cells re-express CD62L and CCR7. This led to the description of two subsets of memory cells. 1st subset is described in human peripheral blood (239), central memory T cells (Tcm) which are similar to naive T cells, and they express CD62L and CCR7 and produce IL-2 after reactivation. 2nd subset, effector memory T cells (Tem) produce effector cytokines (such as IFNγ or IL-4) and are unlikely to pass through lymph nodes due to their low expression of CD62L and CCR7 (234) (240)(241) (242)(243) (244). In mice, $CD8^+$ Tcm appears to provide the best protection against reinfection(245) . Whether the same occurs with $CD4^+$ T cells is a controversial issue(234)(246).

1.11 Tissue resident memory T cells

It has been demonstrated that there are latent Th2 memory cells that resides near small, medium, and large airways in mice who have recovered from their first episode of allergen-induced experimental asthma (247) . These T cells react to an inhaled allergen, causing a disease relapse, with eosinophilic airway inflammation, mucus hypersecretion, and AHR (181). Although these long-lived lung Th2 memory cells are thought to be harmful, little was known about them. Tissue resident memory cells (Trms) have recently been discovered in a variety of tissues, including the lungs, and can be identified from memory cells in the blood by their inability to freely circulate and their resistance to intravenous anti-CD4 monoclonal antibody (mAb) treatment(248) (249) (250).

Turner and colleagues found that both $CD4^+$ and $CD8^+$ T cells infiltrate lung tissue during acute HDM exposure, but only $CD4^+$ Trms, not $CD8^+$ Trms, survive long after HDM treatment is stopped. $CD4^+$ Trms in the lungs cluster around airways and reactivate fast in response to allergen

re-exposure, causing AHR without the involvement of circulating T cells. In response to HDM exposure, increased DCs recruitment and activation in the lungs is also linked to lung $CD4^+$ Trm activation. (251) .

Also, Bosnjak and his colleagues demonstrated a subset of $CD3^+$ $CD4^+$ lung cells that express $CD44^{hi}CD62L^-CD69^+$ $ST2^+$, produce Th2 cytokines, and mediate allergen-induced disease recurrence even after treatment with anti-CD4 antibodies. These cells are presented in the lungs during the lifetime of the mouse (> 665 days), indicating long-lasting pathogenic Th2 Trm cells, which sustain "allergic memory" in the lungs(248).

Lung $CD4^+$ Trms can maintain long term allergen-specific sensitization and direct early inflammatory signals that promote rapid response to allergens (252) (253). Trm cells can provide more direct protection. and several reports have shown that they provide the host with the most effective immune protection(254) (255) (256) (257)(258).

Tissue-resident memory cells are identified as $CD69^+$ cells, which remain in peripheral tissues even after the pathogen is cleared(258) (259) (260). CD69 is considered a retained signal because it inhibits surface expression of sphingosine receptor 1- phosphate (S1P). S1P is a signal phospholipid that can regulate the transfer of immune cells from lymph nodes to efferent lymph vessels and guides the migration of cells from tissues to draining lymph nodes(261) (262) (263).

It has been known that IL-2 promotes the growth of activated $CD4^+$ T cell effectors and transmits signals that are required for $CD4^+$ Trm cell production. The absence of IL-2R signaling on activated $CD4^+$ T cells in a Th2 model of allergic asthma resulted in $CD4^+$ T cells failing to move into the lung and establish residency (252) (264,265). Both naive and lymphoid homing $CD4^+$

memory cells require IL-7 signals to survive. In a Th2 allergy model, IL-7R blockage resulted in a decrease in the number of airway resident $CD4^+$ T cells (252) (266). Furthermore, $CD4^+$ Trm cells failed to survive long term in a skin model of contact hypersensitivity after ablation of IL-7 in the skin (252) (267). This suggests that IL-7 signaling is critical for $CD4^+$ Trm recruitment and survival (252).

The immune protection cycle of circulating memory $CD4^+$ T is slower when compared to Trm-mediated because they must transfer to the site of infection. The immune protection of circulating cells may require cooperation with other members of the memory immune response such as Trms cells (254). Implying that, targeting lung $CD4^+$ Trms could be beneficial in the treatment of recurrent asthma attacks.

1.12 Rationale, Hypothesis and Aims

PAR-2 is important not just in allergic sensitization but also in the development of allergic airway inflammation, as we've shown in this chapter (3) (206) (205) (3). This will lead us to a question, which cell/s (structural or hematopoietic) in the airways are mediating PAR-2 activation to induce allergic sensitization and allergic airway inflammation.

It has been shown that PAR-2 activation on airway epithelial cells induces the production of inflammatory mediators essential for allergic sensitization and allergic airway inflammation (73,113). Inhibiting PAR-2 activation in the airways has been found to reduce allergic sensitization and allergic airway inflammation (2,3,19,206) . Furthermore, confocal imaging has shown that Fitc-conjugated SAM-11 (monoclonal antibody against PAR-2) given intranasally, binds mainly to airway epithelium (206).

The reduction of PAR-2 activation on airway epithelium is thought to be the main cause of decreased allergen sensitization and allergic airway inflammation. So, we **hypothesize** that PAR-2 is activated on airway structural cells possibly, airway epithelial cells, which induce the production of pro-inflammatory mediators with subsequent allergic sensitization and allergic airway inflammation.

Understanding how memory T helper type 2 (Th2) triggers recall responses to aeroallergens could change the way allergic asthma is managed. Approximately 5-10% of effector Th2 cells recruited into the lungs mature into long-lived tissue resident memory cells ready to respond to allergen re-exposure. As a result, targeting memory Th2 cell activation as a therapeutic method appears appealing. However, the mechanism by which allergen inhalation triggers memory Th2 response in the lungs is not fully clarified. The possible involvement of molecules such as PAR-2 in Th2 memory cells development is a novel and exciting topic that requires more attention by researchers.

According to studies from our lab, PAR-2 has a critical function in allergic sensitization to allergens that have serine proteinase activity (3) (19). More importantly, the studies delve into the mechanisms underlying this effect, revealing that it is possibly mediated by altered T cell responses. Furthermore, the fact that animals exposed to HDM extract had lower *ex vivo* splenocyte proliferation when PAR-2 was blocked, suggests that systemic sensitization to inhaled HDM allergens requires PAR-2 activation in the airways. This observation is consistent with lower lung Th2 cytokine (IL-4, IL-5, and IL-13) production, which is likely attributable to a decrease in antigen-specific Th2 cells (3).

We **hypothesize** that allergens possessing serine proteinase activity and/or endogenous proteinases, activate PAR-2 on airway epithelial cells, which induce the release of pro-inflammatory mediators with subsequent allergic sensitization, allergic airway inflammation and

development of antigen specific CD4 $^{+}$ T memory cells in the lungs and spleen, which is missing in the case of *Par-2* $^{-/-}$ mice.

Aim 1: To study if PAR-2 expression on airway structural cells or/ and hematopoietic cells is required for the development of allergic airway inflammation using chimeric mice between WT and *Par-2* $^{-/-}$ mice (chapter 2).

Aim 2: To study the role of PAR-2 expression in the airways on the development of CD4^{+} T memory cells upon exposure to HDM (chapter 3).

Chapter 2: PAR-2 expression on airway structural cells is essential for development of allergic airway inflammation

2.1 Introduction:

Airway epithelium is the 1st organ to interact with inhaled allergens possessing serine proteinase activity and, in many cases, with endogenous serine proteinases released from immune and structural cells in the airways (268) (269). The interaction between allergens and airway epithelium induces the release of inflammatory mediators which create a favorable environment for dendritic cells (DCs) maturation and activation with Th2 skewing of the immune response (270)(271) (272)(73)

PAR-2 activation on airway epithelium promotes the release of GM-CSF, eotaxin(113), TSLP (134), and IL-6 (273)(critical mediators for allergic sensitization). In addition, our lab demonstrated that functional inhibition of PAR-2 in the airways alleviates allergen-induced airway inflammation by using SAM-11(monoclonal antibody against PAR-2), which showed intense binding to airway epithelium by confocal microscopy (10).

Furthermore, lack of PAR-2 expression as in *Par-2*$^{-/-}$ mice or blocking PAR-2 activation in the airways during allergen sensitization attenuates lymphocytes sensitization, confirmed by defective lymphocytes *ex-vivo* proliferation upon re-exposure to the allergen, decrease in Th2 cytokines expression in the lungs, and the decrease in serum antigen specific IgG(3) (188) (128). Data mentioned above suggests a major role for PAR-2 expression in airway structural cells on allergic airway development.

PAR-2 is also expressed on immune cells in the airways (274) . Furthermore, TSLP and GM-CSF can be released from airway epithelium stimulated by TNF, which can be produced by PAR-2 activation on DCs and alveolar macrophages (116)(117)(118).This data suggests that PAR-2 expression in immune cells (hematopoietic cells) may play a role on the development of allergic airway inflammation.

Our lab findings suggest that I.N treatment of the anti-PAR-2 antibody SAM-11, which binds primarily to the airway epithelium, reduces allergic airway inflammation (206) . As a result, we hypothesize that airway epithelial cells are the primary cells activated by PAR-2 to induce asthma. This chapter will investigate whether the expression of PAR-2 on airway tissue resident cells, including airway epithelium, is required for the development of allergic airway inflammation.

2.2 Materials and methods:

2.2.1 Animals

Par-2$^{-/-}$ mice were purchased from UC DAVIS KOMP (Knockout mouse project) repository (strain: C57BL/6N-F2rl1tm1a(EUCOMM)Wtsi/J). Mice were bred and housed at University of Alberta mouse facility. Mice were housed in virus- and Ab-free conditions and maintained on a 12-h light-dark cycle. Balb/c mice from Jackson lab were used in the cytokine analysis experiment (materials and methods section: 2.2.2). The University of Alberta Health Sciences Laboratory Animal Ethics Committee (Edmonton, AB, Canada) approved all experiments described. Mice were genotyped via ear punch at weaning (21-28 days) and a gene-specific polymerase chain reaction (PCR) on DNA taken from ear tissue using primers recommended by the UC DAVIS KOMP was performed by TAGC facility (University of Alberta, Katz building).

Our KO mice were generated by using the gene trapping technique. After fertilization, embryonic stem (ES) cells were harvested from early-stage mouse embryos. Because ES cells can develop into practically any type of adult cell, the impact of knocking out a gene in an ES cell can be seen in every tissue in an adult mouse. Researchers use the gene trapping to modify a gene in an ES cell once more. A reporter gene is embedded in a piece of artificial DNA that is designed to insert randomly into any gene. By inserting a "trapping cassette" into an intron of the target gene (275,276) , the upstream exons are trapped by splicing to the cassette and the mRNA is truncated. A LacZ reporter is flanked by a splice acceptor and polyadenylation signal in the trapping cassette, as well as site-specific recombination sites for additional genetic alteration of the allele (277). The artificial DNA injected into the cell hinders the cell's RNA "splicing" machinery from working properly, blocking the existing gene from creating its intended protein and thereby deactivating it. A modified viral vector or a linear segment of bacterial DNA are frequently utilized to transport

artificial DNA into ES cells. The genetically altered ES cells are cultivated in a lab dish for several days after the artificial DNA is added before being injected into early-stage mouse embryos. The embryos are put in a female mouse's uterus and allowed to grow into mouse pups. Some tissues in the subsequent mouse pups - those produced from the altered ES cells - have had a gene knocked out. They do, however, have some normal tissues generated from non-altered embryos into which the ES cells were injected. As a result, they aren't fully knockout mice. Crossbreeding such mice to obtain lines with both copies of the gene (one on each chromosome) knocked down in all organs is required until homozygous knockout mice developed. Adapted from https://www.genome.gov/about-genomics/fact-sheets/Knockout-Mice-Fact-Sheet.

Our mice 'knockout-first' allele (tm1a) contains an *IRES* (internal ribosome entry site): *lacZ* (β-Galactosidase) trapping cassette and a floxed (LoxP, locus of X-over P1) promoter-driven *neo* (neomycin) cassette inserted into the intron of our gene, disrupting gene function. Mating our mice with mice expressing recombinase flippase (FLP) recombinase converts the 'knockout-first' allele to a conditional allele (tm1c), restoring gene activity (floxed mice, which we already housing at the animal facility) by acting on flippase recognition target (FRT) and excise the FRT-flanked vector. Mating the floxed mice with mice expressing Cre recombinase can convert the floxed mice to tissue specific knockout mice according to which cells are expressing Cre recombinase. Moreover, mating our mice 'knockout-first' allele (tm1a) with mice expressing Cre, deletes the promoter-driven selection cassette and floxed exon of the tm1a allele to generate a *lacZ*-tagged allele (tm1b), [Reporter-tagged deletion allele (post-Cre)] (276). Look figure 1 for more details about our *Par-2* $^{-/-}$ mouse.

Fig. 1

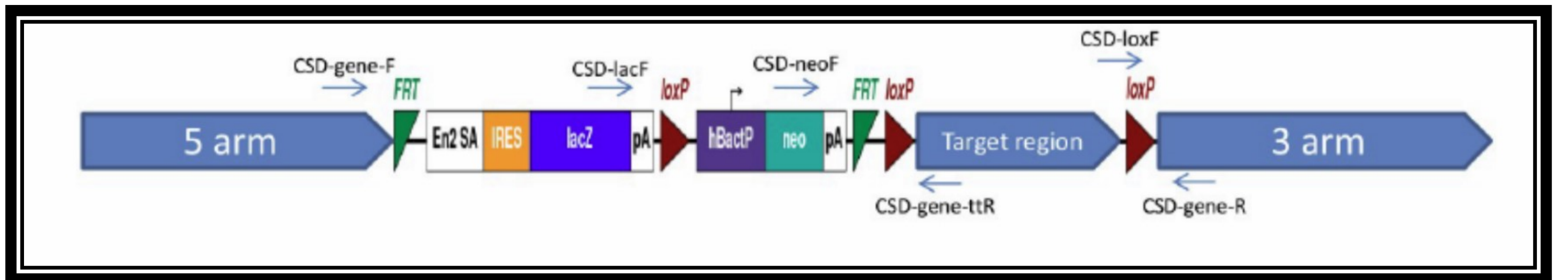

Fig. 1 (construction of *Par-2* $^{-/-}$ mouse): The L1L2-Bact-P cassette was inserted into Chromosome 13 at position 95647954, upstream of the crucial exon(s) (Build GRCm39). An FRT site is followed by a lacZ sequence and a loxP site on the cassette. A neomycin resistance gene under the control of the human beta-actin promoter, SV40 polyA, a second FRT site, and a second loxP site follow this first loxP site. At position 95651266, a third loxP site is added downstream of the targeted exon(s). As a result, loxP sites border the crucial exon(s). FLP recombinase expression in mice bearing this allele can result in a "conditional ready" (floxed) allele. Cre expression after that results in a knockout mouse. A reporter knockout mouse will be developed if Cre expression happens without FLP expression. Adapted from, http://www.informatics.jax.org/allele/MGI:4460480"

2.2.2 PAR-2 activation and cytokine analysis

To analyze inflammatory mediators in the lungs following PAR-2 activation we challenged WT Balb/c mice I.N for 3 consecutive days with 25 ul of saline, PAR-2 activating peptide (PAR-2 AP), or PAR-2 control peptide (PAR-2 CP). The peptides had the following sequence and were both used as 100-uM solution in saline: [PAR-2 AP: (SLIGRL-NH2) and PAR-2CP: (LSIGRL-NH2)]. PAR-2 AP and PAR-2 CP were provided as a gift from Dr. Hollenberg (Department of Pharmacology and Therapeutics, University of Calgary, AB, Canada). On day 4, mice were

euthanized and the whole lungs were stored dry at -80°C and a small piece of the lungs was taken for quantitative PCR, (qPCR) and was stored in RNAlater (Invitrogen by Thermo Fisher Scientific) for 24 hrs at 4°C and then stored dry at - 20°C to be used for multiplex cytokine analysis or qPCR respectively. Lung tissue for the multiplex cytokine assay was thawed and lysed in 1 mL of PBS with 0.5% NP-40 and a proteinase inhibitor cocktail (Sigma) per 100 mg of tissue. Lung tissue for q-PCR was homogenized and total RNA was extracted using RNeasy mini kit and Qiashredders (Qiagen, Venlo, Limburg, the Netherlands). Reverse transcription was performed on 1 ug of RNA per reaction using oligo dT primers and M-MLV (Invitrogen, Carlsbad, CA, USA).

Lung lysates was analyzed for the levels of 31 cytokines and chemokines using a multiplex bead-based assay (Eve Technology, Calgary, AB, Canada). Cytokines analyzed by multiplex bead-based assay : Eotaxin, G-CSF, GM-CSF, IFN gamma, IL-1alpha, IL-1beta, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17A, IP-10, KC, LIF, LIX, MCP-1, M-CSF, MIG, MIP-1alpha, MIP-1beta, MIP-2, RANTES, TNF, VEGF.

Lung tissues were analyzed for the levels TSLP, IL25 and IL-33 using qPCR which was done in an Eppendorf realplex4 using Taqman Gene Expression Master mix and Taqman Gene Expression Assays from ThermoFischer Scientific. The PCR protocol consisted of 10 min at 95°C followed by 40 cycles of 30 s at 95°C and 60 s at 60°C. All samples were run in triplicate and Hprt1 was used as a reference gene to correct for cDNA input. The relative expression of the target genes was determined using the ddCt method, for which the average dCt for the saline samples was set at 1 and all samples were compared to this value (3) .

2.2.3 Ovalbumin (Ova) mouse model of asthma

Male mice on a C57 BL/6J background (6–8 weeks of age) were used. Mice were sensitized via intraperitoneal injection (I.P) of ovalbumin (10ug) +Al $(OH)_3$ (2mg) or saline on days 1 and 6 and challenged intranasally (I.N) via ovalbumin (50 ug) or saline on days 12 and 14. Mice were euthanized on day 14 and BAL fluid was collected for assessment of allergic airway inflammation(fig.2).

Fig. 2

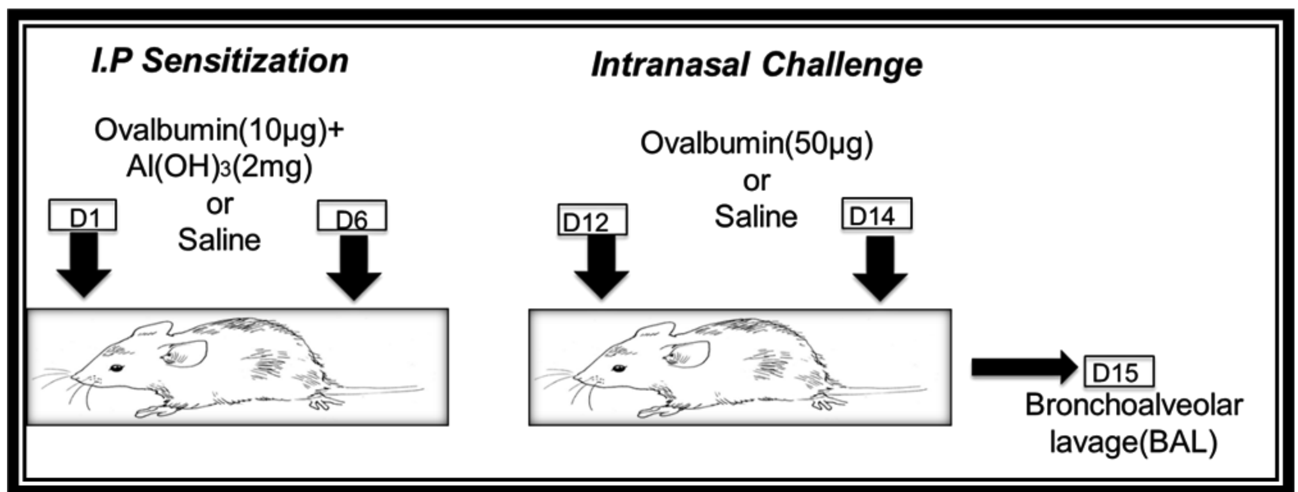

Fig. 2: Model for ovalbumin - induced allergic airway inflammation in mice.

2.2.4 Administration of Ovalbumin (Ova):

Sensitization phase (Day 1 and Day 6): 1-2 mg of ovalbumin (Endograde ovalbumin, lyophilized, Cat#321001) was dissolved in sterile saline to make a solution of 1 mg/ml. 200µl of this solution was added to 10 ml of sterile saline to make a 20µg/ml solution. 40 mg of aluminum hydroxide (Thermo Fisher Scientific, cat# 77161) were added to the 20µg/ml solution of ovalbumin and 500µl of the ovalbumin-alum was injected intraperitoneally (I.P) / mouse, or 500µl sterile saline as control to sensitize the mice on days 1 and 6(fig. 2).

Challenge phase (Day 12 and Day 15): Weigh out 1-2mg of ovalbumin and dissolve it in sterile saline to make a solution of 2 mg/ml. Inject every 20g mouse with 0.125 ml anesthetic cocktail via I.P injection [To prepare 5 ml of the anesthetic cocktail, we add 0.75ml ketamine (100mg/ml) + 0.25 ml xylazine (20 mg/ml) and dilute to 5 ml with 0.9% sterile saline]. After injection of the anesthetic cocktail, we wait for 5-10 minutes until the mouse has stopped moving and is mildly sedated. Pipet 25µl (50µg) ovalbumin into the right nostril using sterile pipet tips for challenging the mice on days 12 and 14(fig.2). Put the mouse back into the cage to recover, line the cage bottom with paper towel to prevent the mice from breathing in the bedding while sedated.

2.2.5 Measurement of airway inflammation

Bronchoalveolar lavage (BAL) was performed 24 hrs after the final I.N allergen challenge to assess airway inflammation. Mice were euthanized with an I.P injection of 2 mg sodium pentobarbital in 100 ul saline. Cardiac puncture was used to collect blood, followed by tracheal intubation with polyethylene tubing. The lungs were washed twice, with 1 mL of phosphate buffered saline (PBS), pH 7.4, and lavage fluid was collected. The BAL fluid (BALF) was spun at 300 g for 5 min, and cytospins were prepared with 5000 cells. The slides were stained with Diff Quick (Fisher Scientific, Co. Kalamazoo, MI, USA), and the inflammatory cells were counted to assess airway inflammation (3) .

2.2.6 BM chimeras

To understand whether the development of allergic airway inflammation requires PAR-2 expression on tissue resident cells or hematopoietic cells we performed bone marrow (BM) chimeras between male WT mice and *Par-2* $^{-/-}$ mice(figure3).

To develop chimeric mice, donor WT and *Par-2* $^{-/-}$ mice, were injected (I.P) with 100ug of 30H12 (anti-Thy1.2) on days 1 and 2 before harvesting the bone marrow to deplete total T cells. The antibody was provided as a gift from Dr. Baldwin's Lab at the University of Alberta. Recipient WT and *Par-2* $^{-/-}$ mice were irradiated with total 10 GY (divided in to 2 doses, 5 GY each and the mice were left to rest for 3-4 hrs between each dose. Recipient mice were allowed to rest overnight following final irradiation before I.V injection of T cell depleted bone marrow cells (according to University of Alberta Institutional SOP for I.V injection). The recipient mice were injected with 200ul of cells (5-10 million of bone marrow cells).

Irradiated mice were provided with Novotrimel containing water, provided by University of Alberta animal facility for 4 weeks following irradiation to prevent opportunistic infections as they are immunocompromised mice. Novotrimel water was changed weekly. Irradiated mice were monitored daily for the first three weeks as this is the time frame when morbidity due to the bone marrow cells failing to reconstitute the recipient or toxicity due to irradiation is observed. After the three-week window, mice were monitored every other day for signs of morbidity.

On the 5th week, tail blood was collected to confirm successful reconstitution of BM cells (figure 4). 100 ul of blood was collected from tail vein by incision with a lancet and RNA was isolated using RNeasy protect animal blood kit (Qiagen, Cat # 73224). RNA was reverse transcribed and PCR for PAR-2 were performed as described above (section 2.2.2). Mouse PAR-2 expression was measured using Taqman gene expression assay (Mm0043360_m1, ThermoFisher Scientific, Waltham, MA) as per manufacturer's instructions; HPRT (Mm03024075_m1, ThermoFisher Scientific) was used as a house keeping gene.

2.2.7 Bone marrow cell isolation protocol

The protocol is adapted from Liu, X., & Quan, N. (2015). "Immune Cell Isolation from Mouse Femur Bone Marrow". *Bio-protocol*, *5*(20), e1631. https://doi.org/10.21769/bioprotoc.1631.

1- Pentobarbital is used to euthanize the mouse, which is then placed on a sterile surgical pad in a sterile hood. Using 70% ethanol, sterilize the mouse abdomen area and hind limb skin. Remove the surface muscles from the abdominal cavity with sterile scissors and locate the pelvic-hip joint.

2- Using sterilized scissors, cut the hind leg above the pelvic-hip junction. Using sterile scissors, cut the tibia from the hind leg below the knee joint. To dissect the tibia, make a cut at the ankle joint. To isolate BM cells, we used both the femur and the tibia.

3- Forceps and scissors are used to remove the muscles and tissues that surround the femur and tibia. Scissors are used to cut both ends of the femurs and tibias. Flush the bone marrow out onto a 70 m nylon cell strainer set in a 50 ml Falcon conical with a 23- or 25-gauge needle and a 10-cc syringe filled with ice-cold DMEM. Use 10 mL or until the flow through turns white, whichever comes first.

4- At 4 °C, centrifuge cells for 7 minutes at 1,500 rpm. Resuspend the cell pellet in 1 ml RBC lysis buffer (BD Biosciences lysis buffer Cat# 555899). Incubate at room temperature for 5 minutes, then add 5 mL DMEM medium to neutralise the lysis buffer (HyClone).

5- At 4 °C, centrifuge cells at 1,500 rpm for 7 minutes. Discard the supernatant and resuspend the cell pellet in 5 mL DMEM medium containing 10% FBS for the next phase of the test. After that, the cells were placed on ice and counted using a hemocytometer.

2.2.8 Statistical analysis: Statistics were performed using Prism 5 (GraphPad Software, La Jolla California USA). Data are presented as Mean ± SEM; ns (not significant), *P<0.05, **P<0.01, ***P<0.001 between the groups and were analyzed for significance using a one-way ANOVA and Tukey's multiple comparison tests.

Fig 3: Bone marrow chimera experiment

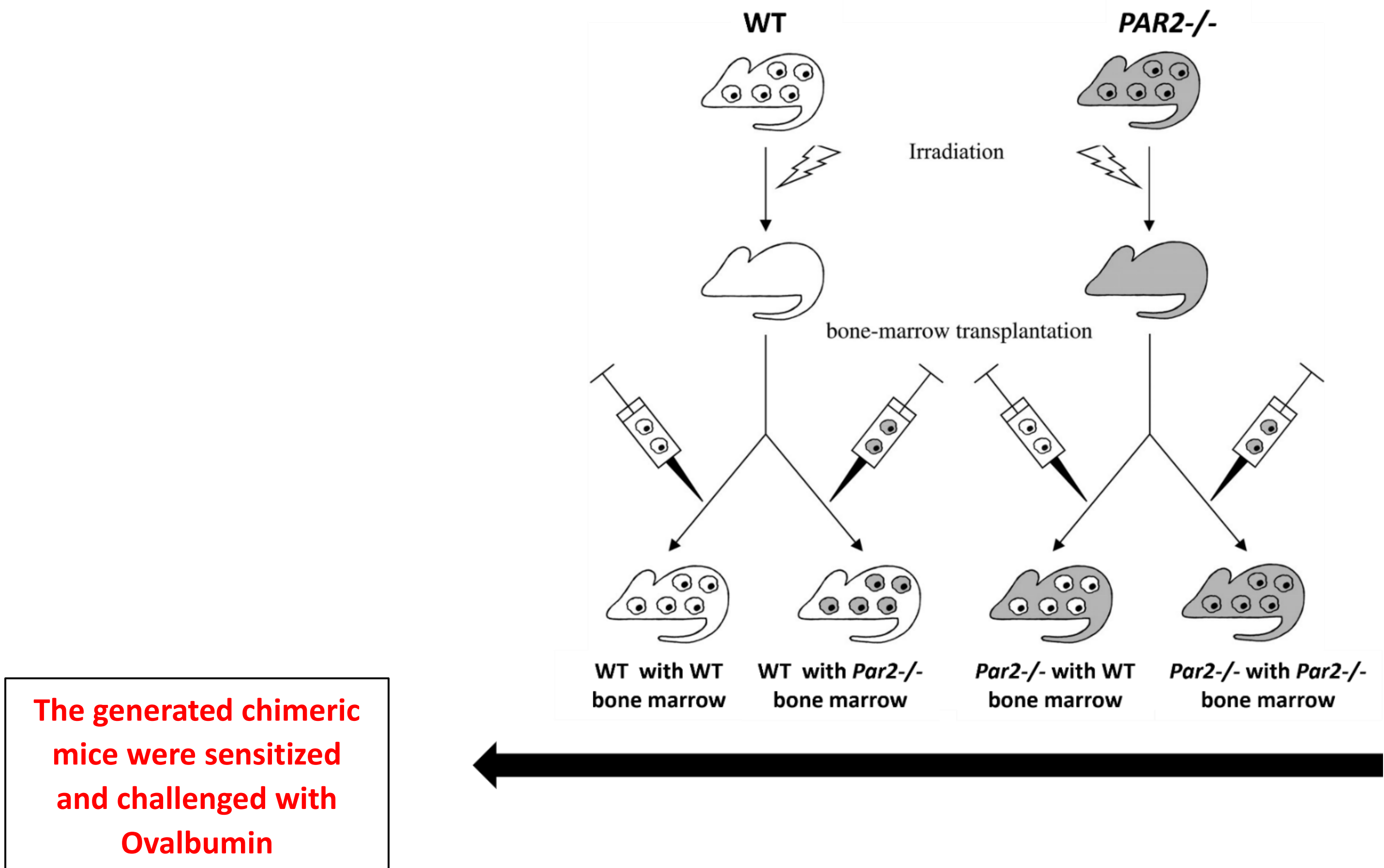

Fig. 3: Mice were irradiated and then administered ovalbumin as described in methods section 2.2.6. This approach generated four different chimeric mice as shown in the figure: WT / WT BM (WT mice transplanted with WT BM, white mouse with white cells), WT / KO BM (WT mouse with *Par-2*$^{-/-}$ BM, white mouse with grey cells), KO / WT BM (*Par-2*$^{-/-}$ mice with WT BM, Grey mouse with white cells) and KO / KO BM (*Par-2*$^{-/-}$ with *Par-2*$^{-/-}$ BM, grey mouse with grey cells). The generated chimeric mice were sensitized and challenged with ovalbumin as described before to induce allergic airway inflammation.

2.3 Results:

2.3.1 Detection of PAR-2 expression in chimeric mice to evaluate the success of BM transplantation

Blood was collected from tail vein and qPCR for PAR-2 was performed as described in methods section 2.6.6. As expected, the mRNA levels of PAR-2 were significantly higher in WT / WT BM chimeric mice compared to KO/KO BM chimeric mice. The mRNA levels of PAR-2 were higher in KO / WT BM chimeric mice compared to WT / KO BM chimeric mice however, the difference was not significant (fig.4). Suggesting that BM cells might not be completely eliminated in recipient mice. So immune cells from the chimeric mice may be contaminated with immune cells from the recipient mice.

Fig. 4

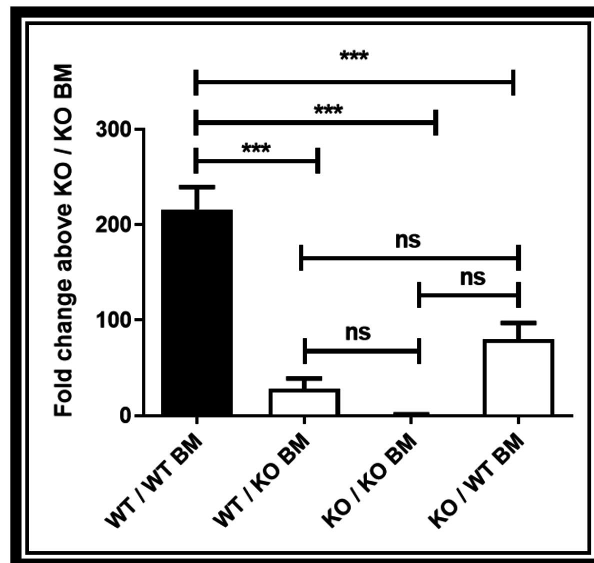

Fig. 4: **mRNA levels of PAR-2 in chimeric mice blood.** Blood from tail vein was collected on the 5th week as described in methods section 2.6.6. qPCR for PAR-2 was performed as described in this chapter. Results are presented as fold change above KO/KO BM chimeric mice. WT / WT BM chimeric mice (216.3 ± 23.37), WT / KO BM chimeric mice (20.85 ± 9.910), and KO / KO BM chimeric mice (1.077 ± 0.2214) KO / WT BM (80.12 ± 17.08). Data are presented as Mean ± SEM; ns (not significant), *P<0.05, **P<0.01, ***P<0.001 between the groups and were analyzed for significance using a one-way ANOVA and Tukey's

2.3.2 To explore inflammatory mediators' production upon PAR-2 activation in the airways

PAR-2 AP significantly increased the lung levels of Eotaxin, IL-6 and KC-Gro, CXCL1 (detected by Multiplex Immunoassay) in WT mice compared to mice treated with saline or PAR-2 CP (fig. 5-A, 5-B, 5-C, and table 1). However, PAR-2 AP did not increase the lung levels of other cytokines in the immunoassay in WT mice compared to mice treated with saline or PAR-2 CP (table 1). IL-3, IL-10, IL-12p40, IL-13, IL-17A, LIX and GM-CSF were not detected (not presented in table 1). PAR-2 AP significantly increased the lung levels of TSLP (detected by qPCR) in WT mice when compared to mice treated with saline or PAR-2 CP (fig 6). However, other epithelial driven cytokine detected also by qPCR (IL-25 and IL 33) did not increase upon PAR-AP treatment in WT mice compared to mice treated with saline or PAR-2 CP (fig 6). The levels of other cytokines /chemokines levels detected by Multiplex Immunoassay are shown in table 1.

Fig. 5-A

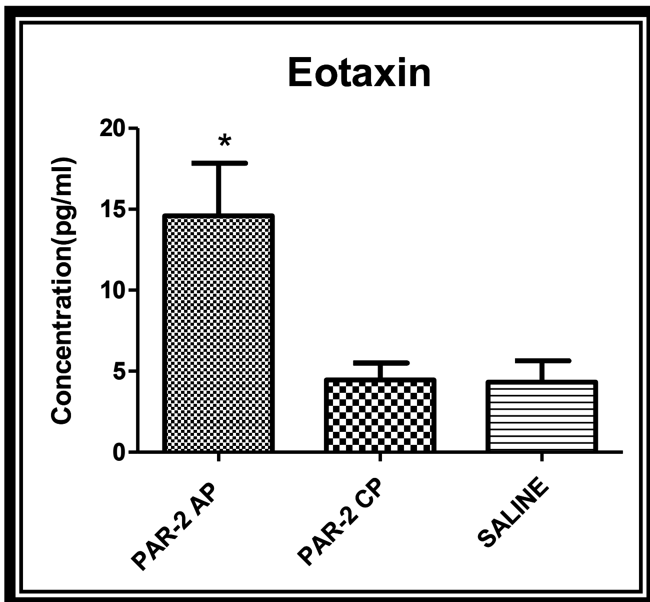

Fig. 5-B

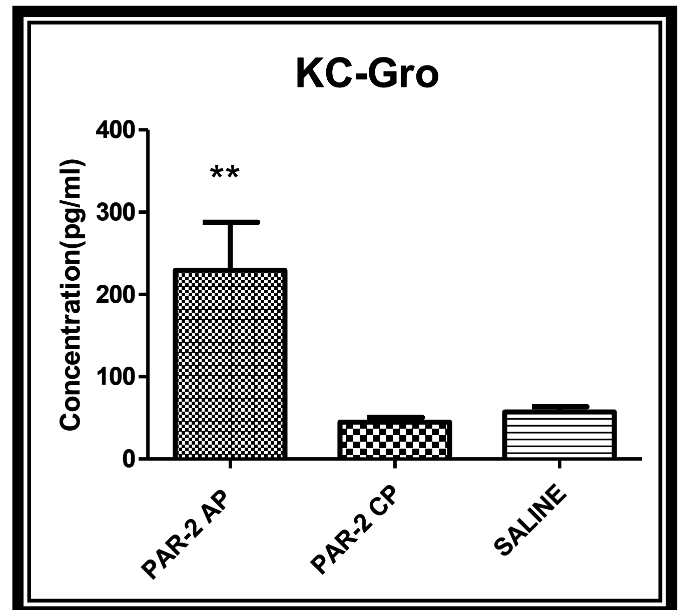

Fig. 5-C

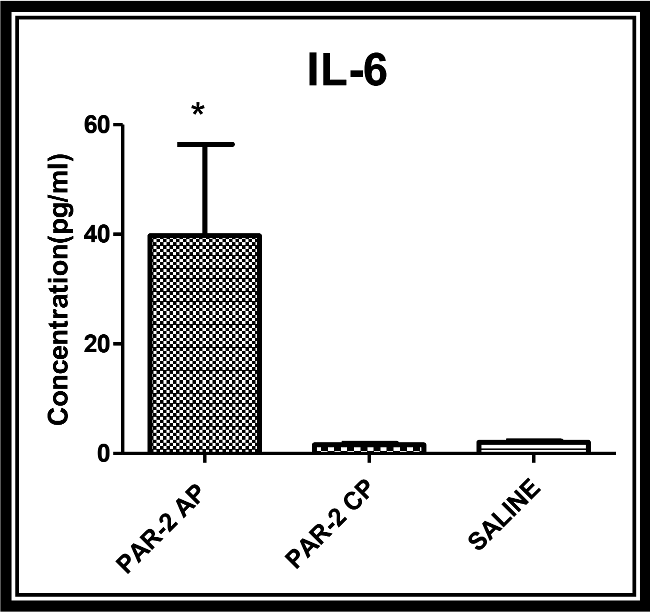

Fig. 5: Levels of eotaxin (5-A), Kc-Gro (5-B) and IL-6 (5-C) in lung lysates from mice administered intranasally with PAR-2 AP or PAR-2 CP or saline. * Represents significant difference between PAR-2 AP and both, PAR-2 CP, and saline. Look table 1 for cytokine /chemokines levels (pg/ml). N= 7

Table 1: Cytokine/chemokine levels in lung lysates

Cytokines	PAR-2 AP	PAR-2 CP	Saline
Eotaxin	48.85±34.36	4.451±1.059	4.33±1.306
IL-6	39.73±16.7	1.574±0.2584	2.040±0.2184
KC-Gro	482.2±257.3	46.63±5.092	56.90±5.478
TNF	3.480±0.7014	1.829±0.3048	1.710±0.1949
MCP-1	40.31±7.827	27.48±2.263	24.05±1.658
M-CSF	5.563±1.082	4.403±0.6374	5.876±0.4401
MIP-2	129.7±29.89	69.58±11.21	64.63±9.024
MIP-1α	22.6±3.621	12.70±2.026	15.72±1.883
MIP-1β	26.09±6.837	12.12±3.408	13.82±4.142
G-CSF	186.2±63.24	188.7±65.90	195±68.45
IL-1β	10.79±3.077	10.58±2.279	10.21±2.189
IL-1α	17.98±4.68	12.47±3.330	11.79±2.206
IL-2	8.627±1.138	7.917±0.4341	7.789±0.7239
IL-4	0.1786±0.03341	0.2029±0.03115	0.2429±0.04779

IL-5	**0.6686±0.1296**	**0.3929±0.05528**	**0.5514±0.1584**
Rantes	**18.27±2.862**	**26.98±3.445**	**40.91±3.112**
MIG	**60.81±12.62**	**65.37±9.528**	**94.25±11.09**
IL-7	**3.506±0.4355**	**4.721±0.759**	**4.689±0.897**
IL-9	**13.22±2.02**	**11.99±1.759**	**16.31±2.851**
IL-15	**10.19±1.900**	**12.51±1.418**	**10.42±1.915**
IP-10	**41.45±9.652**	**29.03±1.961**	**35.86±1.958**
VEGF-A	**39.15±7.444**	**35.72±3.934**	**46.13±5.975**
LIF	**2.207±0.3807**	**0.818±0.148**	**0.9586±0.13**
IL-12p70	**4.334±1.261**	**3.628±0.598**	**4.615±0.759**

Data is presented as mean of concentration(pg./ml) ± SEM, n = 7/group.

Fig. 6-A

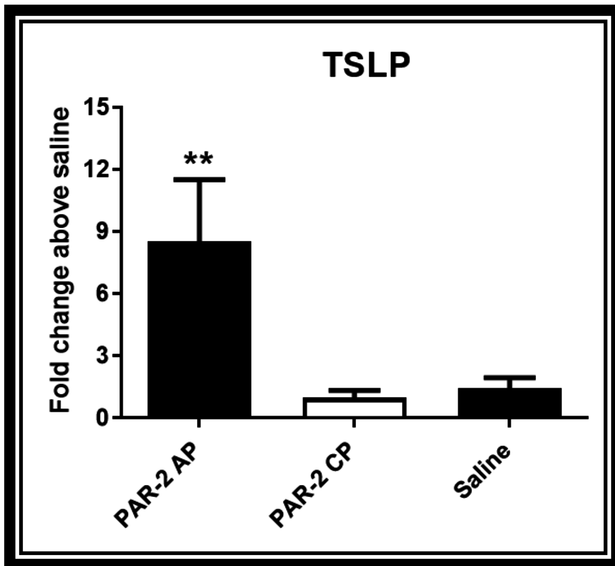

Fig. 6-B

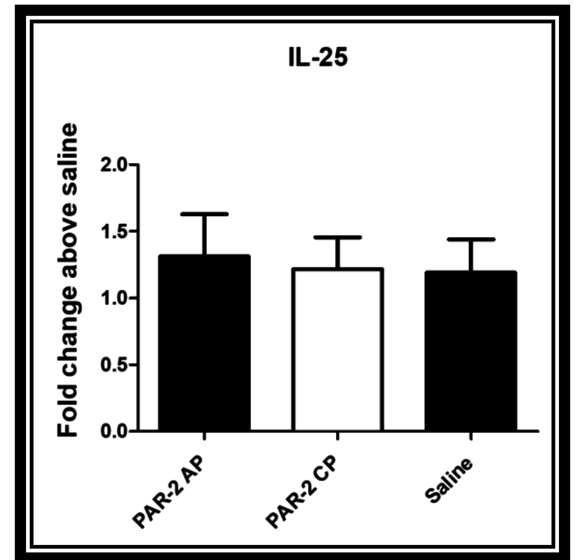

Fig. 6-C

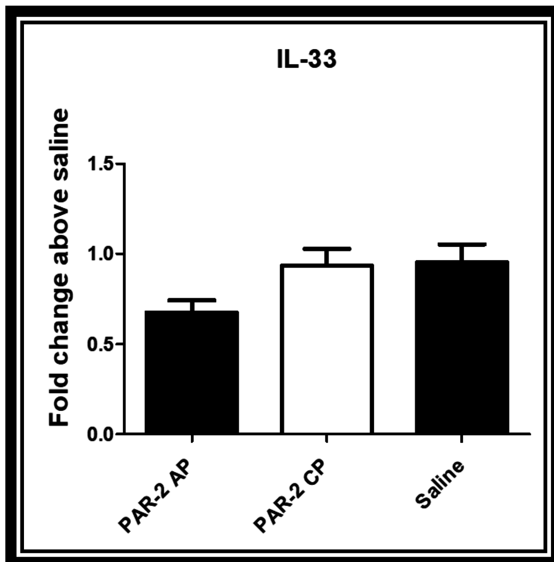

Figure 6*:* mRNA levels of TSLP (fig 6-A), IL-25(fig 6-B) and IL-33(fig 6-C) in lung tissues detected by qPCR (data presented as fold change above saline) from mice administered intranasally with PAR-2 AP or PAR-2 CP or saline. * Represents significant difference between PAR-2 AP and both, PAR-2 CP, and saline. N=7 for all experiments

2.3.3 Allergic airway inflammation in response to Ova in chimeric mice

We developed BM chimera between WT and *Par-2*$^{-/-}$ mice and allergic airway inflammation was induced in the chimeric mice using ovalbumin. WT/WT BM chimeric mice sensitized and challenged with Ova, as expected, had inflammation characterized by increased number of total cells (fig. 7-A) and eosinophils (fig. 7-B) neutrophils (fig. 7-C), lymphocytes (fig. 7-D) and macrophages (fig. 7-E) compared to mice that were sensitized and challenged with saline (fig.8). Saline treated mice did not develop allergic airway inflammation in all mice groups (fig. 8) and differences between numbers of macrophage was not significant between all groups treated with Ova (fig. 7-E). KO/KO BM chimeric mice sensitized and challenged with Ova, as expected, had lower inflammation characterized by decreased number of total cells (fig. 7-A), eosinophils (fig. 7-B), neutrophils (fig. 7-C), lymphocytes (fig. 7-D) and macrophages (fig. 7-E), similar to what have been shown with *Par-2*$^{-/-}$ mice (206).

WT/KO BM chimeric mice sensitized and challenged with Ova had increased allergic airway inflammation characterized by increased number of total cells (fig. 7-A), eosinophils (fig. 7-B), lymphocytes (fig. 7-D) and macrophages (fig. 7-E) compared to mice sensitized and challenged with saline (fig. 8). However, the numbers were lower than WT / WT BM chimeric mice.

KO /WT BM chimeric mice sensitized and challenged with Ova showed increased allergic airway inflammation characterized by increased number of total cells (fig. 7-A), eosinophils (fig. 7-B), neutrophils (fig. 7-C), lymphocytes (fig. 7-D) and macrophages (fig. 7-E) compared to mice sensitized and challenged with saline.

Allergic airway inflammation represented by the number of total cells and eosinophils was higher in WT/KO BM chimeric mice sensitized and challenged with Ova compared to KO/WT BM

chimeric mice (7-A and B). Suggesting that the loss of PAR-2 expression on structural cell reduced allergic airway inflammation more than with the loss of PAR-2 expression on hematopoietic cells, however, the differences was not statistically significant. This may mean that PAR-2 expression on both airway structural cells and hematopoietic cells are essential for the development of allergic airway inflammation.

Interestingly, the loss of PAR-2 expression on structural cells significantly reduced allergic airway inflammation represented by the numbers of eosinophils in BAL compared to WT/ WT BM chimeric mice (fig 7-B). This may mean that PAR-2 expression on structural cells is critical for the development of allergic airway inflammation, however, from findings mentioned above, PAR-2 expression on hematopoietic cells contribute to the full effect.

Fig. 7-A

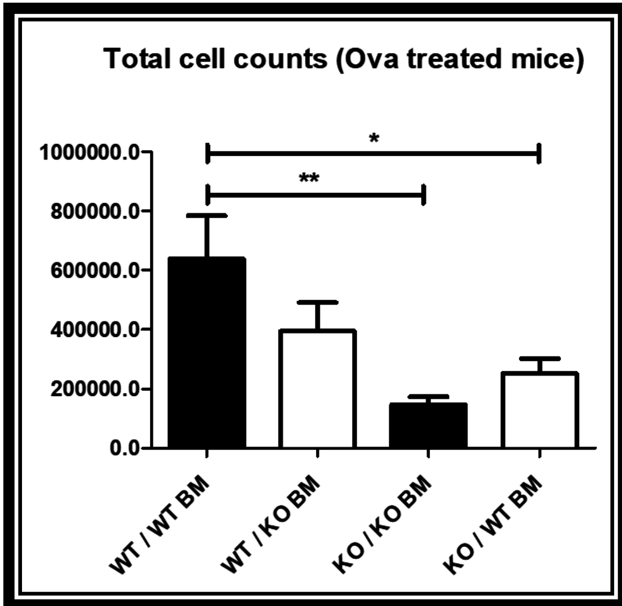

Fig. 7-B

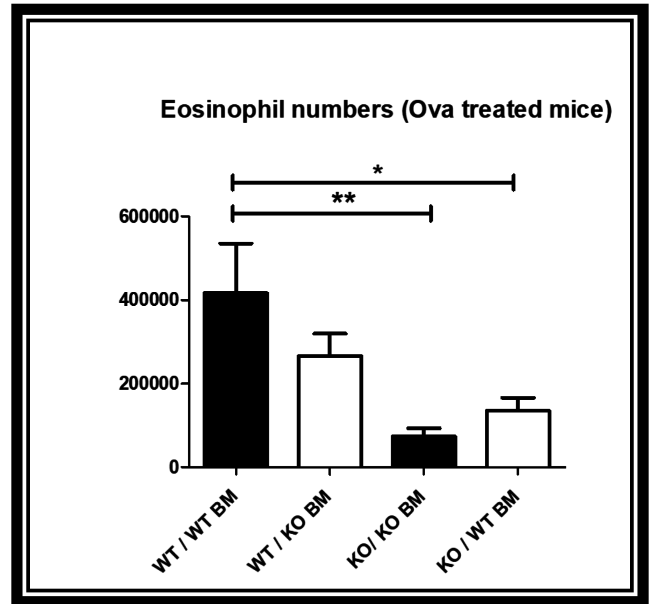

Fig. 7-C

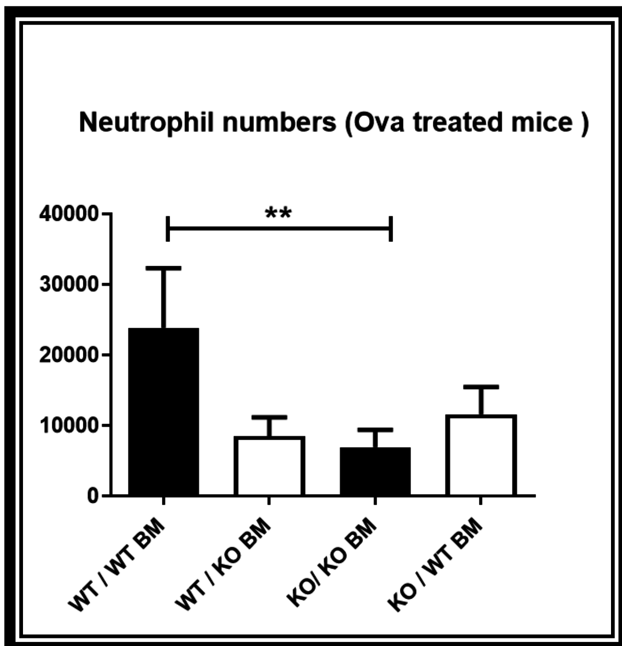

Fig. 7-D

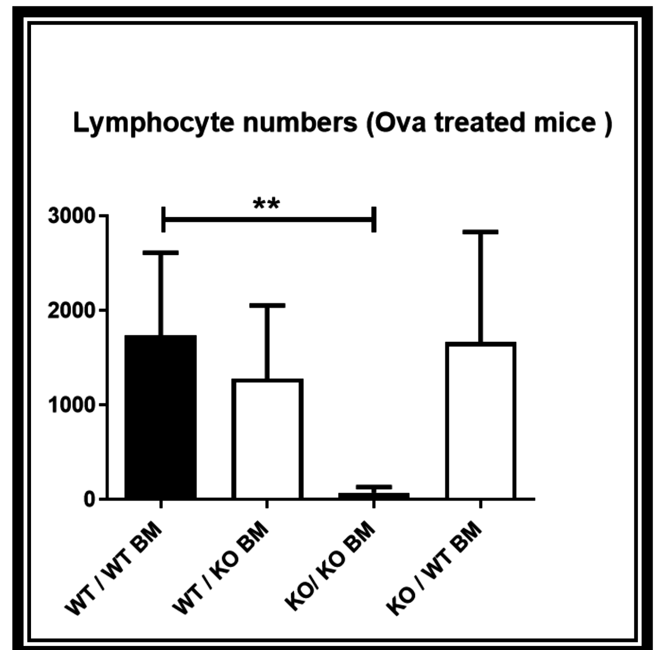

Fig. 7-E

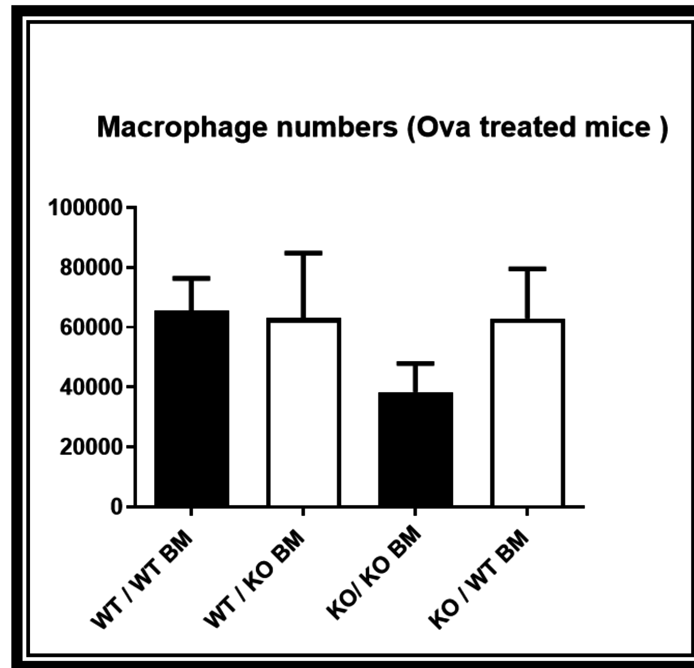

Fig. 7*:* Levels of airway inflammation in response to Ova in chimeric mice. Fig. 7-A, show the total cell counts in BAL fluid. Chimeric WT mice with WT hematopoietic cells (638, 182 ± 146, 212) and *Par-2*$^{-/-}$ with *Par-2*$^{-/-}$ hematopoietic cells (145,852 ± 27,471) (black columns). WT chimeric mice with *Par-2*$^{-/-}$ hematopoietic cells (394,242 ± 96,671) and *Par-2*$^{-/-}$ chimeric mice with WT hematopoietic cells (251,667 ± 50,384) (white columns). Fig 7-B, show Eosinophil numbers in BAL fluid. Chimeric WT mice with WT hematopoietic cells (416,658 ± 119211) and *Par-2*$^{-/-}$ with *Par-2*$^{-/-}$ hematopoietic cells (73,578 ± 19,452) (black columns). *Par-2*$^{-/-}$ chimeric mice with WT hematopoietic cells (135,080 ± 31, 157) and WT chimeric mice with *Par-2*$^{-/-}$ hematopoietic cells (265, 603 ± 54, 211) (white columns). In Fig 7-C, D and E we are showing the numbers of Neutrophil, Lymphocyte and Macrophage (differences between groups are not significant) in BAL fluid respectively. Data are presented as Mean ± SEM; *P<0.05, **P<0.01, ***P<0.001 between the groups indicated by horizontal lines. N=14-17

Fig 8-A

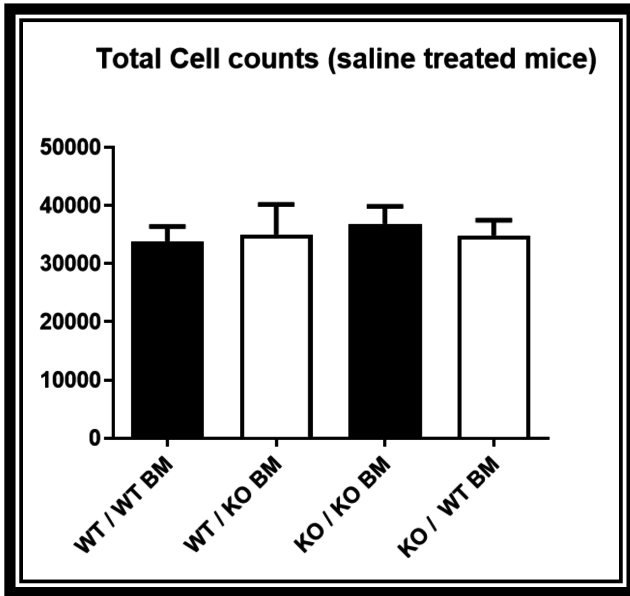

Fig 8-B

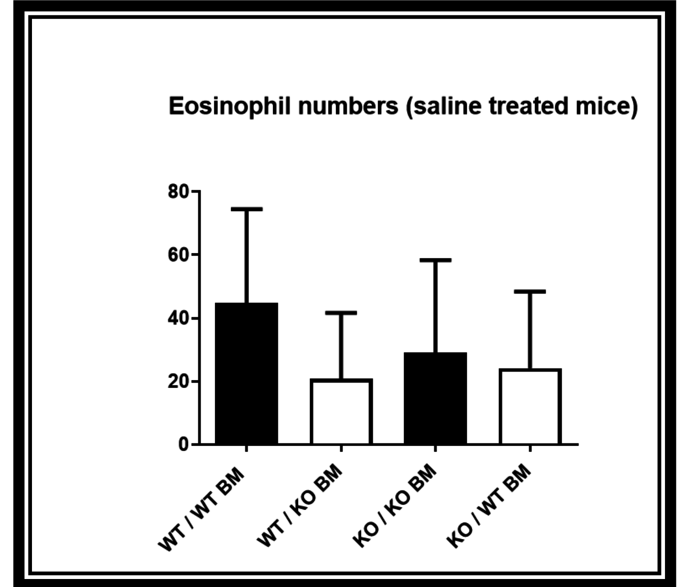

Fig 8-C

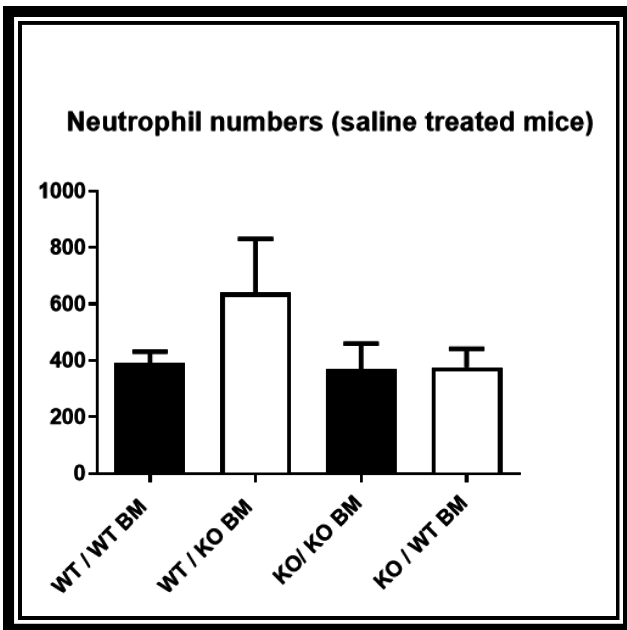

Fig 8-D

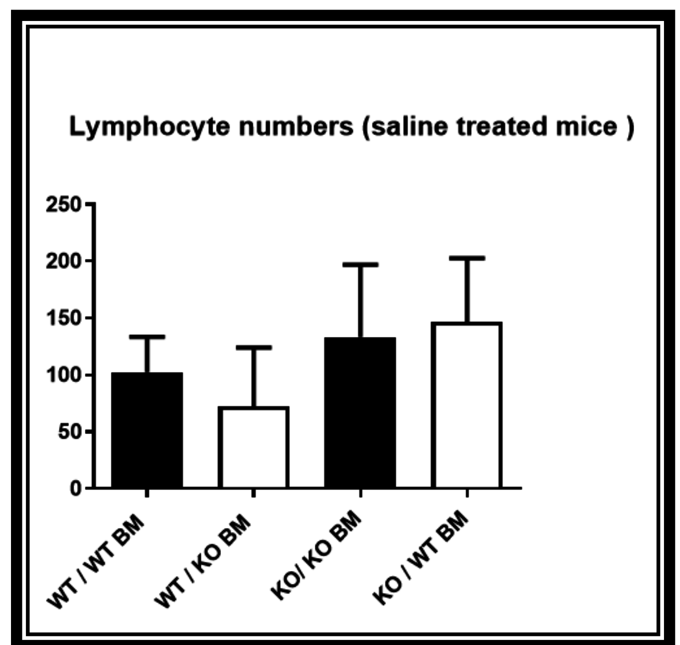

Fig 8-E

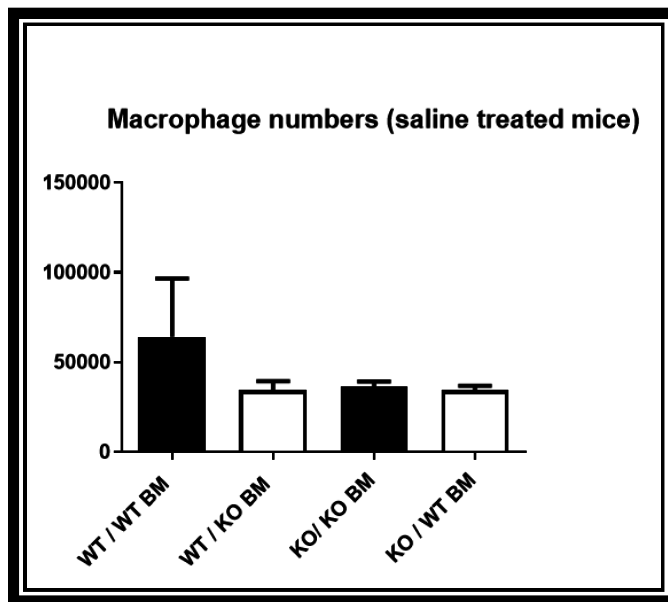

Fig. 8 Levels of airway inflammation in response to saline in chimeric mice. Fig. 8-A, show the total cell counts in BAL fluid. Chimeric WT mice with WT hematopoietic cells (33833± 2579) and *Par-2*$^{-/-}$ with *Par-2*$^{-/-}$ hematopoietic cells (36833± 3027) (black columns). WT chimeric mice with *Par-2*$^{-/-}$ hematopoietic cells (35000± 5196) and *Par-2*$^{-/-}$ chimeric mice with WT hematopoietic cells (34833 ± 2651) (white columns). Fig. 8-B, show Eosinophil numbers in BAL fluid. Chimeric WT mice with WT hematopoietic cells (44.69± 29.84) and *Par-2*$^{-/-}$ with *Par-2*$^{-/-}$ hematopoietic cells (29.17± 29.17) (black columns). WT chimeric mice with *Par-2*$^{-/-}$ hematopoietic cells (20.83± 20.83) and *Par-2*$^{-/-}$ chimeric mice with WT hematopoietic cells (24.17± 24.17) (white columns). In figures 8-C, D and E we are showing the numbers of Neutrophil, Lymphocyte and Macrophage in BAL fluid respectively (differences between all groups are not significant). Data is presented as mean ± SEM.

2.4 Discussion:

Bone marrow chimera is a useful and reliable tool for determining the biological contribution of the hematopoietic and stromal components and they have been used widely in the immunology field (278) (279) (280). This model was used to determine whether the development of allergic airway inflammation requires PAR-2 activation on airway structural cells or hematopoietic cells.

For the first time in this chapter, we show that PAR-2 expression on structural cells is critical for the development of allergic airway inflammation; however, PAR-2 expression on hematopoietic cells contributes to the full effect.

In vitro studies from our lab showed that activating PAR-2 induces airway epithelial cells to produce GM-CSF and eotaxin (113). In addition, other laboratories have shown that in vitro PAR-2 activation induce the release of a variety of mediators from airway epithelial cells, including IL-6, IL-8 (273) , and TSLP (134). However, we show in this chapter that in vivo activation of PAR-2 increases the levels of TSLP, IL-6, KC-Gro, and Eotaxin in mouse lungs. This suggests that PAR-2 activation in the airways causes the production of inflammatory mediators that are required for the Th2 skewing of the immune response. Other epithelial driven cytokines, IL-25 and IL-33 levels in the lungs did not change upon PAR-2 activation (detected by qPCR), implying that IL-25 and IL-33 may not be required to initiate allergic responses in response to PAR-2 activation by allergens with serine proteinase activity, and TSLP production may probably be sufficient (281).

TSLP is a pluripotent cytokine that was known to enhance B and T cells survival and maturation. TSLP is mainly produced by epithelial cells, and it has been demonstrated that it is upregulated in asthmatic patients. Interestingly, allergens with proteinase activity activate PAR-2, which causes the release of TSLP from airway epithelial cells(282) (132). In addition, TSLP induces the

maturation and activation of DCs with subsequent Th2 shifting of the immune response (114). Furthermore, allergic airway inflammation in mice lacking the TSLP receptor cannot be induced. Therefore, TSLP may play a critical role in allergic asthma. According to data focusing on the role of TSLP in allergic inflammation, we propose that TSLP production from airway epithelium induced by PAR-2 activation with exogenous or endogenous serine proteinase is a critical step for the development of allergic airway inflammation, which is missing in *Par-2*$^{-/-}$ mice and KO / WT BM chimeric mice.

IL-6 is a pleiotropic cytokine secreted by inflammatory cells and epithelial cells (139), but it has been shown that elevated levels of IL-6 mRNA are present in mouse primary lung epithelial cells but not in lung resident immune cells. (140). Studies suggested that IL-6 plays an essential role in deciding the type of adaptive immune response. It has been demonstrated that IL-6 induces Th2 differentiation of $CD4^{+}$ T cells while suppressing Th1 differentiation. IL-6 enhances the differentiation of Th2 cells by activating endogenous IL-4 production by CD4 T cells (141) (142). In addition, IL-6 has a regulatory role on $CD4^{+}$ T cells by supporting its activation with anti-apoptotic signals (143) (142) (144). Interestingly, it has been demonstrated that IL-6 can be secreted from airway epithelium (283) .

Asthmatic patients have higher levels of IL-8 and GM-CSF in their bronchial epithelial cells (284). KC-Gro, is a neutrophil chemoattractant; (IL-8-related protein in rodents)(285). Free IL-8 has been found in the serum and bronchial tissue of people with severe atopic asthma but not in healthy or mild atopic asthma patients, implying that IL-8 could be a marker for severe asthma (285) .

GM-CSF is a hematopoietic cytokine linked to Th2 immune responses (286) (287). It has been demonstrated that GM-CSF can be induced by HDM in vitro and vivo models. Interestingly,

blocking GM-CSF during the sensitization phase or loss of GM-CSF expression as in KO model, (288)abrogates allergic sensitization (289) (290) (291). Exogenous GM-CSF also increased allergic sensitization and airway eosinophilic inflammation in response to otherwise innocuous antigens. (292) (37).

According to data mentioned above, PAR-2 activation on airway epithelium by exogenous (such as HDM and cockroach) or endogenous serine proteinases (in case of ovalbumin model) produce mediators mentioned above which induce Th2 skewing of the immune response and create an inflammatory environment that enhances allergic responses. However, we cannot exclude proinflammatory mediators induced by PAR-2 activation on hematopoietic cells such as dendritic cells and macrophages which also play an important role in developing the full effect.

Interestingly, activation of PAR-2 on alveolar macrophages and DCs can cause the release of TNF, which causes the release of proinflammatory mediators like TSLP and GM-CSF from the airway epithelium, promoting allergic sensitization (116) (117) (118). In addition, our lab published that PAR-2 promotes allergic sensitization to an inhaled antigen through the production of TNF (205). Because we expect PAR-2 expression on hematopoietic cells to play a role in allergic sensitization based on data from the BM chimaera experiment in this chapter. So, studying the role of PAR-2 expression on other immune cells, particularly alveolar macrophages, will be one of our main future directions, as they are the most abundant cells in the alveolar spaces and conducting airways (293), they play a major role in allergic sensitization (164) (294), and they can produce large amounts of TNF.

Other tissue resident cells, such as smooth muscle and endothelial cells, express PAR-2, and the data presented in this chapter suggest that airway epithelium may be the primary cells that are

PAR-2 activated to cause allergic airway inflammation. This does not rule out the possibility that activation of PAR-2 on other tissue resident cells plays a role in allergic airway inflammation. (295,296).

Future work can include cell specific deletion of PAR-2 to better understand the role of PAR-2 on different cells. To prove our hypothesis, we will induce asthma in mice lacking PAR-2 expression in airway epithelium only. We already have PAR-2 floxed mice and crossing the floxed mice with mice expressing cre recombinase only on airway epithelium such as the commercially available CC10-Cre mouse (297) will produce mouse that has no PAR-2 expression on airway epithelium. This mouse would allow us to differentiate between PAR-2 expression on airway epithelium and expression on other tissue resident cells in the lungs.

Clara cells are non-ciliated secretory cells that line the lung's bronchioles. The CC10 gene encodes the Clara cell 10-kDa protein, which is produced in airway epithelial cells (297) (51).

Although the Cre-lox system gives exceptional control over gene expression, but some restrictions must be considered. In adult mice, Cre recombinase is mainly directed at genes expressed in a small number of cells. However, Cre recombinase expression in the germline or during early development may be unexpectedly transient. Many genes are normally expressed in the germline or at various stages of development, though the significance of this expression is generally unknown, it doesn't take much Cre-recombinase expression to operate on the loxP sites and cause recombination events. As a result, it's critical to devise tests capable of detecting these unanticipated and undesirable recombination events (299) .

PAR-2 activation by exogenous proteinases inhaled with allergens or endogenous proteinases generated by inflammatory and other cells plays a key role in allergen-induced airway

inflammation. As a result, PAR-2 antagonists, or the neutralization of mediators released after PAR-2 activation, could be an appealing therapeutic approach for preventing asthmatic airway inflammation. The capacity to apply targeted therapeutic techniques will be enhanced if the involvement of airway epithelial cells is determined.

Chapter 3: The role of PAR-2 expression on the development of CD4$^+$ T memory cells following exposure to house dust mite

3.1 Introduction:

In chapter 2, we showed that PAR-2 expression on structural cells possibly, airway epithelium is critical for the development of allergic airway inflammation. Also, we indicated that activation of PAR-2 in the airways induced the production of inflammatory mediators essential for Th2 skewing of the immune response. In this chapter, we are investigating the role of PAR-2 expression on the formation of Th2 memory cells upon exposure to house dust mite (HDM).

Our lab demonstrated that blocking PAR-2 activation during the sensitization phase attenuated allergic airway inflammation (19) (3). Also, blockage of PAR-2 activation in the airways during HDM sensitization decreases HDM-specific splenic cells *ex-vivo* proliferation. In addition, CD4$^+$ T cells from the spleens of mice sensitized to HDM after PAR-2 inhibition in the airways were not able to induce airway inflammation in naive mice, unlike CD4$^+$ T cells from mice exposed to HDM in the absence of the blocking antibody (3).

These findings imply that PAR-2 activation plays a significant role in allergic sensitization. This raises the question of whether the attenuation of allergic sensitization mediated by loss of PAR-2 expression or preventing PAR-2 activation is linked to a decrease in CD4 $^+$ T memory cells following allergen exposure with subsequent disease transfer failure. This is what we're going to investigate in this chapter.

This chapter will report for the first-time results from the adoptive transfer of CD4$^+$ T cells between WT and *Par-2* $^{-/-}$ mice in order to better understand the impact of PAR-2 activation in the airways

on allergic sensitization. We will also present results from WT and *Par-2* $^{-/-}$ mice to study the role of PAR-2 expression in the development of CD4^{+} T memory cells following exposure to HDM. Adoptive transfer of T cells is a popular method that has been used widely in asthma studies (300) (301) (302)(303). It has been known that during an immune response to allergens, specific memory Th2 cells are presented. When mice recover from a single episode of allergic asthma, allergen-specific memory Th2 cells persist in their lungs (304) (305) (306). When these allergen-specific memory T cells are exposed to the specific allergen, they respond quickly and vigorously, resulting in eosinophilic lung inflammation (305). Furthermore, recurrent allergen exposure will stimulate memory Th2 cells, resulting in chronic airway inflammation and asthma flare-ups (307).

Naïve CD4^{+} T cells, to be efficiently activated, must engage with antigen, presented by dendritic cells (DCs). This interaction takes place in secondary lymphoid, which allows antigen-specific T cells and DCs to interact. CD4^{+} T cells differentiate into activated effector cells that can migrate to infection sites. These activated effector CD4^{+} T cells expand and contract, forming the memory pool with 90-95 percent of lymphocytes dying during contraction and the remaining 5-10% develop into memory T cells (central memory T cells (Tcm), effector memory T cells (Tem), and tissue-resident memory T cells (Trm).

CD45RO, CCR7, CD62L, and CD44 are molecules located on the surface of central memory T cells, which can be found in lymph nodes and tonsils (308) . Tem cells, unlike Tcm, do not express surface molecule, CD62L. Tem is primarily located in non-lymphoid peripheral tissue such as the lungs, liver, and intestines (309,310), and are capable of blood-tissue recirculation (308). CD4^{+} Trm cells expressing CD44, CD69 and not expressing CD62L are the first line of defense against infections since they occupy specific sites without recirculating (308,309,311,312). They are found in the digestive tract, the female reproductive system, the lungs, the skin, and the brain. Also, they

respond faster and more effectively than other memory T cells (252,308). However, the significance of $CD4^+$ Trm cells in tissues is less well defined than that of $CD8^+$ Trm cells (252,308).

Since, $CD4^+$ T cells from the spleens of mice sensitized to HDM after blocking PAR-2 in the airways were not able to induce airway inflammation in naive mice, unlike $CD4^+$ T cells from mice exposed to HDM in the absence of the blocking antibody (3). So, we hypothesize that the loss of PAR-2 expression as in *Par-2* $^{-/-}$ or inhibition of PAR-2 activation using antibodies, abrogates allergic sensitization with attenuation of antigen-specific $CD4^+$ memory T cells formation upon exposure to HDM.

3.2 Materials and Methods:

3.2.1 Animals: *Par-2*$^{-/-}$ mice were purchased from UC DAVIS KOMP (Knockout mouse project) repository (strain: C57BL/6N-F2rl1tm1a(EUCOMM)Wtsi/J). Mice were bred and housed at University of Alberta mouse facility. For more details see methods in chapter 2.

3.2.2 House dust mite asthma model

Male on a C57 BL/6J background (6–8 weeks of age) were used. Mice were sensitized and challenged using HDM according to the protocol shown in figure 1. Mice were sensitized intranasally (I.N) with 25 ug HDM for 5 consecutive days from day 1 to day 5. Mice were challenged I.N with 25 ug HDM for 4 consecutive days from day 11 to day 14. On day 15, mice were euthanized, and BAL fluid was collected to analyze allergic airway inflammation

Fig. 1

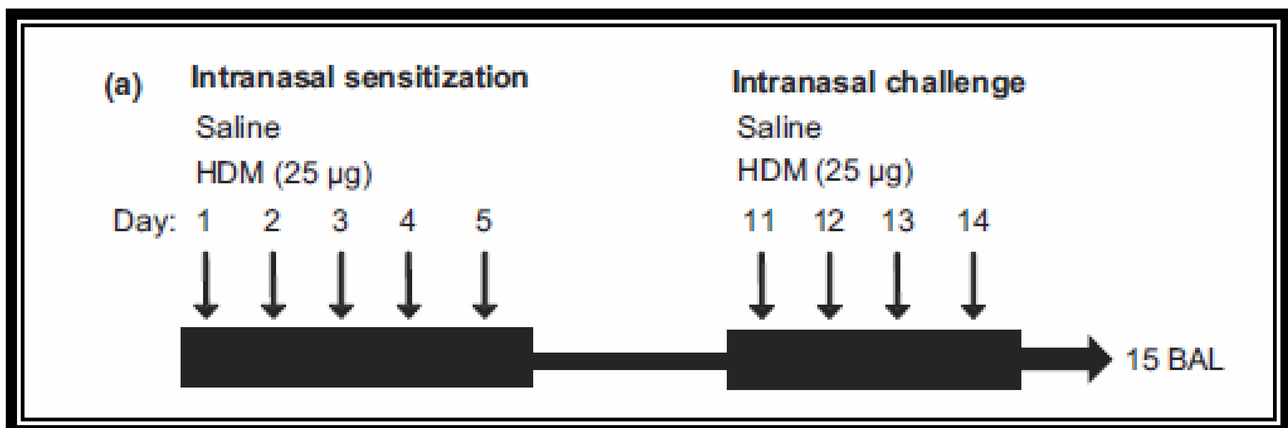

Fig. 1: Model of HDM- induced allergic airway inflammation in mice. Mice were sensitized with I.N 25 ug HDM for 5 consecutive days. Mice were challenged I.N with 25 ug HDM for 4 consecutive days. On day 15 mice were euthanize and Bal fluid was collected to analyze airway inflammation.

3.2.3 Administration of HDM

Sensitization Phase (day 1 to day 5): Lyophilized, whole-body extracts of HDM (Dermatophagoides pteronyssinus) were purchased from Stallergenes Greer (Cat # XPB70D3A2.5) and re-suspended at a concentration of 6 mg/mL of total protein in 0.9% sterile saline and a dilution of 1mg/ml was prepared. Anesthetic was prepared by mixing 0.75ml ketamine (100mg/ml) + 0.25 ml xylazine (20 mg/ml) and diluted to 5 ml with 0.9% sterile saline. Every 20 g mouse was injected with 0.125 ml anesthetic cocktail by I.P injection and 25µl (25µg) HDM was pipetted into the right nostril once daily for 5 days using sterile pipet tips. The mouse was returned to its cage to rest, and the bottom of the cage was lined with paper towel to keep the mouse from breathing in the bedding while sedated.

Challenge phase (day 11 to day 14): Same as sensitization phase but the mice were challenged once daily for 4 consecutive days.

3.2.4 Measurement of airway inflammation:

Bronchoalveolar lavage (BAL) was performed, 24 hrs after the final I.N allergen challenge to assess airway inflammation. For more details see methods in chapter 2.

3.2.5 CD4^{+} T cell transfer

Mice were euthanized as mentioned above and spleens were collected on day 10 from mice that received I.N saline or HDM from days 1 through 5. Spleens were pooled and disrupted using 2 glass slides in RPMI 1640 media (Gibco) containing 10% foetal bovine serum (FBS), 1% Penicillin-streptomycin, 1% L-Glutamine, 1% Non-essential amino acids, 0.01% b-Mercaptoethanol, and a dispersed suspension was obtained by passing the cell suspension through a 40um cell strainer. Red blood cells were lysed by using RBC lysis buffer from BD Biosciences,

Cat# 555899. Cells were re-suspended in complete RPMI 1640 media to create single cell suspensions. CD4+ T cells were purified from splenocytes using an EasySep Mouse CD4+ T Cell isolation negative selection kit, that yielded a population with ~ 85% purity (StemCell Technologies, Cat# 19852). Naïve mice (unsensitized mice) were given 3 million of these T cells intravenously by tail vein injection. These mice were then challenged twice with 25 ug HDM or saline I.N, 6 hrs following the $CD4^{+}$ T cell transfer, and again 2 days later. BAL were performed 24 hr after the final challenge (fig. 2).

Fig. 2

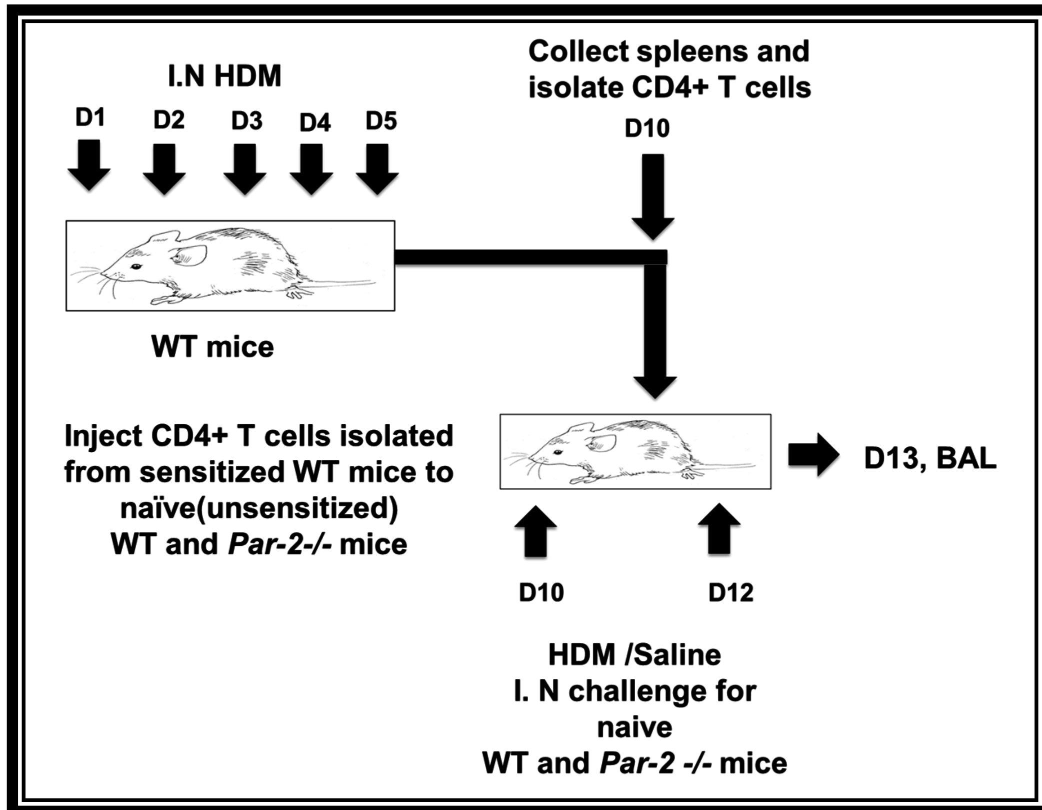

Fig 2: ($CD4^{+}$ T cells adoptive transfer model): WT / *Par-2*$^{-/-}$ were sensitized intranasally (I.N) with 25 ug HDM in 25 ul saline for 5 consecutive days (day1 to day 5). On Day 10, spleens were collected and $CD4^{+}$ T cells were isolated as mentioned in methods section 3.2.5. Three million $CD4^{+}$ T cells were injected via tail vein injection to naïve (unsensitized mice) WT / *Par-2* $^{-/-}$ mice. The naïve mice were challenged I.N with 25 ug HDM or saline, 6 hrs after the transfer on day 10 and on day 12.

3.2.6 Flow cytometry for lung and spleen cells

WT and *Par-2*$^{-/-}$ mice were treated with I.N 25 ug HDM in 25 ul saline for 5 consecutive days or 5 consecutive days every week for 3 weeks. Also, we did a long-term study analysis to investigate the presence of tissue resident memory cells after 5 months. The mice were treated with I.N 25 ug HDM for 3 weeks as mentioned above. Then the mice were left for 5 months in the animal facility. After 5 months, the mice were challenged twice with I.N 25 ug HDM before harvesting (day 1 and day 3).

Mice were euthanized on day 10 or day 22 or after 5 months respectively. Lungs were harvested and cut into small pieces in HBSS with 10 % FBS + collagenase and Dnase. Lung cells were collected using single MACS C tubes and gentleMACS Dissociator. The spleens were disrupted in RPMI 1640 media (Gibco, Carlsbad, CA, USA) containing 10% foetal bovine serum (FBS), 1% Penicillin-streptomycin, 1% L-Glutamine, 1% Non-essential amino acids, 0.01% b-Mercaptoethanol, and a dispersed suspension was obtained by passing the cell suspension through a 50-um cell strainer. Red blood cells were lysed by adding 20 mM Tris and 140 mM ammonium chloride, re-suspended in complete RPMI 1640 media. Cells were blocked by adding mouse Fc blocking reagent (Mitenyi Biotec Cat# 130-092-575) up to 10 X 10^{6} in 90 ul PBS FACS+ 10ul Fc blocker in 4 degrees for 10 minutes. Cells were washed with 5 ml PBS Facs and centrifuged 5 minutes in 300g at 4 degrees. Cells were resuspended at concentration of 200,000 cells /200ul in PBS Facs and we proceeded to antibody (Ab) staining. Abs concentrations were suggested according to manufacturer's recommendations.

Flow cytometry for detection of CD4 $^{+}$Trm in the lungs (CD3^{+} CD4^{+} CD44^{+} CD69^{+} ST2^{+} CD62L^{-}) were performed using following markers (fig.3): CD3 - eFluor450 (ThermoFischer Scientific, cat. # 48-0032-82), CD4 - PerCP-eFluor710 (ThermoFischer Scientific, cat # 46-0042-82), CD44

- PE-eFluor610 (ThermoFischer Scientific, cat # 61-0441-82), CD69 - AlexaFluor700 (ThermoFischer Scientific cat # 56-0691), ST2(IL-33R) - PE (ThermoFischer Scientific catalog # 12-9335-82) and CD62L- BV-711 (BioLegend, cat#104445).

Flow cytometry for lung and spleen cells for detection of $CD4^+$ T memory cells subtypes were performed using following markers: anti-CD4 (APC) Biolegend, cat#100516, anti -CD3(BV-605) Biolegend, cat # 100237, anti-CD44(PE efluor 610) Thermo Scientific cat#61-0441, anti CD62L(BV-711) Biolegend cat#104445 and anti-CD69 (Alexa Fluor 700) Invitrogen Thermo scientific cat # 56-0691 markers (fig. 4).

$CD4^+$ T Memory cells subtypes were detected as follow (fig.4): **central** ($CD4^+$ $CD3^+$ $CD44^+$ $CD62L^+$ $CD69^{-)}$, **effector** ($CD4^+$ $CD3^+$ $CD44^+$ $CD62L^-$ and $CD69^-$) and **tissue resident** ($CD4^+$ $CD3^+$ $CD44^+$ $CD62L^-$ $CD69^+$).

3.2.7 Statistical analysis: Statistics were performed using Prism 5 (GraphPad Software, La Jolla California USA). Data are presented as Mean ± SEM; ns (not significant), *P<0.05, **P<0.01, ***P<0.001 between the groups and were analyzed for significance using a one-way ANOVA and Tukey's multiple comparison tests. Two-tailed unpaired *t*-test was performed for comparison between two groups only.

Fig. 3

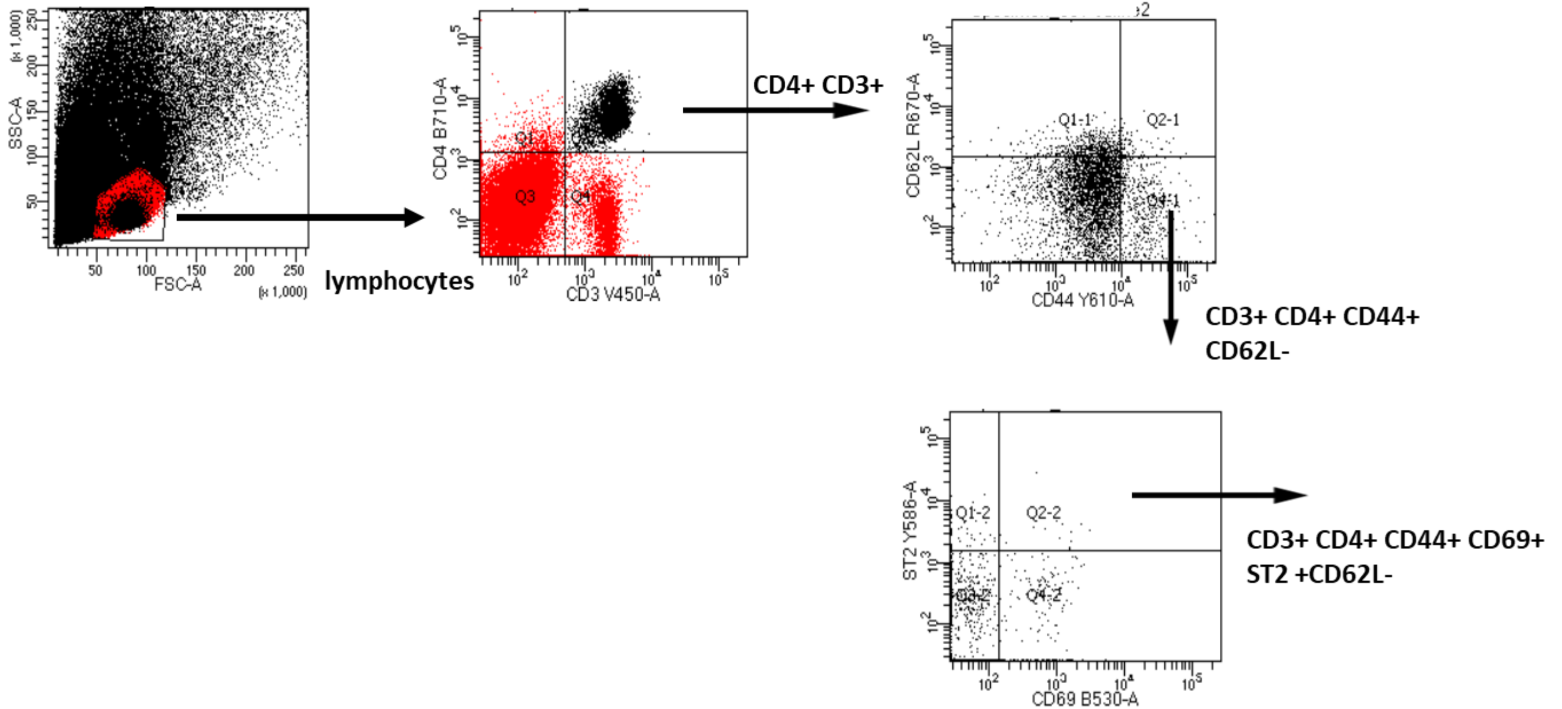

Fig. 3: Gating strategy for detection of CD4$^+$ Trm in the lungs. Total lymphocytes were gated as low SSC and low FSC from total population (top left plot). Gated lymphocytes were plotted against CD3(eFluor450) and CD4(PerCP-eFluor710) to detect CD4$^+$ T cells population (top middle plot). CD4$^+$ CD3$^+$ lymphocytes were plotted against CD62L (BV-711) and CD44 (PE-eFluor610) to determine CD44$^+$ CDD62L $^-$cells (effector and tissue resident memory CD4$^+$ T cells) (top right plot). CD4$^+$ CD3$^+$ CD44$^+$ CDD62L$^-$ population were plotted against CD69 (AlexaFluor700) and ST2(PE) and double positives used to determine CD4$^+$ Trm. Same strategy was done for detection of CD4$^+$ Trm in the spleens.

Fig. 4

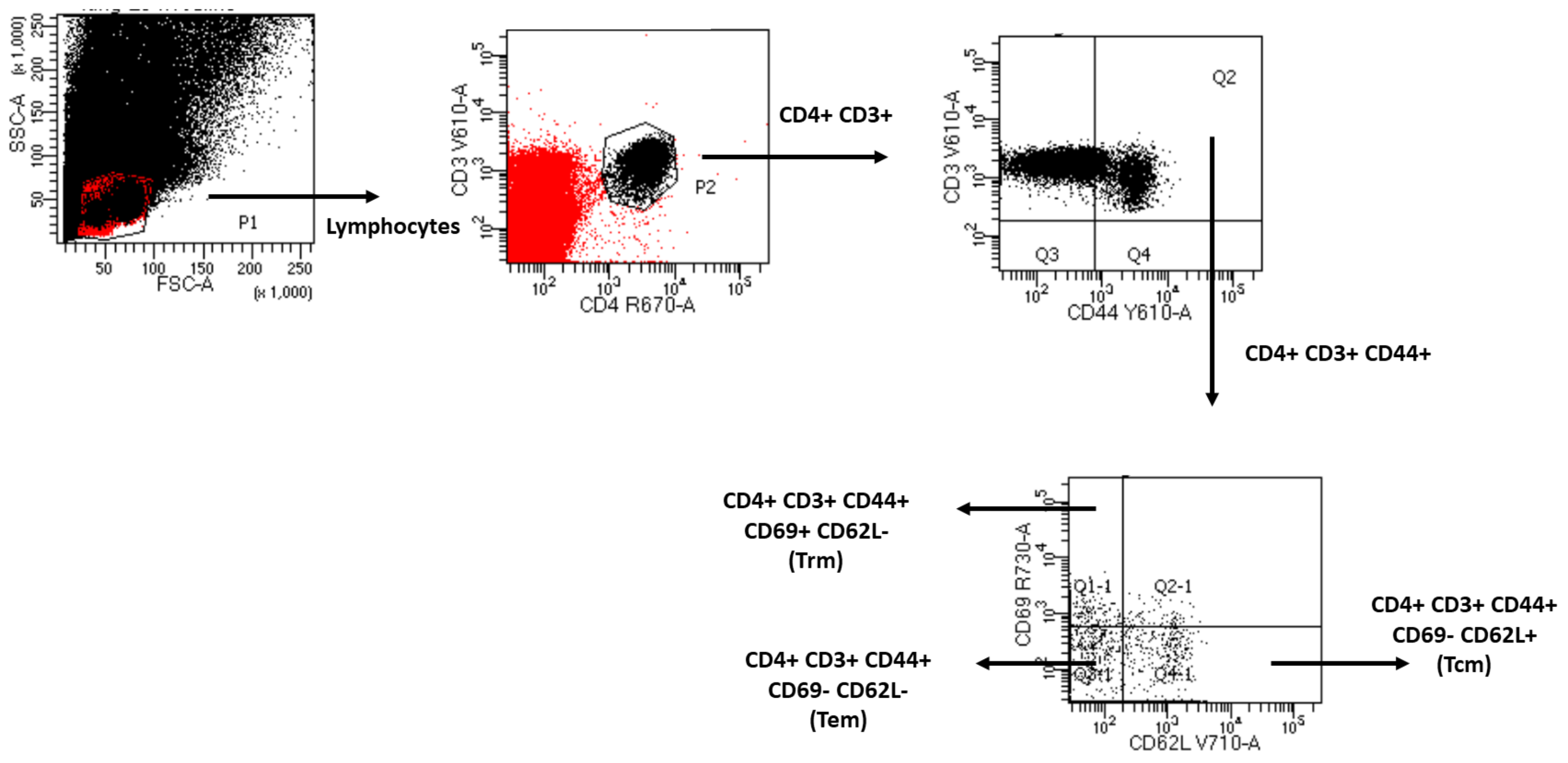

Fig. 4: Gating strategy for detection of $CD4^+$ T memory cell subsets in the lungs**.** Total lymphocytes were gated as low SSC and low FSC from total population (top left plot). Gated lymphocytes were plotted against CD3(BV-605) and CD4(APC) to detect $CD4^+$ T cells (top middle plot). $CD4^+$ $CD3^+$ lymphocytes were plotted against CD3 (BV-605) and CD44 (PE) and the double positive used to determine $CD4^+$ T memory cells (effector, central and tissue resident memory $CD4^+$ T cells) (top right plot). $CD4^+$ $CD3^+$ $CD44^+$ population were plotted against CD69 (AlexaFluor700) and CD62L (BV-711) to determine CD4+ T memory cells subtypes as follow: CD4+ CD3+ CD44+ CD69+ CD62L- (Trm), CD4+ CD3+ CD44+ CD69- CD62L- (Tem) and CD4+ CD3+ CD44+ CD69- CD62L+ (Tcm). Same strategy was done for detection of $CD4^+$ T memory cell subsets in the spleens.

3.3 Results:

3.3.1 CD4 $^{+}$ T cells from WT mice treated with HDM can transfer the disease to naïve WT and Par-2$^{-/-}$ mice

Naïve WT mice (unsensitized mice) transferred with CD4^{+} T cells from WT mice treated with HDM and challenged with HDM showed increased allergic airway inflammation characterized by increased total cell counts(fig.5-A) and numbers of eosinophil (fig. 5-B) in BAL fluid compared to mice challenged with saline. Naïve *Par-2*$^{-/-}$ mice transferred with CD4^{+} T cells from WT mice treated with HDM and challenged with HDM showed increased allergic airway inflammation characterized by increase in total cell counts (fig.5-A) and numbers of eosinophil (fig. 5-B) in BAL fluid compared to saline challenged mice. Naïve WT and *Par-2*$^{-/-}$ mice challenged with saline did not develop allergic airway inflammation. The results indicate that, transfer of CD4^{+} T cells from WT mice treated with HDM can induce allergic airway inflammation in naïve WT and *Par-2*$^{-/-}$ mice after challenging with HDM and interestingly, *Par-2*$^{-/-}$ mice developed allergic airway inflammation with similar levels to WT mice (fig. 5-A and 5-B).

Fig 5-A: Total cell counts in BAL Fluid

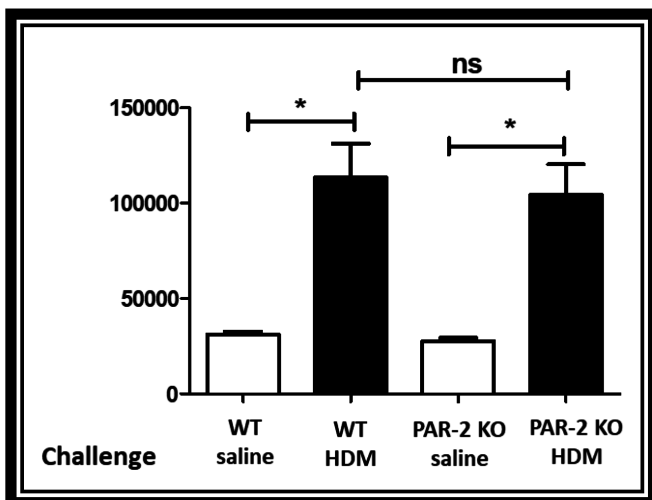

Fig 5-B: Eosinophil counts in BAL fluid

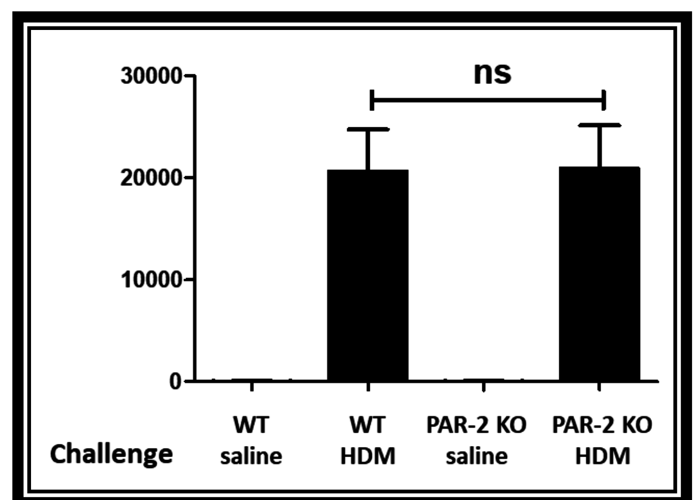

Fig 5: Levels of airway inflammation following adoptive transfer of $CD4^+$ T cells from WT mice. (Fig 5-A) WT and *Par-2$^{-/-}$* mice challenged with saline and received $CD4^+$ T cells from WT mice treated with HDM showed total number of cells in BAL fluid: (31,000 ± 1,871) and (27,600 ± 1,720) respectively (white columns). WT and *Par-2$^{-/-}$* mice challenged with HDM and received $CD4^+$ T cells from WT mice treated with HDM showed total number of cells in BAL fluid: (113, 333 ± 17, 888) and (104,333±15,996) respectively (black columns). (Fig 5-B) WT and *Par-2$^{-/-}$* mice challenged with HDM and received $CD4^+$ T cells from WT mice treated with HDM showed total number of eosinophils in BAL fluid: (20,593 ± 4,130) and (20,877 ± 4,232) respectively (black columns). WT and *Par-2$^{-/-}$* mice challenged with saline and received $CD4^+$ T cells from WT mice treated with HDM showed total number of cells in BAL fluid: (32.00 ± 32.00) and (31.00 ± 31.00) respectively (white columns). N=12 for all. Data are presented as Mean ± SEM; ns (not significant), *P<0.05, **P<0.01,

3.3.2 CD4 $^+$ T cells from Par-2$^{-/-}$ mice treated with HDM can not transfer the disease to naïve WT mice

As the experiment in figure 5 , naïve WT mice transferred with $CD4^+$ T cells from HDM treated WT mice and challenged with HDM showed increased allergic airway inflammation characterized by increased total cell counts (fig.6-A) and numbers of eosinophil (fig. 6-B) in BAL fluid compared to mice challenged with saline. But naïve W*T* mice transferred with $CD4^+$ T cells from *Par-2$^{-/-}$* treated with HDM mice and challenged with HDM showed significant decreased in allergic airway inflammation characterized by decrease in total cell counts (fig.6-A) and numbers of eosinophil (fig. 6-B) compared to naïve WT mice transferred with $CD4^+$ T cells from HDM sensitized WT mice and challenged with HDM. Donor WT and *Par-2$^{-/-}$* mice sensitized with HDM for 5 days and harvested on day 10 developed allergic airway inflammation, however, the levels were lower than challenged mice(fig. 6- A and B).

The results indicate that, transfer of $CD4^+$ T cells from *Par-2$^{-/-}$* mice sensitized with HDM were not able to transfer the disease to naïve WT mice . However, the transfer of $CD4^+$ T cells from WT mice sensitized with HDM was able to transfer the disease to naïve WT mice (fig. 6)

Fig 6-A: Total cell counts in BAL fluid

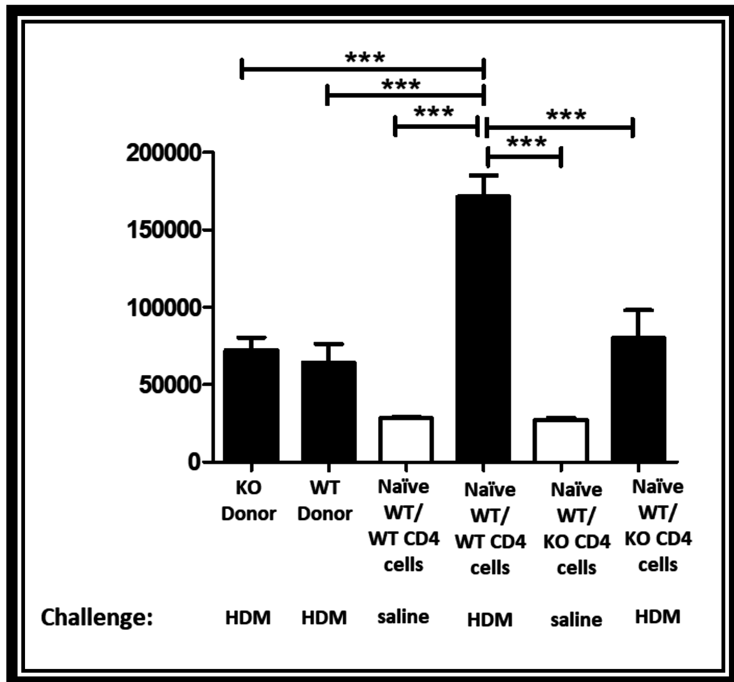

Fig 6-B: Eosinophil numbers in BAL fluid

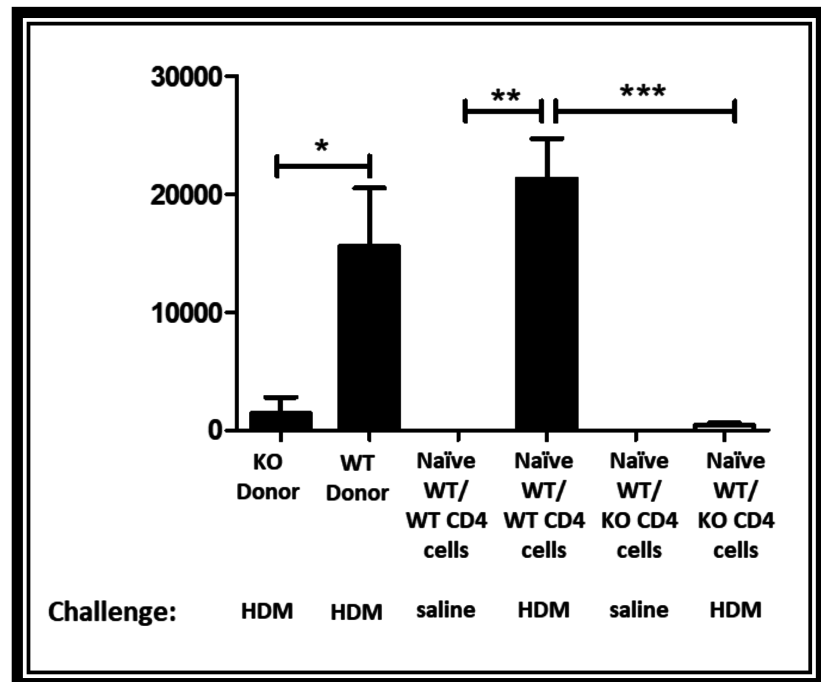

Fig 6: Levels of airway inflammation following adoptive transfer of $CD4^+$ T cells from *Par-2$^{-/-}$* mice**.** (Fig 6-A) Donor *Par-2$^{-/-}$* mice showed total cell counts in Bal fluid on day 10: (72,000 ± 8,602) (1st column). Donor WT mice showed total cell counts in Bal fluid on day 10: (64,000 ± 12,49) (2nd column). Naïve WT mice challenged with HDM after receiving $CD4^+$ T cells from WT mice treated with HDM showed total cell countsin BAL fluid: (171,400 ± 13,891) (column #4). Naïve WT mice challenged with HDM after receiving $CD4^+$ T cells from *Par-2$^{-/-}$* mice showed total cell counts: (80,000 ± 18,166) (column #6). The total cell counts in BAL fluid for all saline groups were very low. (Fig 6-B**)** Donor *Par-2$^{-/-}$* mice showed total numbers of eosinophil in Bal fluid on day 10: (1,400 ± 1400) (1st column). Donor WT mice showed total numbers of eosinophil in Bal fluid on day 10: (10, 938 ± 2, 142) (2nd column). Naïve WT mice challenged with HDM after receiving $CD4^+$ T cells from WT mice treated with HDM showed total numbers of eosinophil in BAL fluid: (21,240 ± 3,471) (column #4). Naïve WT mice challenged with HDM after receiving $CD4^+$ T cells from *Par-2$^{-/-}$* mice showed total numbers of eosinophil: (420.0 ± 200.4) (column #6). The total numbers of eosinophil in BAL fluid for all saline groups were very low. N=8 for all. Data are presented as Mean ± SEM; ns (not significant), *P<0.05, **P<0.01, ***P<0.001 between the groups and were analyzed for

3.3.3 HDM induced the development of CD4 $^{+}$ Trm in treated mice

WT mice treated with HDM for 3 weeks showed significnat increase in the percentage of $CD4^{+}$ Trm ($CD4^{+}$ $CD3^{+}$ $CD44^{+}$ $CD69^{+}$ $ST2^{+}$ $CD62L^{-}$) in the lungs and spleens compared to saline treated WT mice (fig.7-A and fig. 7-B). WT mice treated with HDM for 5 days showed significant increase in the percentage of $CD4^{+}$ Trm in the lungs and spleens compared to saline treated WT mice (fig. 7-C and fig. 7-D).

Moreover, WT mice treated with HDM for 3 weeks showed significant increase in the percentage of $CD4^{+}$ Trm in the lungs after 5 months compared to saline treated WT mice (fig. 7-E).

The results indicate that HDM was able to induce $CD4^{+}$ Trm cells in the lungs and spleens of treated mice and it can be maintained in the lungs for long term (5 months).

Fig 7-A: Percentage of lung $CD4^+$ Trm over total $CD4^+$ T cells (mice treated with HDM for 3 weeks)

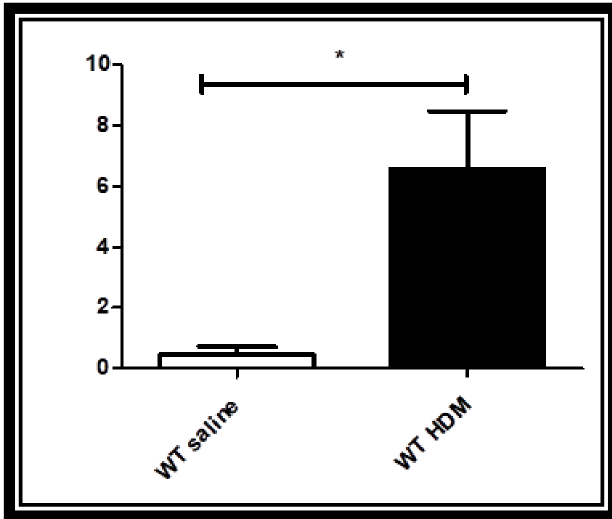

Fig 7-B: Percentage of spleen $CD4^+$ Trm over total $CD4^+$ T cells (Mice treated with HDM for 3 weeks)

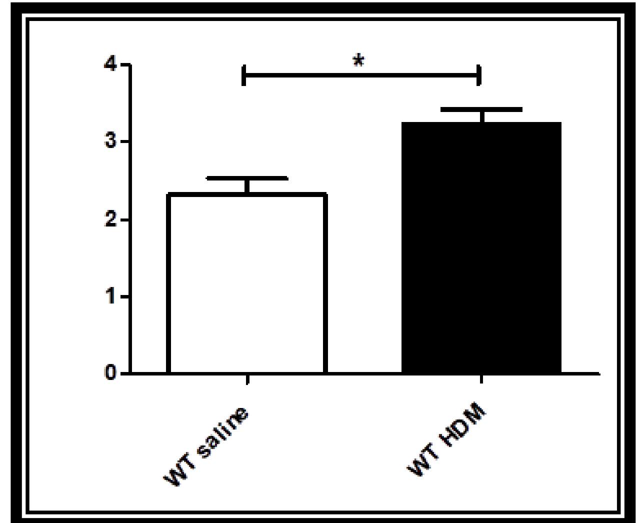

Fig 7-C: Percentage of lung $CD4^+$ Trm over total $CD4^+$ T cells (Mice treated with HDM for 5 days)

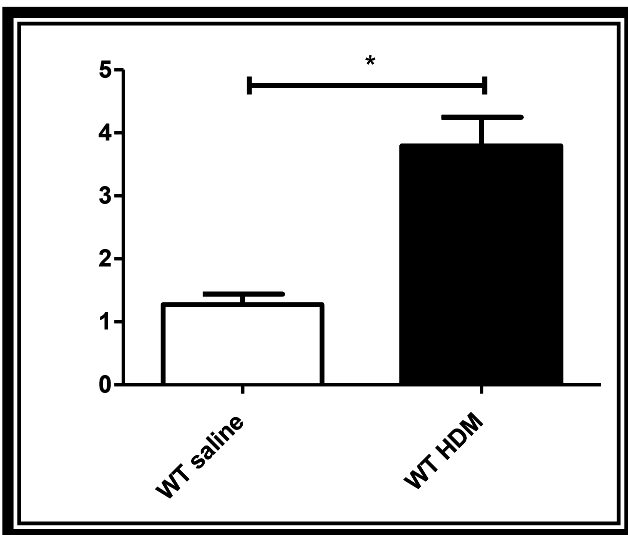

Fig 7-D: Percentage of spleen $CD4^+$ Trm over total $CD4^+$ T cells (Mice treated with HDM for 5 days)

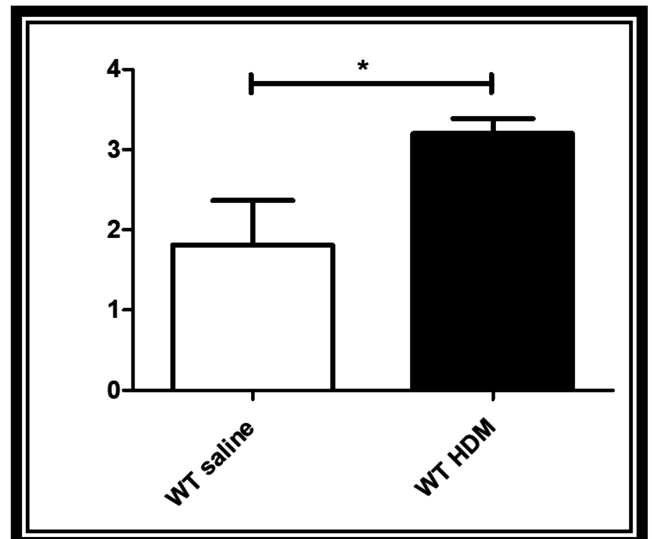

Fig 7-E: Long term study for 5 months (Percentage of lung CD4⁺Trm cells over total CD4⁺ T cells)

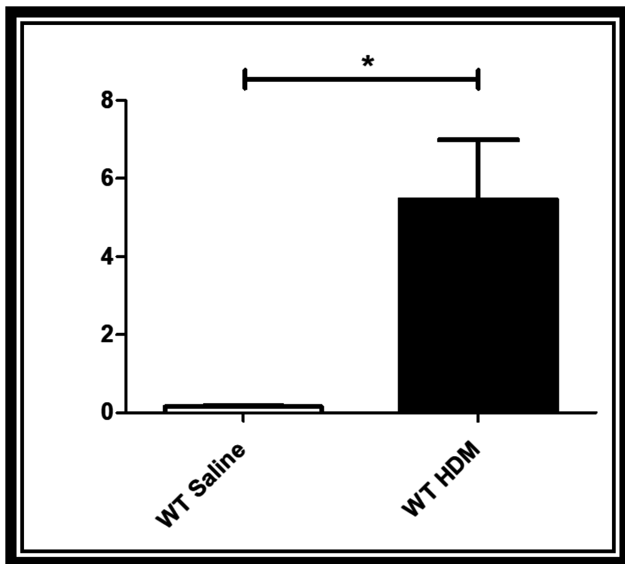

Fig 7: Percentages of $CD4^+$ Trm in the lungs and spleen of HDM treated WT mice. All data are presented as the percentages of $CD4^+$ Trm over total $CD4^+$ T cells. The percentage of Trm cells in the lungs of WT mice treated with saline or HDM for 3 weeks is (0.4473 ± 0.2047) and (8.124 ± 1.788) respectively, N=6 (Fig 7-A). The percentage of Trm cells in the spleens of WT mice treated with saline or HDM for 3 weeks is (2.325 ± 0.2050) and (3.230 ± 0.1912) respectively, N=4 (Fig 7 -B). The percentage of Trm cells in the lungs of WT mice treated with saline or HDM for 5 days is (1.275 ± 0.1652) and (3.792± 0.4579) respectively, N=6 (Fig 7-C). The percentage of Trm cells in the spleens of WT mice treated with saline or HDM for 5 days is (1.813 ± 0.5509) and (3.198 ± 0.1907) respectively, N=6 (Fig 7-D). The percentage of Trm cells in the lungs of WT mice treated with saline or HDM after 5 months is (0.1480 ± 0.03597) and (5.460 ± 1.528) respectively, N=5(Fig. 7-E). Data are presented as Mean ± SEM; ns (not significant), *P<0.05, **P<0.01, ***P<0.001 between the groups and were analyzed for significance using two-tailed unpaired *t*-test was performed for comparison.

3.3.4 Five days treatment with HDM induced the development of lung CD4 $^{+}$ Tem in treated mice

The percentage of lung CD4^{+} Tem (CD44^{+}, CD69^{-} and CD62L^{-}) was significantly higher in WT mice treated with HDM for 5 consecutive days compared to saline treated mice (fig 8-A). The difference in the percentages of spleen CD4^{+} Tem between WT mice treated with HDM for 5 days (fig.8-B) or 3 weeks (fig. 8-D) and saline-treated WT mice were not significant. The difference in the percentages of lung CD4^{+} Tem between mice treated with HDM for 3 weeks and saline treated mice was not significant (fig. 8-C).

The findings indicated that HDM treatment for 5 days, rather than 3 weeks, was able to generate CD4^{+} Tem in the lungs but not in the spleens of WT mice.

Fig. 8-A: ***Percentage of lung CD4^{+} Tem over total CD4^{+} T cells (mice treated with HDM for 5 days)***

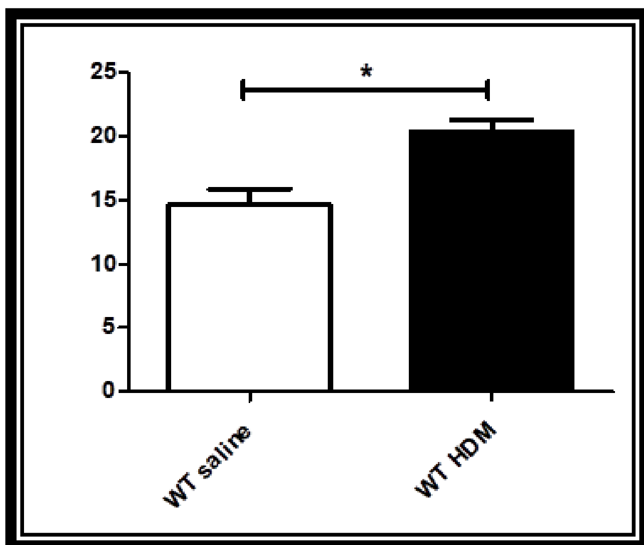

Fig. 8-B: Percentage of spleen CD4^{+} Tem over total CD4^{+}T cells (mice treated with HDM for 5 days)

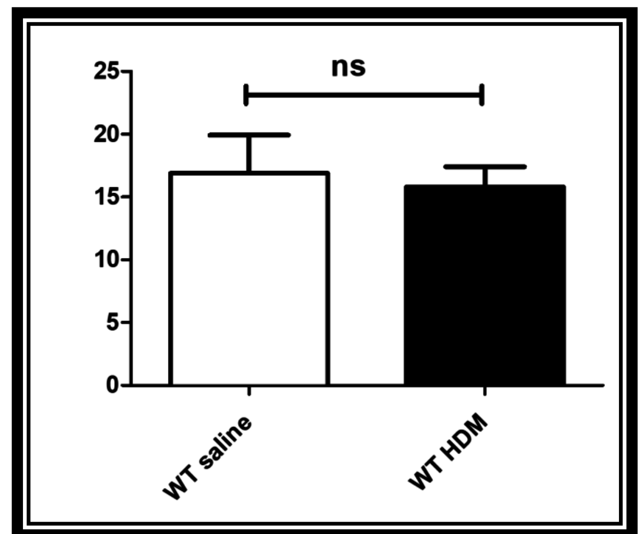

Fig. 8-C: ***Percentage of lung CD4⁺ Tem over total CD4⁺ T cells (mice treated with HDM for 3 weeks)***

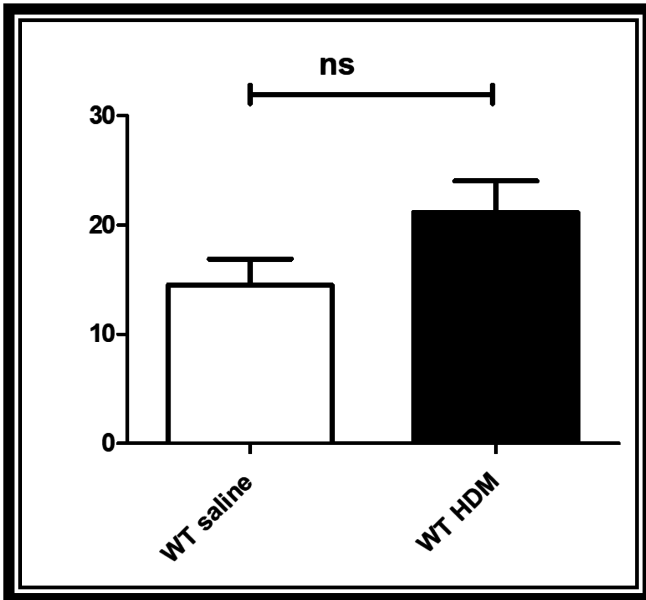

Fig. 8-D: ***Percentage of spleen CD4⁺ Tem over total CD4⁺ T cells (mice treated with HDM for 3 weeks)***

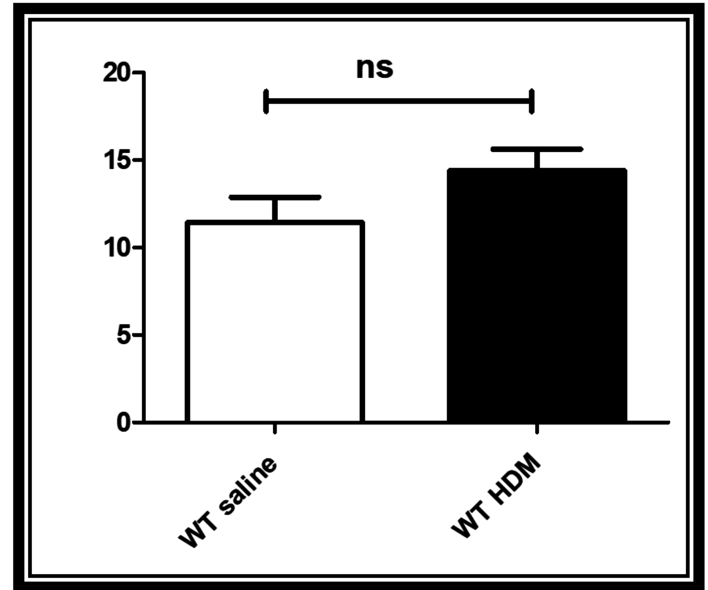

Fig 8: Percentages of $CD4^+$ Tem in the lungs and spleen of HDM treated WT mice All data are presented as the percentages of $CD4^+$ Tem over total $CD4^+$ T cells**.** Percentage of Tem cells in the lungs of WT mice treated with saline or HDM for 5 days is (14.63± 1.237) and (20.33 ± 0.9301) respectively, N=6 **(**Fig 8-A). Percentage of Tem cells in the spleens of WT mice treated with saline or HDM for 5 days is (16.89 ± 3.029) and (15.81 ± 1.593) respectively, N=6 (Fig 8-B). Percentage of Tem cells in the lungs of WT mice treated with saline or HDM for 3 weeks is (14.50 ± 2.400) and (21.18 ± 2.857) respectively, N=2-4 **(**Fig 8-A).Percentage of Tem cells in the spleens of WT mice treated with saline or HDM for 3 weeks is (11.44 ± 1.435) and (14.39 ± 1.230) respectively, N=2-4 (Fig 8-B)Data are presented as Mean ± SEM; ns (not significant), *P<0.05, **P<0.01, ***P<0.001 between the groups and were analyzed for significance using two-tailed unpaired *t*-test.

3.3.5 HDM did not induce the development of CD4 + Tcm in treated mice

The difference in the percentages of lung (fig. 9-A and C) and spleen (fig. 9-B and C) $CD4^+$ Tcm between mice treated with HDM for 5 days or 3 weeks and saline treated mice was not significant. The results indicate that HDM was not able to induce $CD4^+$ Tcm in the lungs and spleens of treated mice.

Fig. 9-A: Percentage of lung $CD4^+$ Tcm over total $CD4^+$ T cells (mice treated with HDM for 5

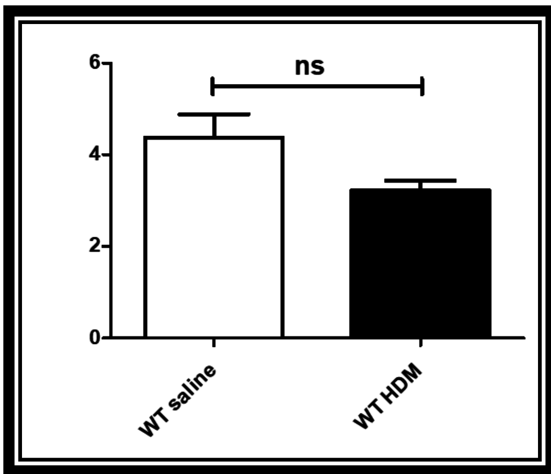

Fig. 9-B: Percentage of spleen $CD4^+$ Tcm over total $CD4^+$ T cells (mice treated with HDM for 5 days)

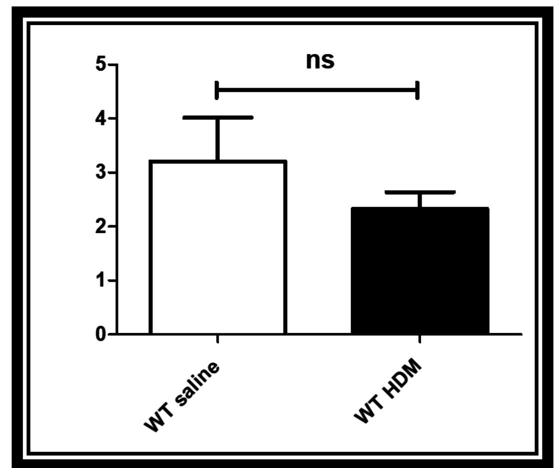

Fig. 9-C: Percentage of lung $CD4^+$ Tcm over total $CD4^+$ T cells (mice treated with HDM for 3 weeks)

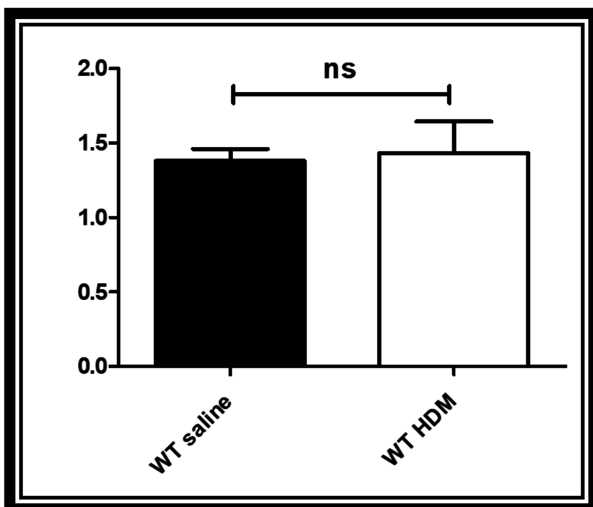

Fig. 9-C: Percentage of spleen $CD4^+$ Tcm over total $CD4^+$ T cells (mice treated with HDM for 3 weeks)

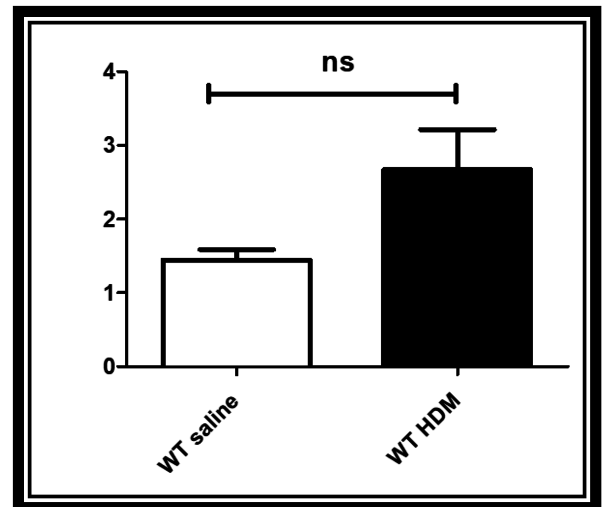

Fig 9: Percentages of $CD4^+$ Tcm in the lungs and spleen of HDM treated WT mice All data are presented as the percentages of $CD4^+$ Tcm over total $CD4^+$ T cells. The percentage of $CD4^+$ Tcm in the lungs of WT mice treated with saline or HDM for 5 days is (4.375 ± 0.5138) and (3.235 ± 0.2039) respectively, N=6 (Fig. 9-A). The percentage of $CD4^+$ Tcm cells in the spleens of WT mice treated with saline or HDM for 5 days (3.205 ± 0.8134) and (2.327 ± 0.3138) respectively, N=6 (Fig. 9 -B). The percentage of $CD4^+$ Tcm cells in the lungs of WT mice treated with saline or HDM for 3 weeks is (1.380 ± 0.08000) and (1.433 ± 0.2114) respectively, N=4 (Fig. 9-C). The percentage of $CD4^+$ Tcm in the spleens of WT mice treated with saline or HDM for 3 weeks is (1.445 ± 0.1450) and (2.668 ± 0.5482) respectively, N=4 (Fig. 9-D). Data are presented as Mean ± SEM; ns (not significant), *P<0.05, **P<0.01, ***P<0.001 between the groups and were analyzed for significance using two-tailed unpaired *t*-test was performed for comparison

3.3.6 The loss of PAR-2 expression did not decrease the percentages of $CD4^+$ T memory cells

The difference in lung $CD4^+$ Trm percentages between WT mice treated with HDM for 5 days (fig. 10-A) or 3 weeks (fig. 10-E) and *Par-2*$^{-/-}$ mice was not statistically significant. The difference in spleen $CD4^+$ Trm percentages between WT mice treated with HDM for 5 days and *Par-2*$^{-/-}$ mice was not statistically significant (fig.10-B). The difference in percentages of lung $CD4^+$ Tem between WT mice and *Par-2*$^{-/-}$ mice treated with HDM for 5 days was not statistically significant (fig.10 -C). The difference in spleen $CD4^+$ Tem percentages between WT mice treated with HDM for 5 days and *Par-2*$^{-/-}$ mice was not statistically significant (fig.10-D It appears that PAR-2 expression is not essential for the formation of $CD4^+$ Trm and Tem in HDM treated mice's lungs and spleens. We must take into account the small number of mice included in this study (n=2-3).

Fig 10-A: Percentage of lung CD4$^+$ Trm over total CD4$^+$ T cell (mice treated with HDM for 5 days)

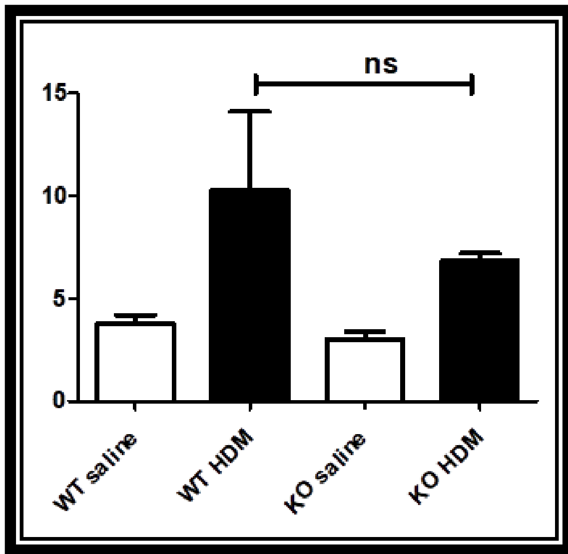

Fig 10-B: Percentage of spleen CD4$^+$ Trm over total CD4$^+$ cells (mice treated with HDM for 5 days)

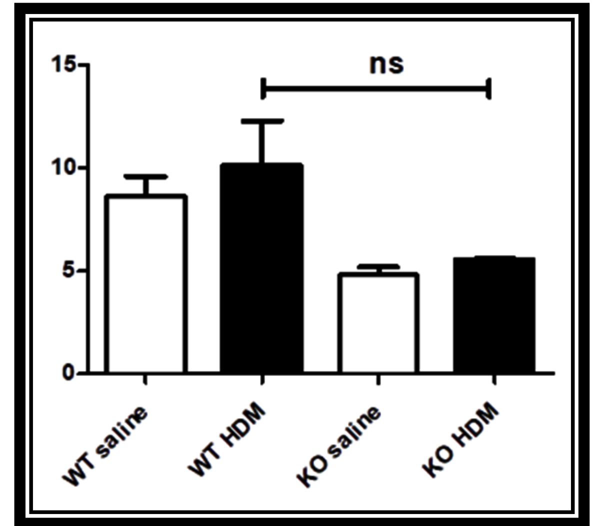

Fig 10-C: Percentage of lung CD4$^+$ Tem over total CD4$^+$ T cell (mice treated with HDM for 5 days)

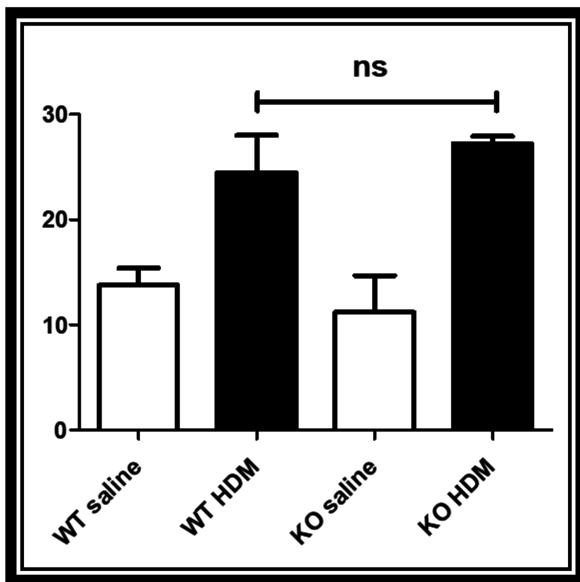

Fig 10-D: Percentage of spleen CD4$^+$ Tem over total CD4$^+$ T cell (mice treated with HDM for 5 days)

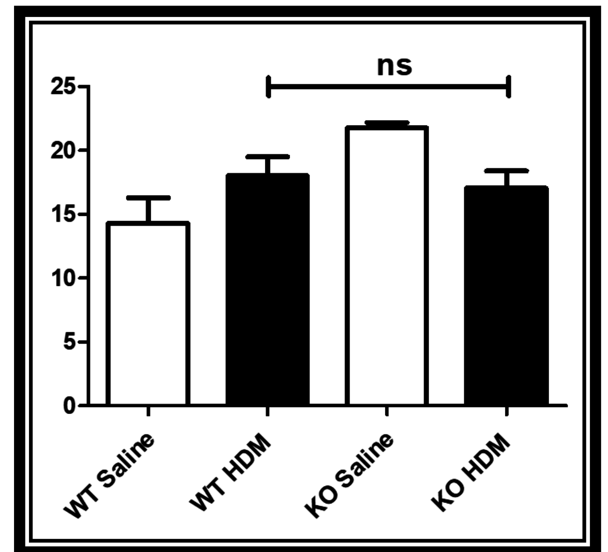

Fig 10-E: Percentage of lung CD4⁺ Trm over total CD4⁺ T cell (mice treated with HDM for 3 weeks)

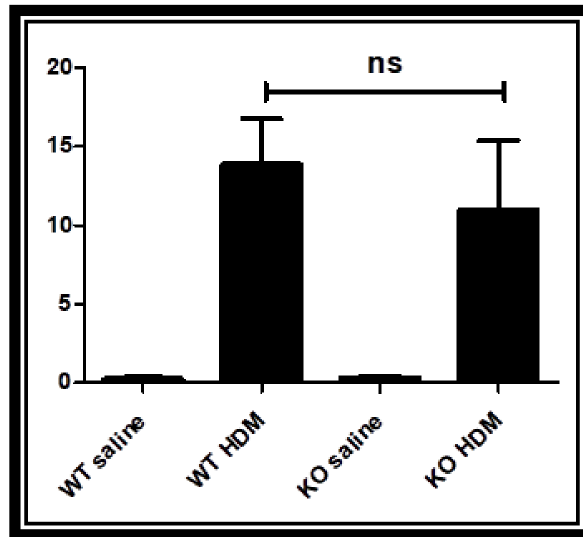

Fig 10: Percentages of $CD4^+$ Trm and Tem in the lungs and spleen of HDM treated WT and *Par-2* $^{-/-}$ mice. All data are presented as the percentages of $CD4^+$ Trm over total $CD4^+$ T cells. The percentage of Trm cells in the lungs of WT and *Par-2* $^{-/-}$ mice treated with saline for 5 days is (3.775 ± 0.4250) and (3.000 ± 0.4000) respectively (white columns) **(Fig 10-A)**. The percentage of Trm cells in the lungs of WT and *Par-2* $^{-/-}$ mice treated with HDM for 5 days is (10.25 ± 3.850) and (6.830 ± 0.3700) respectively (Black columns) **(Fig 10-A)**. The percentage of Trm cells in the spleens of WT and *Par-2* $^{-/-}$ mice treated with saline for 5 days is (8.650 ± 0.9500) and (4.790 ± 0.4100) respectively (white columns) **(Fig 10-B)**. The percentage of Trm cells in the spleens of WT and *Par-2* $^{-/-}$ mice treated with HDM for 5 days is (10.15 ± 2.150) and (5.550 ± 0.05000) respectively (Black columns) **(Fig 10-B)**. The percentage of Tem cells in the lungs of WT and *Par-2* $^{-/-}$ mice treated with saline for 5 days is (13.77 ± 1.630) and (11.19 ± 3.510) respectively (white columns) **(Fig 10-C)**. The percentage of Tem cells in the lungs of WT and *Par-2* $^{-/-}$ mice treated with HDM for 5 days is (24.45 ± 3.550) and (27.20 ± 0.7000) respectively (Black columns) **(Fig 10-C)**. The percentage of Tem cells in the spleens of WT and *Par-2* $^{-/-}$ mice treated with saline for 5 days is (14.30 ± 2.000) and (21.75± 0.4500) respectively (white columns) **(Fig 10-D)**. The percentage of Tem cells in the spleens of WT and *Par-2* $^{-/-}$ mice treated with HDM for 5 days is (18.00 ± 1.500) and (17.05 ± 1.350) respectively (Black columns) **(Fig 10-D)**. The percentage of Trm cells in the lungs of WT and *Par-2* $^{-/-}$ mice treated with saline for 3 weeks is (0.2500 ± 0.1 100) and (0.3250 ± 0.1150) respectively (white columns) **(Fig 10-E)**. The percentage of Trm cells in the lungs of WT and *Par-2* $^{-/-}$ mice treated with HDM for 3 weeks is (13.90 ± 2.854) and (10.97 ± 4.424) respectively (Black columns) **(Fig 10-E)**. n= 2 for saline treated mice and n=3 for HDM treated mice for all experiments. The results are expressed as Mean ± SEM and two-tailed paired *t*-test was performed; ns (not significant), *P<0.05, **P<0.01, ***P<0.001 between the groups.

3.4 Discussion:

The primary findings of our study were that adoptive transfer of $CD4^+$ T cells from spleens of *Par-2*$^{-/-}$ mice treated with HDM, unlike $CD4^+$ T cells from spleens of WT mice treated with HDM, failed to transfer the disease to naïve WT mice. Also, we discovered that HDM could only induce $CD4^+$ Trm in the spleens of WT mice but not Tem or Tcm, implying that Trm may be the memory cells responsible for disease transfer to naive mice in the adoptive transfer models. Furthermore, the loss of PAR-2 expression did not attenuate the percentages of $CD4^+$ T memory cells. However, we attribute this to the small number of mice participating in these trials (n=2-3), and we believe that increasing the number of mice will result in significant results.

Studies showed that HDM and Ova- specific $CD4^+$ T cells can transfer the disease to naive mice in adoptive transfer models (230) (300), and significant lung inflammation can be induced in naive mice by the transfer of <100,000 allergen specific cells (159) (300). In addition, our lab published a paper in 2013 suggesting that the role of PAR-2 in allergic sensitization could be mediated by altered T cell responses. Where, they showed that mice exposed to HDM after PAR-2 inhibition in the airways had decreased ex vivo splenocyte proliferation and failure of spleen $CD4^+$ T cells to transfer the disease, which they attribute to lower numbers of antigen-specific Th2 cells (3).

As we mentioned above, we propose that increasing the number of mice will induce significant results showing that the loss of PAR-2 expression attenuates the formation of memory $CD4^+$ T cells with the subsequent abrogation of disease transfer to naïve mice. We propose that inhibition of PAR-2 activation on airway epithelium or the loss of PAR-2 expression on airway epithelium (*Par-2*$^{-/-}$ mouse), attenuate the formation of inflammatory mediators crucial for Th2

polarization of the immune response with subsequent reduction in the formation of $CD4^+$ T memory cell (possibly Trm) and failure of disease transfer.

Future research should be done to demonstrate that the spleen Trm identified in our trials are HDM specific and they are capable on transferring the disease to naive mice.

To support our findings, we were able to establish allergic airway inflammation in *Par-2*$^{-/-}$ mice to levels comparable to WT mice by transferring already sensitized $CD4^+$ T cells from WT mice. As a result, we believe that in this experiment, we bypassed the key signal from PAR-2 activation on airway epithelium that is essential for the development of $CD4^+$ T memory cells, presumably Trm in *Par-2*$^{-/-}$ mice.

It has been discovered that a $CD3^+CD4^+$ subset in murine lungs that express CD44hi CD62L-ve CD69+ve ST2+ve (phenotype similar to tissue resident memory cells detected in our experiments) generate Th2 cytokines and causes allergen-induced illness (247) . These cells are considered long-lived pathogenic Th2 Trm that keeps “allergic memory” in the lungs, residing in the tissue for the duration of a mouse's life (>665 days) (247). In addition, according to studies in secondary lymphoid organs, $CD4^+$ and $CD8^+$ Trm can be detected in the lymph nodes and spleens of mice and humans (313) (314) (315) but unfortunately, there haven't been many studies on the role of spleen Trm in asthma.

The previously mentioned data suggests that lung and spleen Trm detected in our experiments may be primed to respond quickly to inhaled allergens and to maintain long-term allergic memory, however, it is missing in the case of *Par-2* $^{-/-}$ mice.

Treatment techniques targeting these cells or blocking PAR-2 activation may bring relief to allergic asthma patients

Chapter 4: General Discussion

Much is known about the consequences of PAR-2 activation in the airways, little is known about cell/s mediating PAR-2 activation in the airways to induce allergic sensitization and allergic airway inflammation. In this study, we showed for the 1st time using chimeric mice between WT and *Par-2*$^{-/-}$ mice that the loss of PAR-2 expression on airway structural cells is critical for the development of allergic airway inflammation.

In chapter 2 we demonstrated that in vivo activation of PAR-2 induced the production of TSLP, eotaxin, IL-6, and KC-Gro (critical mediators in allergic asthma) from the lungs of mice treated with I.N PAR-2 AP and it has been published that in vitro PAR-2 activation on airway epithelial cells induces the production of GM-CSF (critical mediator in allergic asthma) (113). In addition, data from our lab indicated that fluorescently labelled antibody against PAR-2 (SAM-11) administered intranasally showed robust binding to airway epithelium by confocal microscopy. Suggesting that in the experiments where PAR-2 was blocked in the airways, the decrease in allergen sensitization was mainly induced by the loss of PAR-2 activation on airway epithelium(206). Furthermore, PAR2 expression on the surface of respiratory epithelial cells is elevated in patients with bronchial asthma (316) .

According to the data mentioned above and since airway epithelium is the first organ to be in contact with allergens., we propose that PAR-2 activation on airway epithelial cells induce the production of inflammatory mediators which induce Th2 shifting of the immune response with subsequent allergic sensitization and allergic airway inflammation.

PAR-2 is expressed by tissue resident cells such as bronchial smooth muscle and endothelial cells. The confocal findings of SAM-11 binding to epithelium following intranasal delivery suggest that

the airway epithelium may be the key cells driving PAR-2 activation in the airways, although this does not rule out the possibility of PAR-2 activation on other tissue resident cells (94) (295).

Also, we demonstrated that the loss of PAR-2 expression on hematopoietic cells reduced airway inflammation, however, the reduction in airway inflammation was not significant compared to the loss of PAR-2 expression on structural cells. Suggesting that PAR-2 expression on hematopoietic cells is essential to have the full effect.

Future studies on the role of PAR-2 activation on hematopoietic cells in the airways should focus on alveolar macrophages. They express PAR-2 and are located all over the airways. They are also one of the first cells to come into contact with inhaled serine proteinases (317). Alveolar macrophages can release substantial levels of inflammatory mediators specially, TNF which is critical for allergic sensitization through DCs activation (318) and induction of epithelium-driven inflammatory mediators important for allergic sensitization such as GM-CSF (319) (116). Interestingly, a study showed that PAR-2 activation on murine macrophages may contribute to the establishment of a Th2 response (320). Moreover, it has been demonstrated that PAR-2 activation on bone marrow-derived macrophages (BMDM) isolated from C57/BL6 mice, induced M1 polarization with the production of inflammatory mediators (IL-6, TNF, IL-1 β and MCP-1) (321). According to the data presented above, alveolar macrophages are an interesting cell for studying the role of PAR-2 activation on airway hematopoietic cells.

It has been discussed thoroughly in literature the role of PAR-2 activation in allergic sensitization and allergic airway inflammation (2,3,19,206), but little is investigated regarding the role of PAR-2 activation on the development of $CD4^{+}$ memory T cells in allergic asthma.

The vital role of $CD4^+$ T memory cells in allergic asthma has been demonstrated recently. Long-lived Th2 memory cells in the spleen and lung plays a role in allergic asthma after re-exposure to antigen (247). A fraction of CD3+CD4+ T cells in the lungs display $CD44^{hi}CD62LCD69^+ST2^+$, generates Th2 cytokines, and causes allergen-induced illness recurrence. These cells are long-lived pathogenic Th2 tissue resident memory cells (Trm) that keep "allergic memory" in the lungs, residing in the tissue for the duration of a mouse's life (>665 days) (248) .

In chapter 3, we demonstrated that the difference in the percentages of $CD4^+$ Trm and Tem in the lungs and spleens of *Par-2* $^{-/-}$ and WT mice following treatment with HDM was not significant. We attribute this to the low numbers of mice participating in this trial (n=2) and we propose that by increasing the numbers of mice participating in this experiment we will achieve a significant reduction in the percentages of $CD4^+$ T memory cells in the lungs and spleens of *Par-2* $^{-/-}$ compared to WT mice. Furthermore, we showed that HDM could only induce $CD4^+$ Trm in the spleens of WT mice, but not Tem or Tcm mice, demonstrating that Trm cells might be responsible for disease transfer to naive mice in adoptive transfer models.

The fact that $CD4^+$ T cells from HDM sensitized *Par-2* $^{-/-}$ mice cannot transfer the disease to naïve WT mice (data from chapter 3) and animals exposed to HDM extract had lower *ex vivo* splenocyte proliferation when PAR-2 was blocked (3). Furthermore, lower lung Th2 cytokines (IL-4, IL-5, and IL-13) production after PAR-2 blockage in the airways (3) are likely attributable to the decrease in antigen-specific Th2 memory cells. So, we are proposing that PAR-2 activation on airway epithelial cells by allergens possessing serine proteinases or by endogenous proteinases induce the production of inflammatory mediators which is essential for Th2 skewing of the immune response with subsequent allergic sensitization and development of antigen specific $CD4^+$ T memory cells presumably Trm in the spleens and lungs.

Interestingly, a study demonstrated that in $CD4^+$ T cells, TSLP signaling is essential not only for Th2 memory formation in vivo, but also for the memory cells' reaction to specific antigen challenge (322). This study provides support to our hypothesis that PAR-2 activation in the airways is required for the development of $CD4^+$ T memory cells via the production of inflammatory mediators, specifically TSLP. Future research should include cell-specific PAR-2 deletion to better understand the role of PAR-2 activation on different airway cells and allergic airway inflammation.

To test our theory, we will induce asthma in mice lacking PAR-2 expression only on airway epithelium. We already have PAR-2 floxed mice, and we're in the process of crossing them with mice that exclusively express cre recombinase on the airway epithelium, like the commercially available CC10-Cre mouse. This mouse will allow us to differentiate between PAR-2 expression on airway epithelium and expression on other tissue resident cells in the airways.

Future studies should confirm that the spleen Trm discovered in our trials are HDM specific and capable of transferring the disease to naive animals. This could be accomplished by studying *ex vivo* proliferation of isolated $CD4^+$ Trm cells only following exposure to HDM. Furthermore, using flow cytometry, we can examine activation markers such as CD154 (323). In addition, transferring $CD4^+$ Trm population will help us to verify that Trm cells are the key cells involved in disease transmission.

According to our study, PAR-2 is a key molecule in the etiology of allergic airway inflammation. As a result, PAR-2 antagonists, or the neutralization of mediators released after PAR-2 activation, may be an appealing treatment approach for asthma. The capacity to apply targeted therapeutic approaches will be enhanced by determining the role of airway epithelial cells.

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