Role of PTX3 in Allergic Airway Inflammation

By

Jyoti Balhara

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Dedication

I dedicate my work to my mother who is suffering from asthma for past 30 years. Every struggle that she made to breathe, inspired me to undertake asthma research.

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Abbreviations

AHR: Airway hyper-responsiveness

AICD: Activation induced cell death

APC: Antigen-presenting cell

ASM: Airway smooth muscle

BALF: Broncho Alveleolr Lavage Fluid

BMDC: Bone marrow-derived dendritic cells

CCR-7: CC chemokine receptor 7

CD: Cluster of differentiation molecules

CFSE: Carboxyfluorescein succinimidyl ester

CXCR: Chemokine, CXC Motif, Receptor

DAMPs: Damage-associated molecular patterns

DC: Dendritic cell

EC: Epithelial cell

GM-CSF: Granulocyte-macrophage colony stimulating factor

HDM: House dust mite

ICOS: Inducible T-cell co-stimulator

IFN-γ: Interferon gamma

Ig: Immunoglobulin

IL: Interlukin

iNOS: Inducible nitric oxide synthase

i.p.: Intraperitoneal

LPS: Lipopolysaccharide

MCP-1: Monocytic chemotactic protein 1

mDC: Myeloid dendritic cell

MHC: Major histocompatibility complex

OVA: Ovalbumin

pDC: Plasmacytoid dendritic cell

PAMPs: Pathogen-associated molecular patterns

PDE: Phosphodiestrase

PMNs: Polymorphonuclear neutrophil

PRR: Pattern recognition receptors

PTX3: Pentraxin 3

RORγt: retinoic acid-related orphan receptor γt

STAT: Signal transducer and activator of transcription

T-bet: T box transcription factor

Tef: Effector T cell

TGF: Transforming growth factor

Th: T helper cell

TLR: Toll-like receptor

TNF: Tumor Necrosis Factor

Treg: Regulatory T cell

TSLP: Thymic stromal lymphopoietin

Thesis Abstract

Pentraxin 3 (PTX3) belongs to family of pentraxins that are specific pattern recognition receptors. PTX3 is a soluble pentraxin that is characterized as long pentraxin due to its protein size. It regulates the innate and adaptive immune systems, playing an important role in providing immunity against various pulmonary inflammatory conditions. Having known its role in fostering lung immunity, aim of my PhD thesis was to examine the role of PTX3 in asthma. Previously, our lab had demonstrated an enhanced expression of PTX3 in bronchial biopsies of allergic asthmatics. Very early in my project, I found an enhanced level of PTX3 in the BALF obtained from severe asthmatics also in the BALF and the lungs of allergen exposed mice. I furthered my interest in understanding the role of PTX3 in asthma and assessed the effect of PTX3 deletion in a murine model of OVA-induced allergic response. Although allergen-induced allergic response in mice is an experimental model, it is widely used to understand pathogenesis of human asthma. Sensitized PTX3 Knockout (PTX3-/-) mice exhibited an enhanced airway and tissue resistance in response to methacholine (MCh) in contrast to their WT counterparts. We observed an increase in the infiltration of inflammatory cells in BALF and the lungs obtained from PTX3-/- mice upon OVA sensitization/challenge as compared to their PTX3+/+ littermates. Further we found an IL-17A dominant inflammation in PTX3-/- mice as compared to PTX3+/+ mice upon OVA challenge. Given that IL-17A dominant inflammation is associated with increased cell survival and reduced IL-2 production, we also observed increased CD4 T cell survival and reduced IL-2 production by these cells. As Th17 phenotype of CD4 T cells is shaped by IL-6 and IL-23 producing dendritic cells, we observed increased preponderance of such DCs in the lungs and the draining lymph nodes of PTX3 KO mice as compared to their PTX3+/+ counterparts, plausibly supporting Th17/ IL-17A dominant inflammation in the former.

Having discovered critical role of DCs in PTX3 deleted mice in my study, second main aim of my thesis was to understand the impact of PTX3 deletion on phenotype and functions of DCs. We observed increased infiltration of inflammatory DCs in the lungs in PTX3-/- mice upon OVA exposure. This observation was in line with increased generation of common myeloid progenitors in the bone marrow of these mice. PTX3-/- DCs showed reduced MHCII expression but increased surface expression of CD80 and CD86 along with increased ability of PTX3-/- DCs to uptake and process OVA. Since these DCs showed increase IL-6 and IL-23 production, co-culture of PTX3-/- DCs with OT II CD4 T cells resulted in greater IL-17A production as compared to those that were co-cultured with PTX3+/+ DCs.

We also assessed the effect of PTX3 deletion on the phenotype of bone marrow derived DCs (BMDCs) and whether BMDCs could be used in lieu of lung DCs in this study. Contrastingly, PTX3-/- BMDCs showed reduced expression of costimulatory markers and production of inflammatory cytokines in response to lipolysaccheride (LPS). PTX3 also resulted in reduced ability to uptake and process OVA in BMDCs, rendering PTX3-/- BMDCs incomparable to lung DCs from the same genetic background.

Altogether, I have observed that deletion of PTX3 resulted in enhanced IL-17 immune response plausibly through increased IL-6 and IL-23 producing DCs in OVA-exposed mice. Also, I identified differential phenotype of bone marrow derived DCs and lung DCs in PTX3 deleted condition in mice. In summary, findings of my thesis provide insights of the novel regulatory role of PTX3 in allergic inflammation, primarily in context with CD4 T cell-DC axis. Although it is

not clear at this stage how such a role of PTX3 would affect the therapeutic potential of PTX3 in asthma management. My thesis have indeed provided information that will be critical in designing experiments/ studies required to understand whether or not PTX3 can be used as a treatment for asthma and if yes, which pathway it would affect.

Chapter 1: Introduction

1. Asthma

According to the World Health Organization, asthma is now a serious public health problem with over 300 million sufferers worldwide. Further, it is estimated that the prevalence of asthma globally increases every decade by 50% (1). Approximately 500 Canadians die from asthma each year (www.asthma.ca). Asthma is a disease of the airways, clinically recognized by excess mucus production, difficulty in breathing due to airway narrowing and airway inflammation. Allergic asthma is a complex representation of an inflammatory response to specific allergens, which otherwise are harmless. According to a report by Braman (1), collateral increase in atopic sensitization and other allergic conditions such as eczema and rhinitis are associated with an increase in asthma pervasiveness. Allergic sensitization may occur as early as *in-utero*. Genetic or hereditary predisposition, environmental factors and gene-environmental interactions also contribute to the development of asthma in susceptible subjects.

1.1 Genetic factors

Susceptibility to asthma is partly associated with genetic predisposition due to which it is seen to run strongly in families (2, 3). Determination of the genetic regions co-inherited with the disease by positional cloning and linkage studies identifies the genes or gene complexes related to genetic predisposition. Vercelli et al. (4) carefully segregated asthma susceptibility genes into four major categories: “genes associated with innate immunity and immunoregulation; genes associated with Th2-cell differentiation and effector functions; genes associated with epithelial biology and mucosal immunity; and genes associated with lung function, airway remodeling and disease severity”.

The first category refers to polymorphism in genes encoding pattern recognition receptors (PRR) or surface receptors, which have marked impact on the development of allergic inflammation. Given the significance of proinflammatory cytokines in the maturation of specific T cell responses, they constitute the second category (4). Genes associated with the functions of epithelial cells also play critical role in regulating susceptibility to allergic asthma (5-9). The last group of candidate genes affects lung physiology and phenotype. This group is indicated to be highly heterogeneous ranging from the adrenergic beta receptor 2 (ADRB2) (10) and extracellular matrix protein tenascin c (11) to proinflamatory molecules such as tumor necrosis factor (TNF) (12), leukotriene C4 synthase (13) and TGFβ1 (14).

Besides genetic factors, epigenetic factors can also modulate the development and progression of the disease. Epigenetics refers to the inheritance of characters that are not encoded by DNA. Such changes may arise before or during the occurrence of the disease. For example, methylation of the promoter and intronic regions of IL-4 gene modulate its expression (15). Similarly, hypermethylation of IFN-γ gene suppresses its production, thereby promoting a Th2 response (16). Although genetic factors play a central role in determining the outcome of the disease, influence of the environmental factors on genetics can act as an added layer of regulation.

1.2 Environmental factors

Asthma is more prominently seen in western countries of the world, particularly in the societies which are less exposed to infections (17). Such reports support 'the Hygiene hypothesis' according to which an environment rich in microbes provides protection against diseases (18).

These also indicate the role of environmental factors in the perpetuation of allergic asthma. Environmental factors may vary from respiratory viruses, tobacco smoke, air pollutants, toxins, allergens in air, diet to variable lifestyle (19).

2. Mechanisms of allergic asthma

Allergic asthma is type I allergic hypersensitivity that requires initial sensitization with inhalant allergens. Dendritic cells in the airways take up the allergen/antigen and migrate to the draining lymph nodes where they process and present antigenic epitopes to naïve CD4 T cells and induce the generation of effector T cells and memory T cells. Subsequent exposure of the same allergen activates immune system at faster and more sustained manner characterized by an immediate allergic response associated with vasodilation and bronchial constriction, followed by cellular infiltration, cytokine production in the airways, AHR and airway remodeling resulting in sustained bronchospasm. Overall allergic inflammation results from a coordinated network of multiple cell types of innate and adaptive immune system in which it is difficult to conclude dominance of an individual pathway.

2.1 Eosinophils

Eosinophils constitute prominent inflammatory cells found in the asthmatic lungs. Eosinophils originate from CD34+ hematopoietic progenitor cells in the bone marrow after which these cells are released into the peripheral blood (20). Th2 cytokines, particularly IL-3, IL-5 and GM-CSF regulates the phenotype and functions of eosinophil. Moreover TNF, eotaxin-1/CCL-11, eotaxin-2/ CCL24, eotaxin-3/CCL23, MIP1-a/CCL-3, RANTES/CCL-5, MCP-3/CCL-7 and CCL22 promote the differentiation, proliferation and recruitment of eosinophils (21, 22). In response to allergen exposure, massive recruitment of eosinophils is observed. In asthmatic individuals, the

magnitude of their recruitment depends on the severity of the disease (23). Upon activation, eosinophils degranulate and release proinflammatory molecules including reactive oxygen species, major basic protein, eosinophils cationic protein, eosinophils peroxidase, eosinophil-derived neurotoxin, cytokines, and chemokines (24-28). Through the release of these molecules, eosinophils can also regulate airway hyper-reactivity (AHR) (29). Eosinophils also display antigen presenting cells (APC)-like properties such as expression of major histocompatibility factor (MHC) II and costimulatory molecules (30) (31). Through the release of proinflammatory molecules, eosi nophils are capable of regulating allergic inflammation however, their sufficiency to present allergen peptides to T cells remains unclear even though these cells express markers of antigen presentation machinery.

2.2 Neutrophils

Neutrophils are polymorphonuclear leukocytes that constitute the first line of defense against foreign invaders. Neutrophil-dominant allergic inflammation is usually associated with severe asthma that is characterized by irreversible lung function impairment (32-34). In response to allergen, neutrophils count increase in the lungs that release an array of inflammatory mediators and cytokines. Through murine as well as human asthma studies, IL-17 has been implicated in neutrophilic inflammation. Neutrophils recruitment is regulated by Th17 cells that altogether result in glucocorticoid resistance (34). IL-17A induces neutrophil infiltration and also activates them to produce neutrophil myeloperoxidase and elastase. Also, it promotes the production of granulocyte monocytes colony stimulating factor (GM-CSF) and IL-8 by pulmonary epithelial cells (35). IL-8 and GM-CSF act as a chemo-attractant and inducer of inflammatory molecules in neutrophils (36, 37).

Neutrophils also express IgE receptors FcεRI, FcεRII and galectin-3, rendering them responsive to IgE in asthmatics (38-40)**.** Neutrophils exhibit increased expression of FcεRI in atopic asthmatics (38, 41, 42). In neutrophils that were isolated from atopic asthmatics, IgE stimulation enhanced their survival that corresponded with increased activation of anti-apoptotic mechanisms. This phenomenon was suggested to aggravate neutrophilic inflammation in such donors. Even though neutrophils express IgE receptor FcεRI but increased survival seemed independent of FcεRI (42). Together, reports suggest that neutrophilic inflammation is furthered by pro-inflammatory asthmatic mechanisms such as preponderance of IL-17A and IgE.

2.3 Basophils

Basophils also originate from CD34+ progenitor cells in the cord blood, peripheral blood and bone marrow in the presence of IL-3 and are released into the circulatory system (43). Basophils express high levels of FcεRI and CD49b (DX5) but lacks stem cell factor cKit (44-49). Interaction between IgE and FcεRI stimulates activation of basophils to release inflammatory mediators including histamine that eventually leads to an immediate hypersensitivity response (50). Adoptive transfer of FcεRI expressing basophils in FcεRI deficient mice resulted in induction of inflammation (51, 52), thus supporting the role of these cells in allergic inflammation.

Asthmatics have shown an increase in basophil count in the airways and the sputum (44-46). Basophils under the effect of proinflammatory factors including IL-3, IL-5, GM-CSF and histamine-releasing factors, IL-18 and IL-33, release Th2 cytokines (20) (53, 54). Further, basophils express MHCII through which these cells can act as antigen presenting cells. *Sokol et al*

(55) demonstrated that coculture of OVA-specific naïve T cells with basophils in the presence of OVA resulted in MHCII-dependent Th2 differentiation. Antigen presenting function of basophils is especially important because of insufficiency of DCs to produce IL-4, rendering former cell type as key cell type supporting IL-4 dependent Th2 differentiation (56). Depletion of basophils impaired IL-4 production (57), whereas adoptive transfer of basophils in MHCII-deficient mice restored IL-4 production by CD4 T cells (58). Furthermore, basophils promote inflammation by releasing chemokines such as CCL-3, CCL-5, CCL-7, CCL-8, CCL-11, CCL-13, CCL-24 and CCL-26 that attracts basophils and other pro-inflammatory cells into asthmatic lungs (59, 60). Together, basophils play a critical role in allergic asthma, especially in IL-4 dependent phenotypes of asthma.

2.4 Mast cells

In response to allergens, mast cells are one of the primary responders and initiate hypersensitivity reactions. Mast cells reside on the airway surface, in submucosa and deep in the airway wall (61). They express FcεRI, which when interacts with antigen-bound IgE activate mast cells and induce degranulation. Upon degranulation, mast cells release an array of preformed and newly synthesized mediators, such as tryptase, heparin and histamine, and prostaglandin 2 (PGD2) and LTC_4, respectively (62, 63). The products released by mast cells are potent bronchoconstrictors and vasodilators. Moreover, activated mast cells secrete proinflammatory cytokines such as IL-1, IL-3, IL-4, 5, IL-6, IL-8, IL-10, IL-13, IL-16, TNF, TGF-β and chemokines like IL-8, CCL-1, CCL2, CCL-3 and CCL-5. These proinflammatory mediators, cytokines and chemokines are involved in causing characteristic features of allergic asthma, suggesting critical role of mast cells in the development of asthma.

In chronic asthma, tissue type-mast cells increase in the smooth muscle layer contributes to airway smooth muscle (ASM) remodeling (64, 65). Tissue type mast cells contain tryptase and chymase in secretary granules and differ from mucosal type mast cells that contain tryptase granules. Mast cells produce IL-19 that regulates migration and hyperplasia of airway smooth muscle (ASM) cells. On the other hand, ASM cells in the airways release Fracktalkine/CX3CL1 and IP-10/CXCL10 that attract mast cells to the airways (66, 67). Airway smooth muscle cells through CADM1, IL-6 and stem cell factor also promotes survival and proliferation of lung mast cells (68).

Murine models of experimental asthma have also validated the critical role of mast cells in asthma development. Mast cell deficient mice showed impaired airway inflammation, mucus production and AHR (69, 70). Although mast cells can induce MHCII-dependent T cell proliferation, their precise role as APCs is poorly known. Altogether, mast cells through release of proinflammatory mediators damage epithelium and promote airway inflammation by attracting other inflammatory leukocytes in allergic asthma.

2.5 Epithelial cells

Epithelial cells (ECs) form the first line of defense against inhaled allergens/antigens and promote tissue homeostasis (71, 72). Psuedostratified epithelium, consisting of secretory and ciliated epithelial cells, of the airways with mucus layer over it forms the mucociliary apparatus in the airways that samples and removes most of the inhaled foreign particles (73, 74). Secretary cells produce two major gel forming airway mucins: MUC5ac and MUC5b and secrete them in the

periciliary layer. Goblets cells are specialized secretory cells that are capable of storing mucins in unique mucin granules while others can only produce them but are devoid of granules. Ciliated cells of the epithelium propel mucous layer up the airways through cilia. They form a distinct mucous layer as their cilia are coated with membrane spanning mucins and tethered mucopolysaccharides (75). The multilayered structure of airway epithelium protects from external stimuli/ allergen that if damaged, can stimulate inflammatory response such as that observed in asthma.

In asthma, an increase in goblet cells count result in enhanced mucus production. (76, 77) Inflammatory cytokines IL-4, IL-13 and IL-9 promotes mucous hyperplasia (78). Also, signs of consistent apoptosis of epithelial cells, damaged tight junctions, and barrier functions have been recorded in allergic inflammatory condition (79-83). Impaired epithelial integrity and consequent barrier breach result in allergen sensitization by facilitating access of allergen to dendritic cells (DCs) (84-88). Proteolytic activity of inhaled allergens especially house dust mite (HDM), cockroach, pollens, and fungi can also disrupt tight junctions in the airway epithelium (89-92). In asthmatic condition, progressive decline in lung functions, possibly due to subepithelial fibrosis have also been suggested (93, 94).

ECs express pattern recognition receptors (PRRs) and receptors for soluble inflammatory mediators through which they recognize allergens and allergen-complexed complements. Such interactions induce production of inflammatory mediators such as IL-6, IL-8, GM-CSF, TNF, IFN-g and chemokines by ECs that play a critical role in regulating functions of other inflammatory cells (95). Production of thymic stromal lymphopoietin (TSLP) by ECs modulates

DCs functions to facilitate Th2 polarization and recruitment into the inflamed lungs (96). It also stimulates proliferation of bronchial ECs and IL-13 production by them (97). Airway ECs release CCL2, CCL20 and IL12P40 homodimers that promote the recruitment of monocytes and DCs (98-100).

In asthmatics, bronchial ECs produce enhanced IL-33 that activates the DCs though ST2 receptor and promotes Th2 immune response (101, 102). Upon allergen exposure, ECs generated ROS activates DCs by promoting NF-κB. In response to allergens, ECs undergo hypoxia and subsequently produce HIF-a induced VEGF; neutralization of VEGF or HIF-a can suppress the development of allergic inflammation. Allergen exposure also induces production of prostaglandin E2 and IL-37, which tend to abrogate inflammatory response (103, 104). Prior exposure to allergen associated pattern associated molecular pattern (PAMP) and damage associated molecular pattern (DAMPs) further enhance the activation threshold of ECs and their ability to respond (105). Together allergen exposure and/ or allergic inflammation seems to affect multiple components of the airway epithelium that further worsens the symptoms of asthma.

2.6 Dendritic cells

Dendritic cells play a key role in sampling air-borne pathogens in the lungs and inducing an immune response against them. DCs act as a connection between ECs and T cells (98, 106). They take up allergen from ECs or directly uptake allergens in situations where EC integrity is disrupted and present them to the T cells and dictate their differentiation. Activation of DCs in the pulmonary compartment is dependent on either direct effect of allergen on them or is supported by inflammatory signals sent by epithelial cells and other innate immune system cells as discussed

above. In non-sensitive/ healthy individuals, lungs DCs contribute in maintaining homeostasis by inducing tolerance, possibly by induction of IL-10/ TGF-β producing Tregs in IL-10 and/or inducible T-cell costimulatory ligand (ICOSL) dependent pathways. Tregs interfere with inflammatory functions of T cells and cDCs.

DCs mediate similar tolerance in response to harmless foreign agents. Exposure to such agents results in unsuccessful activation of lung DCs (107). These insufficiently activated DCs or immature DCs induce Tregs response through IL-10 and ICOS-dependent mechanisms (108) (109). However, the presence of innocuous environmental agents in the airways breaks tolerance and induces the inflammatory phenotype of DCs. In sensitive subjects such as allergic asthmatics, DCs can be activated and exhibit inflammatory phenotype even upon encountering harmless foreign agents such as allergens. Allergen exposure that induces the production of GM-CSF, TSLP, and IL-33 by ECs, regulate phenotype and functions of DCs. GM-CSF promotes the maturation of DCs and enhances their inflammatory functions. IL-33 and TSLP program DC functions to promote Th2 polarization. TSLP activated DCs induce the expression of prostaglandin receptor D2 on Th2 cells in asthmatic airways, a mechanism through which DCs promote maintainence of Th2 commitment and expansion of Th2 memory cells (110). The function of TSLP is enhanced by IL-25, a cytokine released in active form by epithelial cells, eosinophils, and basophils upon allergen exposure.

Lung DCs shows a faster turnover as compared to other peripheral and central lymphoid tissues. In resting state, their turnover is approximately 3 days as compared to Langerhans cells in skin epidermis that take 21 days to renew (111, 112). In response to pathogens/ antigens, lungs DCs

can show turnover as rapid as 2 days (111) that is also associated with an increased migration to the lymph node to sustain their continuing demand to mount an appropriate T cell mediated immune response (113).

Mechanism of antigen uptake, processing and presentation

DCs are in the immature stage when they encounter antigens at mucosal surfaces. At this stage, DCs are sufficiently capable of detecting and uptaking antigens of picomolar to nanomolar concentrations through three main mechanisms. The first mechanism through which DCs recognize specific entities/ patterns (pathogen associated molecular pattern) on the surface of foreign antigen is called as receptor-mediated endocytosis. Immature DCs express specialized receptors for such PAMPs known as (PRR) through which they interact with antigens and internalize them. PRRs on DCs include toll-like receptors (TLRs), C-type lectin carbohydrate receptor (langerin, BDCA-2, DC-SIGN, macrophage mannose receptor) (114-116), NOD-like receptors and RIG-1 like receptors. DC PRRs can also recognize DAMPS expressed by neighboring ECs, infected cells, and apoptotic cells. Macropinocytosis is the second mechanism in which DCs engulf large amounts of soluble antigens through pinocytic vesicles and concentrate them in endocytic compartments (117). Particulate antigens including whole virus, bacteria, and apoptotic cells are internalized by phagocytosis that is the thirds mechanism through which DCs can internalize antigens (118-121). Once antigens are internalized via any of three mechanisms, they are degraded by proteases in endocytic vesicles. Thereafter, degraded antigenic peptides associate with MHCII in MHC II bearing compartments (MIIC) and antigen peptide-MHCII complex is then displayed on the cell surface of DCs to encounter antigen-specific T cells (122, 123). When DCs undergo maturation in response to exogenous antigen, the number and the half-

life of MHCII-peptide complexes increase, which was suggested to facilitate maximal encounter with allergen/antigen-specific T cells (122). As DCs express peptide-MHCII complex on their cell surface, they tend to loose ability to capture and process antigens. At this stage, expression of co-stimulatory molecules such as CD80 and CD86 is increased (124) and production of T cell polarizing cytokines is induced. DCs through MHCII complex form immunological synapse with TCR on T cells that is further fostered by interaction between costimulatory molecules on DCs and co-receptors on T cells. CD80 and CD86 on DC cell surface belong to B7 family and interact with CD28 on CD4 T cells, regulate survival and proliferation of T cells (124). ICOS-L and PDL-1 of DCs also regulate inflammatory functions of interacting CD4 T cells (125, 126). TNF molecule CD40 (on DCs) interacts with CD40 ligand (CD40L) on T cells and stimulates the production of inflammatory cytokines and expression of CD80 and CD86 on DCs (124, 127-129). Expression of another TNF family molecule OX40L is upregulated upon stimulation of DCs with CD40L and Th2 cytokines IL-33 and TSLP. OX40-OX40L interaction regulates T cell activation and survival (130, 131). OX40L also plays critical role in the maintenance and functions of memory T cells (132, 133).(134)

Maturation of DCs upon allergen uptake is also associated with their migration to lymphoid tissue that initiates with antigen uptake and ends with DC-T cell interaction. Since immature DCs have to travel to the peripheral tissue to detect antigens, they respond to chemokines RANTES/ CCL5 and MIP-2α/ CXCL2 by expressing tissue-homing chemokine receptors CCR1, CCR2, CCR5 and CCR6 (135-141). However, as they internalize antigen at the mucosal surface in the lungs, expression of tissue homing receptor downregulates (142) but the expression of lymph node homing receptors CCR7 and CCR4 upregulate that guides maturing DCs towards CCL19 and

CCL21 containing/producing T cell regions in mediastinal lymph nodes (MLN) (143-145). In MLNs, mature DCs attract T cells through production of chemokines including MIP3b, DCCK-1, TARC (CCL17) and MDC. As compared to T cells, a number of migrated DCs in the MLN is low; therefore DCs continuously engage and disengage with T cells. Interaction between ICAM-1 and DC-SIGN on DCs and LFA-1 and ICAM-2/3 play a critical role in such adhesive associations. It allows sufficient opportunity to neighboring T cells to interact with antigen-bearing DCs in MLN.

Role of lung DCs in regulating T cell mediated allergic inflammation

Although MLN constitutes the primary site of DC-T cells interaction, which dictates phenotype and functions of differentiated T cells, DCs also interact with peripheral tissue T cells. Removal of myeloid DCs during OVA challenge has been shown to abrogate characteristic features of allergic asthma including airway inflammation, airway hyperreactivity and mucus production (146, 147). During effector phase CD11b+ DCs form clusters with activated T cells and regulate T cell functions in the lung interstitium (148, 149). Through production of TARC and MDC, these DCs attract memory CCR4+ Th2 cells to the pulmonary tissue (150, 151). Also, these interactions regulate maturation of local DCs (148). Since recovery of mesenchymal lymph node (MLN) DCs is limited, these reports support the use of lung DCs to study the experimental role of DCs in murine asthma studies.

DC location in the lungs

Since DCs primarily act as antigen presenting cells, these cells are located in areas where these are most exposed to antigens/ allergens such as the lungs, skin, and gut. In the lungs, DCs are found in

every compartment including airways, lung parenchyma, interstitium, visceral pleura, alveolar space, and pulmonary vasculature (111, 152-156) (157). Especially in mucosa and sub-mucosa of conducting airways that serve as principle site for capturing inhaled allergens/ antigens, there exist an extensive network of DCs. In order to sense antigens in the airways effectively, DCs form long dendrites through epithelium to the airway lumen. Their frequency in airway epithelium may range from 500 to 1000 DCs per square millimeter of epithelial surface (155, 158, 159). Although DCs cover a substantial surface area in the lungs, their density depends on the magnitude of antigen exposure. Their density that is greater in proximal airways and diminished towards the periphery of the airways is significantly increased in response to external stimuli such as inhaled allergens, microbial infections (160, 161) and pollution (162). Extensive coverage of DCs in mucosal surfaces and their ability to expand in numbers emphasize the importance of DCs in immune surveillance.

DCs in asthma

Lung DCs regulate sensitization to allergens and play a key role in inducing T cells-mediated immune response that ultimately results in the development of asthma. Adoptive transfer of OVA-pulsed DCs into the lungs resulted in induction of robust Th2 response in the murine lungs (163). Within 12 hrs of allergen administration, lung DCs are capable of taking antigen and migrating to the lymph nodes. In the mediastinal lymph nodes, antigen-bearing DCs attract and induce vigorous polarization and proliferation of T cells within 48 hrs (145, 164). Besides sensitization, DCs also regulate secondary inflammatory response. In an elegant murine study by Lambrecht and group, suicide gene thymidine kinase expressing lineage committed DCs were depleted by treatment with anti-viral ganciclovir (146). Upon OVA challenge, systemic depletion

of DCs resulted in abrogated airway inflammation, mucus production and airway hyperresponsiveness (146). Also, selective depletion of lung DCs led to the elimination of feature of asthma including eosinophilic inflammation, goblet cell hyperplasia and AHR (147). Moreover, reconstitution of these mice with an ntratracheal injection of CD11c+DCs restored these defects. Interestingly, injection of other APCs such as macrophages failed to restore impaired OVA-induced effects (147), suggesting a key role of DCs but not other APCs in regulating development of allergic inflammation and associated feature of asthma.

Conventionally, lung DCs were suggested to be a single population of highly dendritic cells expressing CD11c and MHCII. However, recent reports have clearly characterized multiple subsets of DCs in the lungs, which may play a differential role in steady state and upon exposure to foreign agents. In steady state, an intraepithelial region of conducting airways is occupied by $CD11c^{high}MHCII^{+}CD103^{+}CD11b^{-}$ DCs that extend through epithelium by forming tight junctions with bronchial ECs, similar to Langerhans cells (165). Such DCs can also be found in the alveolar space, however, alveolar DCs project their dendritic projections across the epithelial layer and perform better in antigen uptake than basolateral DCs. Underneath epithelial layer localized are inflammatory $CD11c^{high}MHCII^{+}CD11b^{+}$ DCs (165). These subclasses of DCs have also been suggested to exist in lung interstitium. Emerging evidence have suggested that both these subsets derive from hematopoietic bone marrow progenitor cells, thus also known as myeloid DCs. In allergic inflammation, both of these subsets contribute to inflammation, however, clear division of these two subsets in allergic inflammation is not known. In response to allergen challenge, the number of DCs increases significantly due to rapid recruitment of $CD11c^{+}$ $CD11b^{+}$ monocyte-derived DCs in the lungs as a result of inflammation-induced emigration of Ly6C

^{high}CCR2^{high}CX3CR1$^{low/int}$ into the lungs (98, 146) (166, 167). Such an increase in the lungs is also associated with increase in the generation of myeloid progenitors in the bone marrow. This accumulation of DCs in the lungs seems to depend on mechanisms taking place in the bone marrow in response to allergen exposure.

Another class of pulmonary DCs is plasmacytoid DCs (pDCs) that express intermediate CD11c, siglec H, bone marrow stromal antigen-1 and share cell surface markers with granulocytes and B cells. pDCs can be found in the similar tissues as mDCs, however, their precise anatomical location is not clear. B-Wikstorm and Stumbles, 2007 showed that pDCs could be found in larger conducting airways and lung interstitium. Unlike myeloid DCs, pDCs are lymphoid in shape and are inefficient in antigen presentation and stimulating naïve T cells (144, 168-171). Instead pDCs mediate induction of inhalation tolerance. Adoptive transfer of OVA-pulsed pDCs prior to sensitization can suppress inflammation, however, depletion of pDCs exaggerates inflammation (144). pDCs promotes the induction of Tregs plausibly through an ICOSL-dependent mechanism (144, 170, 172, 173). Inefficient antigen processing and presenting machinery in pDCs have also been suggested to induce Tregs (174-176). Direct inhibition of T cells by pDCs is mediated through production of tryptophan catabolizing enzyme indoleamine 2, 3-dioxygenase (IDO) (177). Expression of PDL-1 on pDCs (178) and anaphylatoxin C5a regulate the suppressive functions of pDCs in experimental asthma in mice. In response to viral infections, pDCs produce a large amount of IFNs (type 1) and induce a cytotoxic response by activating CD8 T cells (179-181). Given that these cells are capable of activating CD8 T cells response in response to viral exposure, tolerance is diminished when allergen challenge is accompanied with adoptive transfer of respiratory syncytial virus-infected pDCs (182).

2.7 CD4 T cells

As DCs interact with naïve CD4 T cells and present allergen epitopes to the later, CD4 T cells are activated and polarized to a specific type of CD4 T cells. In response to allergen loaded DCs, naïve CD4 T cells may polarize to Th1, Th2, Th17 or Tregs. Activated and differentiated CD4 T cells secrete inflammatory cytokines through which they regulate secondary inflammatory mechanisms including altered functions of structural cells and other leukocytes.
(183)

2.7.1 Th2 cells: Frequency of Th2 cytokines is increased in asthmatic subjects (183). Traditionally Th2 cells through cytokines produced by these cells such as IL-4, IL-5, and IL-13 have been primarily implicated in causing allergic airway inflammation. A study showing induction of allergic inflammation upon adoptive transfer of effector Th2 cells into naïve mice followed by allergen exposure suggested the importance of Th2 cells in development of asthma phenotypes. Differentiation of naïve CD4 T cells to Th2 cells and secretion of Th2 cytokines are primarily dependent on phosphorylation and activation of transcription factor GATA 3 (184). Following activation, GATA3 translocates from cytoplasm to the nucleus and induce transcription of genes associated with Th2 phenotype (185). GATA3 positive T cells increase in asthmatic subjects, as compared to healthy human donors (186). T-cell specific transcription factor NFAT promotes GATA3-induced transcription of il4 genes (184). IL-33 also regulates Th2 inflammation by attracting Th2 cells. It also translocates to the nucleus and promotes the differentiation of Th2 cells by altering chromatin structure (187, 188).

IL-4 plays a crucial role in allergen sensitization. IL-4 also promotes differentiation of naïve CD4

T cells to Th2 cells. It along with another Th2 cytokine IL-13 act on B cells and regulate isotype switching of IgG-producing B cells to IgE-producing B cells. The fact that IL-13 is able to share functions of IL-4 is because IL-13 binds to IL-4Ra, which is also a receptor for IL-4. In addition to IL-4Ra, IL-13 also signals through IL-13Ra and mediate its functions through STAT6-dependent pathways. Allergen challenge results in an increase in IL-4 level and IL-13 in BAL; where IL-4 increase is transient but increase in IL-13 remains sustained (189). Increased IL-13 secretion in asthmatic condition positively correlates with increased eosinophilia, enhanced level of chitinase-like proteins in the lungs and the blood, goblet hyperplasia, airway hyperplasia and fibrosis in the subepithelial layer, indicating its importance in producing inflammatory and structural changes that are commonly observed in chronic asthma (189, 190). Direct administration of IL-13 can induce airway hyperresponsiveness. It promotes secretion of chemokine eotaxin from structural cells including epithelial cells. Proinflammatory effects of IL-13 mediate through STAT6-induced acid mammalian chitinase (AMC)-dependent pathways. Inhibition of AMC downregulates IL-13-induced AHR and inflammation (191, 192).

IL-5, a Th2 cytokine plays crucial role in allergen-induced eosinophilia. IL-5 regulates maturation of eosinophils from their bone marrow progenitors. Also, it is involved in regulation of eosinophil survival during inflammation. In asthmatic patients, systemic and local administration of IL-5 results in increase in eosinophils and their precursors (193, 194). On the other hand, inhibition of IL-5 using anti-IL-5 antibody abrogates eosinophilia in human and allergen-induced experimental murine model of asthma (194).

IL-9 level and IL-9 receptor expression are also increased in the airways of asthmatic

patients (195, 196). It promotes mucus production and eosinophilic inflammation through IL-13 secretion. However, in B cells and mast cells, IL-9 has been suggested to act directly (197). Over expression of IL-9 in murine model of asthma caused increased inflammation that was characterized by eosinophils, Th2 cytokines and IgE, mucus hyperplasia and AHR (195). Parallel to this finding, Cheng et al (198) discovered that inhibition of IL-9 reverted the symptoms of asthma in mice, thus validating a critical role of IL-9 in asthma.

Recently other Th2 cytokines such as IL-25, IL-33, and TSLP have also been suggested to regulate pathogenesis in asthma. IL-25 is a member of IL-17 superfamily but is produced by Th2 cells. IL-25 regulates the secretion of IL-4, IL-13 and IL-5 and results in inflammation that is characterized by eosinophilia, IgE production, and AHR (199). In human, it promotes the production of Th2 cytokines (200). Production of IL-33, an IL-1 family cytokine, by pulmonary structural cells including airway smooth muscle cells, fibroblasts and epithelial cells also increases in asthmatics (201, 202) (201). It translocates to the nucleus and regulates the structure of chromatin to induce differentiation of Th2 cells (187, 203). IL-33 selectively attracts Th2 cells to the target tissue. It acts on DCs to induce production of Th2 cytokines IL-5 and IL-13 but not IL-4 (102). Both IL-25 and IL-33 promotes production of TSLP in epithelial cells (204). TSLP belongs to the IL-7 family of cytokines. TSLP levels increased in allergen-exposed mice. Deletion of TSLP abrogated the hallmarks of allergic inflammation atleast in mice (96). Expression of TSLP has been experimentally shown to increase in asthmatic airway epithelial cells and mast cells (205). It regulates function of DCs so as to program them to produce Th2 chemoattractants CCL17 and CCL22 and regulate migration of Th2 CD4 T cells (205). Also, TSLP directly acts on CD4 T cells and induce secretion of Th2 cytokines (206, 207). Although these cytokines are primarily

produced by Th2 polarized CD4 T cells, Th2 cytokines can also be produced by innate lymphoid cells.

Since Th2-dominant inflammation and resultant airway remodeling is well characterized, patients with Th2 dominant endotype asthma can be treated by typical treatments such as the use of steroids (208). Therapies which are targeting Th2 cytokines, IgE or their receptors have provided key insights on newer ways to manage asthma.

2.7.2 Th1 cells: Studies that suggest Th2 cells and Th2 cytokines are the most prominent players of inflammation and airway remodeling in asthma have shown a reduction in T-bet expression in asthmatic T cells as compared to T cells from non-asthmatic donors (209). On the contrary, there are convincing evidence that support key role played by Th1 cells. In addition to Th2 cells, number of Th1 cells also increases in blood and induced sputum in response to allergen exposure in human. Differentiation of naïve CD4 T cells to Th1 cells and secretion of Th1 cytokine IFN-g is regulated by transcription factor t-bet. Deletion of Th1 transcription factor t-Bet in mice resulted in enhanced AHR and IL-13 dependent eosinophilia. Phosphorylated T-bet form an association with GATA3 and inhibits its (of GATA3) function of inducing transcription of Th2 cytokines (210). Furthermore, IFN-g promotes T-bet activation through STAT1-dependent mechanisms and induces and/maintains Th1 phenotype (211). Based on these reports, Th1 cells were suggested to suppress the development of Th2 cells and associated mechanisms.

IL-12 also regulates Th1 phenotype and secretion of IFN-g. Antigen presenting cells such as DCs and macrophages and structural cells such as epithelial cells, which are typically found in the

environment surrounding CD4 T cells, produce IL-12 (212). IL-12 induces the differentiation and activation of Th1 cells. IFN-g, which is induced by IL-12, regulates the expression of IL-12 receptor beta2, thus sustains IL-12 function of CD4 T cells (212). IL-12 mediates its functions by activating STAT4-dependent mechanisms. Asthmatic blood showed reduced IL-12 production, which has been linked to reduced Th1 inflammation in asthmatic condition (213). Since IL-12 promotes Th1, which suppresses Th2 inflammation, recombinant IL-12 was administered to mild asthmatics to see whether it would suppress Th2 dominant airway inflammation and other features of asthma. Interestingly, recombinant IL-12 abrogated circulating eosinophilia, however, failed to affect other mechanisms including AHR (214). Another member of the IL-12 family, IL-27 promotes Th1 differentiation through STAT1 dependent signaling mechanisms (215, 216), however, its role in asthma is not determined yet. IL-27 have been shown to downregulate GATA3 expression and promote expression of T-bet, thereby favoring Th1 differentiation (216). IFN-g secretion (Th1 phenotype) is also regulated by IL-18. IL-18 promotes IFN-g secretion but suppresses Th2-dependent phenotypes (217). Its function may or may not dependent on IL-12 (217, 218).

Although typical paradigm suggests that Th1 cells antagonize Th2 differentiation, however, there have also been contradictory reports that suggest the role of Th1 in promoting Th2 trafficking and eosinophilia (219, 220). *Martin et al* showed increase in lymphocyte accumulation in the lungs upon IFN-g inhalation in human subjects (221). Altogether it seems that whether Th1 and IFN-g play a positive or negative role, depends on the environmental factors.

2.7.3 Th17: Recently, role of Th17 cells in asthma have also gained attention. Differentiation of

Th17 cells is regulated by a network of cytokines and intracellular transcription factors, independent of cytokines and transcription factors required for Th1 and Th2 differentiation (222-224). *In vitro*, naïve CD4 T cells are exposed to a mixture of anti-IL-4, anti-IFN-g, IL-6, IL-23 and TGF-b. In human as well as in mouse, IL-6 and IL-23 play a key role in the induction of Th17 polarization. Initially, IL-23 was suggested to be the most important inducer of IL-17 production (223). IL-23 is a heterodimer that consists of IL-23p19 and IL-12p40 (component shared with IL-12) (225). IL-23p19-deficient mice exhibited normal IFN-g production, however, lacked IL-17 positive cells (223). Such cells acquired the ability to produce IL-17 upon IL-23 exposure. Moreover, treatment of wild-type memory T cells with IL-23 resulted in inhibition of Th1 expansion and promoted Th17 differentiation (226, 227). In context with allergic asthma, Wills-Karp and group showed that deletion of complement C5a promotes severe allergic inflammation with prominent Th17 response possibly due to increased production of IL-23 by DCs. On the other hand, mice deficient in C3 receptor exhibited reduced Th17 cells and AHR, plausibly due to reduced IL-23 production (228). Undoubtedly, IL-23 plays a critical role in Th17 differentiation. Given that IL-23 receptors are expressed in low levels on naïve CD4 T cells and that expression of IL-23 R and IL-12Rb2 (components of IL-23 receptor complex) required activation of T cells (229), relevance of IL-23 in the induction stage was questioned. It implied the role of other factors in the regulation of initial commitment of naïve CD4 T cells to Th17 lineage. IL-6 production by DCs in response to TLR ligands was shown to suppress Tregs (230). TGF-b controls differentiation of Tregs, however, when TGF-b combines with IL-6, it inhibits this phenomenon instead and promotes differentiation of naïve CD4 T cells to Th17 cells (231). On the contrary, the synergy between TGF-b and IL-2 promotes Tregs polarization, suggesting an inverse correlation between Tregs and Th17 development. *Oh et al* (232) showed that STAT3 activation, which is

involved in Th17 differentiation, downregulates IL-2. Stimulation with proinflammatory cytokines such as IL-6 has been reported to inhibit Treg phenotype and increase IL-17 production. IL-1β and TNF promoted this effect (222, 231, 233). In mice, deficiency of TGF-b results in impaired Th17 responses, which reverted upon exogenous administration of TGF-b. Accumulating evidences suggest that IL-6 with TGF-b upregulates the expression of IL-23R through activation of STAT3-dependent pathways (224, 234). Based on *in vitro and in vivo murine studies,* it seems that IL-6 with TGF-b initiates the process of Th17 differentiation, which is then sustained by IL-23-dependent mechanisms (222, 224). Interestingly, IL-6-/- mice can also yield Th17, suggesting the involvement of IL-6-independent pathways in Th17 development. IL-21, a Th17 cytokine promotes IL-17 production (235) plausibly by upregulating RORγt and IL-23 through STAT3 dependent mechanism (236). In mice models lacking IL-21 or its receptor, Th17 responses have been observed to be impaired. Production of IL-21 is induced by IL-6 through a STAT3-dependent signaling pathway. However, once it is produced, it can act on T cells, without needing IL-6 to stimulate IL-17 production**.** IL-15 has also been shown to promote IL-17 production in activated PBMCs and spleen CD4 T Cells (237, 238).

Intracellularly, STAT3 plays a critical role in Th17 differentiation. Th17 promoting effects of IL-6, IL-23 and IL-21 depend on the activation of STAT3-dependent pathways (236). Phosphorylation of STAT3 enables its translocation to the nucleus where its binds to *IL-17* gene locus. Similarly, it interacts with *IL-21* promoter and promotes their transcription (234, 239, 240). Deletion of STAT3 impairs induction of Th17 responses. Furthermore, overexpression of STAT3 stimulates this phenomenon. In hyper IgE syndrome, missense mutation in STAT3 gene results in impaired Th17 phenotype (241, 242). SOCS3 is a negative regulator STAT3 activation, absence/

deletion of which is associated with increased STAT3 phosphorylation and amplified IL-17 production (240). Furthermore, transcription factor ROR-γt has been identified as master regulation of Th17 differentiation, which through STAT3 activation, promoted differentiation of naïve CD4 T cells to Th17 cells. Deletion of ROR-γt renders T cells defective in IL-17 production. This phenotype could be reversed upon overexpression of ROR-γt. Like IL-17 production, ROR-γt expression is also reduced in STAT3-/- T cells, suggesting a possible regulation of ROR-γt expression. ROR-γt also synergize with RUNX to promote production of Th17 cytokines through negative regulation of FOXP3 (243). Deficiency of ROR-α either alone or with ROR-γt also affects IL-17 expression. Coexpression of both of these transcription factors facilitates augmented Th17 development (244). Additionally, IRF-4 that is activated by IL-1 also promotes IL-17 production. IRF-4-/- T cells show defected Th17 differentiation in response to Th17 polarizing condition (245).

Development of Th17 response is also regulated by negative regulatory mechanisms. Retinoic acid suppresses Th17 differentiation by inhibiting ROR-γt through the SMAD-3 dependent mechanism (246, 247). IL-2 activates STAT5, through which it can constrain Th17 phenotype (248). Similarly, IL-27 can also suppress IL-17 production through activation of the STAT1 pathway (249). Collectively, Th17 response is regulated by coordinated function of cytokines and transcription factors.

Th17 in asthma

Th17 CD4 T cells and Th17 cytokines have been reported to increase in asthmatic airways (250-252). Studies showed increased IL-17A, a Th17 cytokine in sputum, BALF, and blood of

asthmatic subjects (253, 254). Typically, Th17 dominant inflammatory response is associated with neutrophilic inflammation than eosinophilic inflammation (253, 255). IL-17A and IL-17F act on airway epithelial cells and airway smooth muscle cells and induce secretion of neutrophilic chemoattractants CXCL1 and CXCL8, thereby promoting neutrophilic infiltration into the lungs in asthma (256, 257). IL-17-induced GM-CSF by bronchial epithelial cells promotes neutrophil accumulation in the lungs (35). Given that severe asthma is characterized by neutrophilic inflammation, which is regulated by IL-17, accumulating evidence have suggested a positive correlation between Th17/ IL-17 with severe asthma in humans (253, 258-261). Increase in the levels of IL-17A, IL-17F, Th17 cells and other IL-17 producing cells have been reported to have a positive association with disease severity in children and adults asthma patients (253, 258-261). A study by *Brandt et al* suggested a positive correlation between diesel exhaust exposure, increased level of IL-17A in the serum and disease severity in atopic asthmatic children (262). Within such a cluster of moderate to severe asthmatics, subjects manifesting a mixed Th2/Th17 inflammation and neutrophilia/ eosinophilia were also observed. In murine models of experimental asthma, allergen sensitization through airways resulted in Th17-dependent neutrophilia and modest Th2 responses in the airways. This phenotype was associated with AHR, which was suggested to depend on both Th17 and Th2 responses (263). Similarly, co-existence of Th2 and Th17 inflammation is also observed in human asthmatics (264, 265).

Th17 dominant inflammation and robust neutrophilia are also associated with steroid resistance in severe asthmatics (266-270). In murine models, reconstitution of SCID mice with OVA-specific Th17 cells resulted in severe steroid-resistant asthma (268). Similarly, allergen sensitization of ROR-γt overexpressing mice led to steroid-resistant neutrophilic inflammation (271).

Furthermore, treatment with dexamethasone supports maintenance of Th17 differentiation *in vitro (267).* Unlike IL-4, dexamethasone treatment of lymphocytes is unable to affect secretion of IL-17A (268). Previous reports explained that Th2/Th17 cells exhibit increased activation of MEK-ERK1 pathways through which they antagonized inhibitory functions of glucocorticoids (261, 272, 273). Moreover, exposure to IL-17 promotes expression of glucocorticoid receptor b in human peripheral mononuclear cells, rendering them steroid resistant (269). Pretreatment with IL-17A inhibits the suppressive ability of budesonide on TNF-induced IL-8 production in human bronchial epithelial cells *in vitro.* This effect was suggested to be mediated through activation of ERK and PI3K pathways (274). In this study, Heijink and colleagues also found that IL-17A reduced HDAC2 activity, which also contributed to IL-17A-induced steroid insensitivity (274). Altogether these reports provide a critical insight of how steroids regulate Th17 differentiation, which then controls sensitivity to steroids in non-T cell compartments.

In human epithelial cells, IL-17A regulates expression of mucus genes *muc5ac* and *mub5b*, plausibly contributing to enhanced mucus production as observed in asthma (275, 276). IL-17 treatment of human epithelial cells has also been shown to upregulate chemokine and cytokine genes such as CXCL2, CXCL3, CXCL5, CXCL6 and IL-19 through activation of a pathway involving JAK, PI3K, ACT1, TRAF6 and TAK1. In airway epithelial cells, IL-17 stimulates gene expression of sPLA2-X, which is associated with AHR and severity of asthma (277). Recently, IL-17A has also been shown to contribute to airway fibrosis related to asthma. IL-17A promotes expression of profibrotic cytokines, collagen and other factors involved in fibrosis (254, 278, 279). CD40-CD40 ligands interaction plays a critical role in mediating profibrotic effects of IL-17A and IL-17F at least in bronchial fibrocytes (280). The fibrotic environment in asthmatic patients also

favors Th17 response (281). *In vitro* experiments on human airway smooth muscle have shown that IL-17A stimulates production of proinflammatory cytokines and chemokines (282). It also promotes their proliferation, survival, and migration, thereby contributing to airway smooth muscle related structural aberration in asthma (283, 284). The fact that these features revert upon inhibition of IL-17 RA or IL-17RC, further corroborates the critical role of IL-17 machinery in affecting airway smooth muscle cells (285). IL-17A activates Rho-ROCK signaling pathway to amplify contraction of smooth muscle of murine tracheal rings and human bronchial tissue (286, 287). Altogether it is evident that Th17 and Th17 cytokines such as IL-17A play the key role not only in promoting inflammation but also exaggerates airway remodeling, finally culminating in severe asthma.

2.7.4 Other sources of Th17 cytokines and their role in asthma

Typically, Th17 cytokines are supposed to be produced by Th17 cells, however, emerging reports have provided us an insight of the Th17 producing capacity of other cell types such as γδ-T cells, ILC-3 and NKT cells and their role in allergic inflammation (288). γδT cells are found in close proximity to lung epithelium where their primary role is to mediate immunosurveillance (289). These IL-17 producing cells can drive accumulation and activation of neutrophils in the tissue. Their role in the regulation of AHR has also been shown (290). γδT cells count has been shown to be increased in the BALF of asthmatic donors as compared to healthy subjects (291). In humans, CD30+ γδ T cells have also been reported to produce IL-4 in response to specific allergen (292, 293). CD122high γδ T cells can also produce IFN. Although γδT cells can regulate some of the mechanisms involved in asthma, their precise role in not studied at least in human asthma (294). IL-17 producing γδ-T cells also express IL-23R and has been shown to produce IL-22 and IL-21

in response to IL-23 and IL-1β irrespective of TCR engagement (295, 296).

Lately, the role of innate lymphoid cells and their IL-17 producing capacity has also been explored in asthma. IL-17A producing innate lymphoid cell type 3 (ILC3) are characteristically identified as non-T, non-B, CD4−, lineage− cells (297). According to an elegant study by Umetsu and group, mice treated with IL-1a and IL-1β induced a significant increase in IL-17A producing ILC3. The ILC3 cells were capable of inducing AHR and allergic inflammation upon adoptive transfer to Rag-/- mice. Expansion and activation of ILC3 were suggested to be dependent on NLRP3-dependent mechanisms at least in obese mice (297). Interestingly, ILC3 do not produce other Th17 cytokines such as IL-17F and IL-22.

iNKT cells have also been implicated as IL-17 producing cells in response to the allergen. iNKT cells express MHC class I-like molecule CD1d through which they recognize lipid antigens (298, 299). These cells express transcription factor ROR-γt and IL-23 (298). iNKT cells increase in response to allergen challenge in the lungs of asthmatic subjects (300-302). In mice lacking in iNKT cells, allergen exposure resulted in impaired eosinophilia and AHR. Together these reports suggest a role of iNKT in the regulation of neutrophilic inflammation and AHR, characteristic features of asthma.

2.8 Treatments in asthma

Asthma is a multifactorial disease that does not have one phenotype for all. As discussed above, there could be different external and internal factors causing asthma. Therefore, it is now clear that one treatment strategy would not be successful for all the patients. Short (SABAs) and long

(LABAs) acting β2-adrenoceptor agonists and corticosteroids are the most commonly used and successful drugs to treat the symptoms of asthma. β2-adrenoceptor agonists bind to β2-adrenoceptor and stimulate adenylate cyclase that induces release of cyclic adenosine 3'5' monophospahte (cAMP) and activation of protein kinase A. As a result of these events smooth muscle relaxation takes place that provides rapid relief from bronchoconstriction (303, 304).

Corticosteroids act by diffusing across the cell membrane, interacting with glucocorticoid receptors that then inhibits the expression of inflammatory cytokines, chemokines and adhesion molecules (304). Corticosteroids are often paired with β2 adrenoceptor agonists to provide more effective relief (305).

Other drugs that are often prescribed to treat allergic reactions are H1-antihistamines and phosphodiestrase inhibitors. Antihistamines such as cetrizine, levocetrizine, loratadine and desloratadine are safer to use and are effective at early stage of allergy (306). Theophyline, a cAMP phosphodiestrase inhibitor, is a xanthine that also acts as an adenosine antagonist. Theophyline has been found effective in treating broncho-constriction but due to the side effects, its use is less desirable. A non-xanthine PDE inhibitor such as rofumulast targets type 4 isoenzyme and have been shown to be more effective as compared to others in this class (307).

Although most of the currently used drugs are effective in treating the asthma symptoms in different combinations, they do not offer long-term relief and neither do they affect the natural history of the disease, including corticosteroids. Also corticosteroids that are considered to be the most effective tool to treat asthma inflammation are ineffective in severe asthma cases including

IL-17A/ neutrophilia dominant severe asthma that calls for novel therapeutic approaches to target severe forms of asthma.

Recently, biopharmaceutical approaches have also been used to target molecular perpetuators of inflammation. Omalizumab is a humanized IgE specific, non-anaphylactic IgG1 that has shown promising results in human clinical trials (308). Humanized anti-IL-5, mepolizumab reduces eosinophilia and these effects may persist for at least 3 months, however does not affect late phase inflammation or airway hyperresponsiveness (309). Blocking functions of other cytokines including IL-4, IL-13 and IL-9 is also been tested (310-313). However use of blocking antibodies or biotherapeutics is still at a very early stage and required more controlled human clinical trials.

2.9 Murine models of experimental asthma

As disussed above, asthma is characterized by structural changes in the airways and inflammation that is the outome of activation of multiple inflammatory mehanisms and involvement of multiple cell subsets. An ideal approach to study these processes and to identify novel mehanisms and therapeutics targeting them, is to perform studies on human asthma patients. However, it is not acceptable due to ethical concerns. Animal models on the other hand, serve as suitable alternative allowing us to perform required experimental studies. Mice are most widely used animals owing to their convenient housing, manipulations and short breeding periods.

Although all charaterstics of human asthma are not possible to be reproduced in mice, use of different allergens, protocols of allergen exposure and strains have been instrumental in modeling

specific features. Acute experimental asthma models are best used for studying inflammation. In acute models, mice are first sensitized through multiple systemic administration of the allergen with adjuvant followed by intranasal or intratracheal challenge with the allergen. Chronic models are achieved by sensitization with low doses of the allergen for upto 12 weeks followed by challenge with the same. Chronic models have been useful in studying airway remodeling which is not seen in acute models (314, 315). OVA is a good choice for acute model but majority reports have used more environmentally relevant allergens in chronic models. Use of model protein allergen such as OVA has a limitation that long-term use of OVA may result in tolerance and inhibition of the expected outcome of the allergen exposure (316).

Although animal models have successfully been used to identify new mehanisms and novel drug targets, final clinical trials have to be done on humans.

3. Pentraxin 3 (PTX3)

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Pentraxin 3: an immuno-regulator in the lungs

Jyoti Balhara[1], Latifa Koussih[1,2], Jingbo Zhang[1,3] and Abdelilah Soussi Gounni[1]*

- [1]Department of Immunology, University of Manitoba, Winnipeg, MB, Canada
- [2]University Saint Boniface, Winnipeg, MB, Canada
- [3]Xinqiao Hospital, Third Military Medical University, Chongqing, China

Pentraxins are a superfamily of evolutionarily conserved, specific pattern recognition proteins that play a salient role in the innate immune system. Based on size, these multifunctional proteins are divided into long and short pentraxins. Long pentraxins include prototypic pentraxin 3 (PTX3), the recently identified PTX4, neuronal pentraxins 1 (NP1) and NP2 whereas short pentraxins consist of C-reactive protein (CRP) and serum amyloid P (SAP) (317, 318).

Phylogenetic analysis has demonstrated conservation of short and long pentraxins in human, mouse, rat, opossum, chicken and some lower vertebrates (319). Although all pentraxins evolve from a common ancestor, PTX3 alone forms a separate cluster and seems to originate directly from the common ancestral pentraxin very early in evolution (319).

PTX3 has been identified as biomarker of several immunopathological states. Its relevance with the resolution of infections and diseases has also been studied. In this review, we detail general

structure, expression and functions of PTX3. Also discussed are current findings, which suggest an important role of PTX3 in immunological states associated with the lungs particularly.

4. PTX3 gene structure

Human and murine PTX3 gene is localized on chromosome 3 (q22-28) (320). The PTX3 gene is organized into three exons: the third exon, which corresponds to the second exon of short pentraxins, encodes pentraxin domain whereas first and second exons translate to leader peptide and N-terminal domain, respectively (320) (figure 1.1). PTX3 in human and mouse displays 92% conservation in amino acids with 82% of identical amino acid residues (321, 322). Significant homology between human and mouse PTX3 renders it reasonable to extrapolate murine studies to address human issues.

PTX3 promoter in human contains several potential cis acting elements including NF-kB site, activator protein 1 (AP-1), AP-2, specificity protein (Sp1) and gamma interferon activation site (GAS) (323) (figure 1.1). In addition to these elements, promoter sequence of the murine PTX3 gene also contains binding sites for Hox-1.3, and transcription factors belonging to Ets family. Mouse PTX3 gene contains a stretch of 44bp of alternating CA residues in its promoter region, which acts as enhancer element. Murine promoter region also contains multiple NF-IL6 binding sites while its human counterpart contain only one, which draws particular attention on the role of NF-IL6 in the regulation of PTX3 expression (324).

Unlike short pentraxins, PTX3 promoter does not contain consensus site for hepatic nuclear factor-1 (HNF-1) that accounts for the absence of its induction in liver (324). Although human and

murine PTX3 protein is homologous, human promoter contains lesser transcriptional elements than murine as evident from figure 1.1, an effect that can be better explained subsequent to greater molecular and phylogenetic analysis.

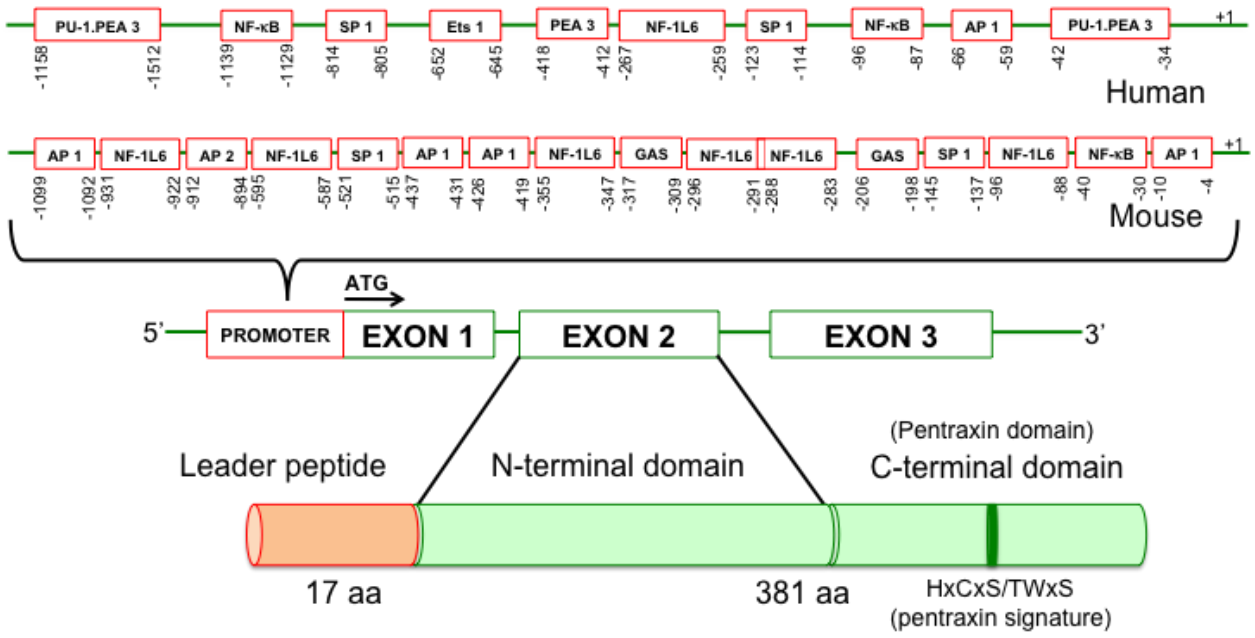

Figure 1.1*: Arrangement of transcription factors of human and murine ptx3 gene. PTX3 gene is organized into promoter region and three exons: the first exon encodes for leader peptide (17 amino acids) while the second and the third exons encode for N- and C-terminal domains of the protein (381 amino acids). Promoter region contains multiple transcription binding sites.*

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5. PTX3 protein structure

The name pentraxin is derived from their pentagonal structure, made up of five subunits, Initially, PTX3 was assumed to acquire quaternary structure similar to short pentraxins that consist of five identical 23 kDa subunits held together by noncovalent interactions. Later, it was observed that amino acid residues at the protomer interface that are required for pentameric structural formation in CRP, are not present in PTX3 (322). PTX3, however, exist mainly as octamers made from two tetramers and each subunit is held together by covalent bonds (325-327). PTX3 octamers are more functionally active as compared to tetrameric oligomers (327).

The protein structure of PTX3 like other pentraxins includes a pentraxins domain containing HxCxS/TWxS (where x is any amino acid) at the C-terminal domain. Its C-terminal domain consists of 203 amino acids, 57% of which are conserved in entire pentraxins superfamily. The unique N-terminal domain consists of 178 amino acids (328). The C- and N-terminal domains of

PTX3 evolved independently which explains why C-terminal domain is widely conserved while N-terminal domain is not (319). Mature PTX3 protein is approximately 40 kDa, however, glycosylation at Asn220 increases the molecular weight to 45kDa (321). Oligosaccharide moieties at Asn220 can be complex or heterogenous sialic and fucosyl sugar moieties. Unique glycosylation patterns are associated with different inflammatory cells and inflammatory stimuli that induce PTX3 production (329). Desialylation of PTX3 results in stronger binding to C1q and activates the classical complement pathway to a sizable extent (329). PTX3 has two conserved cysteine residues at C- terminus (Cys 210 and Cys 271) [20] in addition to seven other cysteine residues, which are exclusive to PTX3: three at N –terminus (Cys 47, 49 and 103) and four in C-terminal region (Cys 179, 317, 318 and 357). These cysteine residues form a network of disulphide bonds that maintains the oligomeric structure of PTX3. Interchain Cys47-Cys47 and Cys49-Cys49 bonds form dimers and these dimers are further held together by Cys 103-Cys103 bonds to loom tetrameric structures [20]. These tetrameric structures are further assembled into octamers by disulfide bonds between Cys317 and Cys 318 which are located on exposed loops of different protomers (327). C- terminal domain of PTX3 is formed by two antiparallel β sheets, stabilized by Cys 210 and 271 organized into a β-jelly roll topology (330, 331). Cys 179 and 357 also form disulphide bonds and seem to limit the flexibility of N and C- terminal region by linking them together (327). N terminal region, on the other hand, is predicted to assume coiled coil conformation because it is made up of four alpha helices (332). Although the crystal structure of PTX3 has not been determined yet; modeling has helped in undertanding the arrangement of β-strands and α-helial segments in PTX domain of PTX3 (333).

6. Proteolytic cleavage of PTX3

Recently, Delneste and coworkers showed that N-terminal domain of PTX3 is susceptible to cleavage by proteases, particularly neutrophil elastase. However, the role of neutrophil Cathepsin G and PR3 was not clear in this report (334). Additionally, *A. fumigatus* proteases also degraded N-terminal domain. The activity of *A. fumigatus* proteases was shown to be inhibited by serine protease inhibitor PMSF, antipain and chymostatin but not affected by aspartic-, metallo-, cysteine- and aminopeptidase protease inhibitors. Degradation of the C-terminal domain was suggested to take place upon prolonged incubation with these agents (334). Proteolytic cleavage of PTX3 has added an interesting aspect to the regulation of PTX3 expression and function, detailed analysis is, however, necessary to validate this phenomenon.

7. PTX3 expression

There is a growing body of evidence suggesting that PTX3 can be produced by a variety of cells and induced by various different stimuli (320, 335). It is due to this reason that PTX3 is capable to serve multiple functions depending upon the condition. It is interesting to note that regardless of the source of its production (immune cells or structural cells), it plays a critical role in regulation of the humoral arm of innate immunity (335).

7.1 Immune cells

Lymphoid cells such as T cells, B cells and NK cells do not express PTX3. This highlights the significance of PTX3's control on the innate immune system (336). However, the action of PTX3 is not limited to the innate immune system, PTX3 activates the adaptive immune system through

indirect mechanisms and provides a mechanism for both arms of the immune system to cooperate and fight against infections.

7.1.1 Dendritic cells: Among cells of immune system, myeloid cells and especially dendritic cells (DCs), are the prime source of PTX3 (322). An intricate network, as demonstrated by Doni et al, regulates its expression in myeloid dendritic cells upon stimulation with TLR ligands, CD40L, IL-10, and IL-1β. However, no such effect was observed in plasmacytoid DCs (337). Similarly, macrophages also express PTX3 and show an augmented response to digest opsonized pathogens (336).

7.1.2 Neutrophils: Neutrophils are the only granular cells reported to release preformed PTX3 in response to TLR agonists and microorganisms. PTX3 exists as a monomer in 'ready to release' myeloperoxidase (MPO) negative granules containing lactoferrin and lactoferrin/gelatinase and assembles into multimers upon release. When neutrophils are activated upon inflammatory stilmulation, they release 25% of their PTX3. Part of the released PTX3 remains associated with neutrophil extracellular traps (NETs), which interacts with certain components of NETs (338, 339). Neutrophils are among the first cells to defend against foreign pathogens and immediate release of PTX3 by these cells may be indicative of its importance in innate immunity.

7.2 Structural cells and other cells

7.2.1 Adipocytes: Induction of PTX3 by TNF has been demonstrated in adipocytes (340). PTX3 expression was shown in preadipocytes, which decreases upon differentiation to adipocytes. In light of differential PTX3 expression in different differentiation stages of adipocytes, function of PTX3 in this process was found irrelevant. Additionally, greater level of PTX3 mRNA was

observed in adipose tissue of obese and obese diabetic mice as compared to WT mice. Although authors suggested this expression resulted from adipocytes, examination of cell-specific PTX3 production in these tissues is requisite (340). Altogether, more studies are required to determine the functional outcome of the role of PTX3 during differentiation and also in obese conditions.

7.2.2 Cardiomyocytes: PTX3 is constitutively expressed in the human heart by cardiomyocytes. (341)**.** However, dying and necrotic cells release it in large quantities, contributing to its increased level in blood of the patients with acute myocardial infarction (AMI) (341). Although its exact role in healthy myocytes is not well understood, it is generally used as an indicator of tissue damage in AMI (341). On the other hand, rodent hearts express high levels of PTX3 upon induction. Unpublished observations of *Peri et al.* suggested induction of PTX3 in rat hearts by physical stress. Whether similar physical stress, which is continuously experienced by normal human myocytes, result in PTX3 constitutive expression is not clear. According to *Introna et al*. (322), LPS exposure did not induce PTX3 expression in cardiac myocytes. PTX3 protein expression was shown to be increased in murine cardiomyocytes after transverse aortic constriction (342). H_2O_2 also induced its expression (342). Interestingly, unlike healthy human heart myocytes, PTX3 expression by healthy myocytes from rodent background remained controversial.

7.2.3 Endothelial cells: In atherosclerosis, high-density lipoprotein (HDL) induces the expression of PTX3 in endothelial cells, lining blood vessels by activating a PI3K/Akt-dependent pathway. It is suggested to manifest an anti-inflammatory and protective function (343). In contrast, PTX3 induction by Lysophosphatidic acid (LPA), the lipid component of oxidized low-density

lipoproteins (oxLDL) through the activation of NF-kβ, has a proatherogenic function (344). These contradictory observations advocate the need of a unifying study to unveil the true role of PTX3 in the development or resolution of atherosclerosis.

7.2.4 Epithelial cells: Alveolar epithelial cells produce PTX3 upon mechanical stretch *in-vitro*. (345). LPS and TNF can also induce the production of PTX3 in epithelial cells (346). Human proximal renal tubular epithelial cells constitutively express PTX3 mRNA, which is further upregulated by IL-1β, TNF, IL-17 and CD40L. IL-6 and IL-4 have no effect on PTX3 expression whereas GM-CSF was shown to diminish the effect of IL-1β (347).

7.2.5 Chondrocytes: IL-1β and oncostatin M (OSM) synergistically induce PTX3 expression in chondrocytes of the synovium (348, 349). Consequently, whether it helps in cartilage repair or degradation is largely unexplored. Previously, enhanced PTX3 expression was observed in rheumatoid arthritis (RA) synoviocyte and in synovial fluid. Expression in RA synoviocyte is unaffected by TNF or IL-1β but is down regulated by TGF-β and IFN-gamma (350).

7.2.6 Brain cells: Although PTX3 and neuronal pentraxins belong to the same family, expression of former is generally not observed in the brain. It is however; induced by inflammatory stimuli in granule cells, presumptive glial cells in the white matter (corpus callosum, fimbria), meningeal pia mater and dentate gyrus hilus. Its role is to provide protection against tissue damage, likely to be induced by LPS, TNF, and IL-1 (351, 352).

8. Mechanism of action

PTX3 exerts diverse functions to provide immuno-protection through multiple ways. It binds to the surface of pathogens and apoptotic inflammatory cells and promotes their opsonization and clearance early in the infection process. Such a role is important to avoid the induction of a deleterious hyper-inflammatory state that could arise due to activation of the adaptive arm of the immune response. Notably, although the N-terminal domain of PTX3 is suggested to be requisite for pathogen binding, the full-length protein is required for opsonization (353). Also, PTX3 when bound to pathogenic components activates DCs. This is instrumental in initiating an appropriate T cell response. Another mechanism by which PTX3 initiates the thread of innate immunity is by activating the complement cascade (354).

8.1 Activation of Complement pathway:

A prototypical paradigm by which PTX3 activates the classical complement pathway is by interacting with C1q**.** PTX3 binds to the globular head of immobilized C1q through its C-terminal domain, subsequently triggering the activation of the downstream cascade. Glycosidic patterns on PTX3 significantly contribute to its interaction with C1q (329). Of note, when C1q is in solution, PTX3 inhibits the activation of complement pathway by blocking C1q interaction with immunoglobulins or other agents (355).

The ability of PTX3 to resolve *Aspergillus* infection in C1q KO mice but not in C3 KO mice indicated that PTX3 might also interact with other members of the complement family to mediate its function. PTX3 amplifies C3b-mediated opsonization and phagocytosis of Aspergillus conidia through inside-out activation of CD11b in macrophages (353). Conidia bound PTX3 binds to

Factor H and promotes C3-mediated activation of the alternate pathway (C3 is also deposited on conidia) and killing of the pathogen. A similar mechanism is employed to remove apoptotic cells which can be a potential cause of unwanted inflammation (356). Factor H binds to PTX3 at two sites: the PTX3 N-terminus acts as the primary binding site and the glycosylated pentraxins domain acts as the secondary binding site (356). While investigating the role of PTX3 in the disposal of *Aspergillus* conidia, Moalli *et al.* (353) found that factor B of the alternate pathway is also necessary for its activity.

Pathogens attempt to evade the immune system so as to infect the host, in turn, activates multiple immune mechanisms to fight against the infection. The work of Moalli *et al* (353) implicated the role of all complement pathways in conidia opsonization. The association of PTX3 with other complement components further strengthens the suggestion. Along the same line, PTX3 is also found to activate the lectin pathway of complement, by interacting with ficolins through their fibrinogen-like domain (357). Ficolins recognize carbohydrate moieties on pathogens and dying cells and provide immuno-protection by activating the lectin complement pathway and prime the adaptive immune response (358). Besides binding to ficolin-L, PTX3 enhances complement activation resulting from Ficolin-L interaction with *Aspergillus* (359, 360). However, the interaction between ficolin-L and PTX3 is critically affected by the polymorphism in the ficolin-L gene causing a T236 amino acid change in the fibrinogen like domain (360). Similarly, ficolin-M also interfaces with PTX3 (360). This may be of even greater significance in this review as ficolin-M is majorly produced and found in the lungs (360, 361). The ficolin-M tetramer binds to four PTX3 molecules where a sialic moiety in an N-linked carbohydrate of the C-terminal domain of later was found to be involved (361).

8.2 PTX3 receptor and Ligands

Since it was evident that PTX3 acts on immune cells, playing a critical role in driving innate immunity against pathogens, it was conceived that it might act through some unknown receptor. A study investigating the binding properties of pentraxins with FcyRs determined that PTX3 could recognize FcyRs (362). The functional significance of such an interaction was later established in an *Aspergillus* infection model (353). The protective effect of PTX3 against *Aspergillus* was found to be diminished in the absence of FcγR. This interaction was suggested to activate CD11b to induce opsonophagocytosis of the pathogen (353). Keeping in mind the diverse functions mediated by PTX3 at the cellular level, it is quite possible that it may interact with additional unknown receptors, which could be the subject of further investigation.

PTX3 binds to fibroblast growth factor-2 (FGF-2) (363, 364) and fibroblast growth factor-8b (FGF-8b) (365) and affects neovascularization by mediating anti-angiogenic and anti-restenotic activity. FGF-2 induces proliferation in vascular smooth muscle cells and endothelial cells, and this is inhibited by PTX3 (363, 366). PTX3 inhibits FGF-2 functions by physically interacting through its N-terminal domain (364). TSG-6 also interacts with PTX3 through the same domain, due to which it competes with FGF-2, thus abolishes the inhibitory effect of PTX3 on FGF-2 mediated angiogenesis (367). The association between TSG-6 and PTX3 is extremely critical in orchestrating ECM in cumulus oophorous, which is important for female fertility (368). Since, PTX3 and TSG-6 were found to be co-regulated in monocytes, macrophages, and myeloid DCs, their association was suggested to contribute to ECM remodeling during inflammation (369).

9. PTX3 and Lung infections

9.1 Pseudomonas aeruginosa infection: The protective role of PTX3 was supported by the observation that PTX3 KO mice are more susceptible to *Pseudomonas aeruginosa. P. aeruginosa* is one of the prominent bacteria colonizing the lungs and causing chronic lung infections observed in Cystic fibrosis (CF) patients. Recombinant PTX3 facilitates the clearance of this pathogen by promoting an appropriate immune response in the lungs of PTX3 KO mice. This effect was demonstrated to be dependent on C3 and Fcγ and independent of C1q (370). The mechanism by which such a protective role is mediated through the complement system is described elsewhere in this review. Treatment of *P aeroginosa* infected CF mice with exogenous PTX3 is of interest as CF environment does not affect PTX3 activity as it does with other natural antimicrobial peptides, such as the β-defensins and the cathelicidin LL-37. In contrast, PTX3 level increases in serum of CF patients as compared to healthy subjects (334). Intraperitoneally administered PTX3 decreased the concentration of inflammatory mediators such as IL-1β, IL-17, CCL-2/MCP1, CXCL1/KC, and CXCL2/MIP-2 in *P aeroginosa* infected CF mice as compared to untreated CF mice. PTX3 treatment also decreased the infiltration of neutrophils and increased the percentage of monocytes in BALF of CF mice. Improvement in vascular leakage was also observed in CF mice upon PTX3 treatment (371). Colonization of *P. aeruginosa* in cystic fibrosis patients is found to be positively associated with two intronic SNPs (rs1840680 and rs2305619) and one exonic SNP (rs3816527) in the PTX3 gene (372).

9.2 Aspergillosis: *Aspergillus fumigatus* is an opportunistic pathogen mainly infecting immunodeficient patients. In Invasive pulmonary Aspergillosis (IPA), conidia of *A. fumigatus* induce the secretion of PTX3 in the lungs which in turn recognizes and binds to the

galactomannan moieties on conidia and facilitates its phagocytosis by macrophages (325). Association of PTX3 with C3b and resulting activation of C11b through FcγR is also involved in this process (353). Additionally, PTX3 is also related to the amplification of the antifungal response by inducing the release of MCP-1/CCL-2 in mononuclear phagocytes thus promoting homing of monocytes to the lung tissue to aid in clearing the pathogen (325). PTX3 also activates the lectin pathway of complement by binding to ficolin-L-bound aspergillus conidia in an attempt to clear the infection (353). Another mode of pathogen clearance is by the activation of DCs and subsequent induction of a Th1 response (325). In PTX3 KO mice, susceptibility to IPA was correlated with increased levels of IL-4 (a Th2 cytokine) and decreased status of IFN-gamma and IL-2 (Th1 cytokine). Since pulmonary *A. fumigatus* infection is a major concern in immuno-compromised patients, particularly in bone marrow transplanted patients, PTX3 allows rapid recovery of myeloid and lymphoid cells into the lung tissue and helps in speeding up the reconstitution of immunity (373). Since cytomegalovirus (CMV) infections can also result in such an immuno-compromised state, PTX3 is able to resolve super infection by *A. fumigatus* even in a CMV infected state (354).

As a matter of fact, PTX3 shows differential binding patterns. It does not bind with hyphae but only with conidia of *A. fumigatus, A. flavus and A. niger*. However, such interaction of PTX3 with fungal strains other than these such as *Candida albicans* is also not observed (325). Due to this, direct action on *Candida albicans* is not possible, however, PTX3 manages to resolve Candida infection through binding with mannose binding lectin (MBL) and subsequent recruitment of C1q and activation of classical complement cascade (374). The precise mechanism of *C. albicans* clearance from the lungs by PTX3 requires further investigation.

9.3 Tuberculosis: PTX3 plasma levels are found to be correlated with the clinical severity of tuberculosis and therefore is seen as an appropriate indicator of the stage of the disease (375). Its level declines with the success of therapeutic treatment against tuberculosis but again increases as soon as treatment fails. This is also suggestive of its suitability as a tool to follow up the efficacy of treatment (375). Analysis of lung samples from human subjects determined that exposure to BCG vaccine leads to increased level of PTX3 (376). This suggests that expression of PTX3 could be a protective mechanism and is not just a marker of the diseased state. One mechanism to confer immunoprotection may be mediated by monocytes that are induced to express PTX3 by mycobacterial component (Mycobacterial lipoarabinomannan (LAM)) (377). Like *P. aeruginosa*, susceptibility to *Mycobacterium* tuberculi is also found to be positively associated with two intronic SNPs (rs1840680 and rs2305619) in PTX3 gene (359). However, there are some unanswered questions, which need further investigation, in particular, the mechanism of action of PTX3 on monocytes and involvement of other cell types in this process. It would also be worthwhile to determine whether PTX3 plays additional roles in context with tuberculosis and its associated outcomes in lungs.

9.4 Pneumonia: *Klebsiella pneumoniae* causes acute pulmonary infection in immuno-compromised subjects and results in pneumonia. Like other bacterial infections, PTX3 levels are found to be associated with the disease progression. A study by Soares et al. (378, 379) determined that in PTX3 transgenic mice, high inoculum of bacteria induces overt expression of PTX3, and culminates in greater lethality. Very high levels of PTX3 inhibit neutrophil influx in the lungs due to inhibition of P-selectin (380) and enhanced NO production and iNOS expression

(378). As a result of this, production of TNF was found inhibited, resulting in greater bacteria count in lung tissue (378). Since TNF induces PTX3 expression, one explanation for reduced production of TNF in this infection model could be a feedback mechanism to regulate PTX3 levels and exacerbations that may result from its very high levels. However, when PTX3 transgenic mice were infected with a smaller inoculum, protective effects of PTX3 were conferred and was attributed to enhanced TNF production, increased neutrophil infiltration to lungs and decreased bacterial load in lungs and blood (378). It is quite possible that such a protective effect is initiated by a mechanism that involves induction of PTX3 by OmpA (Outer membrane protein A of *K. pnuemoniae*) through TLR2, which in turn binds OmpA and amplifies the activation of the complement cascade and promotes the opsonophagocytosis of OmpA containing bacteria (381) (382). In humans, a positive correlation is observed between plasma levels of PTX3 and severity of Community acquired pneumonia (CAP) and that PTX3 concentration decreased upon treatment with antibiotics. PTX3 level is also found to be significantly correlated with hospital stay of CAP patients (383).

9.5 Cytomegalovirus (CMV) infection: Cytomegalovirus is a herpes virus, present in the majority of the general population. It exists in latent form even after the infection is treated and may reactivate in an immuno-compromized state (384). PTX3 not only provides protection at very early stages of the infection, but also suppresses reactivation (354). PTX3 mediates its action by binding to hemagglutinin on the surface of the virus and blocks its entry to host cells, particularly DCs (354). It can also induce antiviral immunity through the activation of mDCs and subsequent up-regulation of appropriate T cells functions (354). Virus bound PTX3 acts through TLR2, 3 and 4 and induces the production of IFN-gamma and IL-12 through the transcription factor IRF3 in

CD11b $^{+}$ DCs C57BL/6 mice (354). mDCS themselves also secrete PTX3 upon exposure to viral particles and maintains the loop of infection followed by immunoprotection. Exogenous PTX3 also shows promise for therapeutic purpose against CMV infection, both alone (354) as well as in combination with Thymosin alpha 1 (Patent: Use of thymosin alpha-1, alone and in combination with PTX3 or Ganciclovir, for the treatment of cytomegalovirus infection).

9.6 Influenza: Influenza virus presents another serious challenge to the respiratory tract due to its ability to mutate and create virulent variants as a mechanism to evade the host immune system. Influenza viruses are RNA orthomyxoviruses, which infect epithelial cells of the respiratory tract (385). A detailed study by *Reading et al* (386) determined that like CMV, a sialic moiety of PTX3 engages hemagglutinin of the influenza virus and hinders attachment of the virus to host epithelial cells. Inhibition of viral neuraminidase glycoprotein by PTX3 could be an additional strategy to limit viral infection as it inhibits the release of newly formed viral particles from the infected host cells (387). Further PTX3 facilitates opsonization and clearance of infected cells. PTX3 -/- mice were more prone to influenza virus infection but attained resistance when treated with exogenous PTX3 (386).

9.7 Severe acute respiratory syndrome (SARS): SARS is caused by SARS coronaviruses (SRAS-CoV) (388). The role of PTX3 is being detailed with respect to murine hepatitis virus (MHV) which is a group 2 coronavirus, closely related to its human counterpart SARS-CoV(388, 389). Airway infection of MHV-1 results in acute lung injury in mice, similar to airways damage observed in the lungs of SARS patients (390). MHV-1 airway infection induces PTX3 expression on lungs. PTX3 bound to MHV-1, reduced its infectivity and accelerated viral clearance.

Consequently, PTX3 KO mice showed greater pulmonary damage as compared to their WT counterparts and the animals, which were treated with exogenous PTX3. In PTX3 KO mice, an early influx of neutrophils and macrophages into lungs is found to exaggerate lung injury due to MHV-1 infection. Other determinants of inflammation such as IL-6, MCP-1 and MIP-1b were found to be enhanced in PTX3 KO condition. Their production reduced upon PTX3 treatment, resulting in protection from airway damage (389).

As a matter of caution, PTX3 is not a general mechanism to guard against all pathogenic infections. Infection due to *Listeria monocytogenes* and *Salmonella typhimurium* is neither controlled nor exaggerated by PTX3 directly or indirectly. Similarly, PTX3 is unable to bind and mediate protection against some variants of Influenza virus such as A/PR8/34 (HINI), H3N3 and type B influenza viruses (386).

10. PTX3 in lung Diseases

The level of circulating PTX3 is low in healthy human condition (< 2ng/ml), however, a rapid increase is observed in inflammatory conditions starting from very early stages. Due to its precocious appearance in various clinical conditions, attention is being given to investigate whether it can be used as an index of systemic inflammatory activation. Another major reason suggestive of its suitability as a marker of the severity of diseases like tuberculosis particularly in parasite endemic regions is the fact that its plasma level is unaffected by helminthic infections. There are several studies described in following sections, which demonstrate an association between different diseased states and higher levels of PTX3.

10.1 Chronic Obstructive Pulmonary Disorder (COPD): COPD is characterized by irreversible airflow limitation associated with an abnormal inflammatory response in the lungs. Status of PTX3 in COPD patients is controversial. According to Van *Pottelberge et al.* (391), no difference was observed in the levels of PTX3 in serum, sputum, pulmonary arteries and alveolar space. However, they claimed reduction in PTX3 positivity in lung sections obtained from moderate and severe COPD patients as compared to mild patients and healthy subjects (391). On the other hand, Delneste and coworkers showed increase in PTX3 level in serum and sputum of COPD patients compared to healthy people (334). Whether such a discrepancy in observation is due to the proteolytic cleavage of PTX3 in subjects participating in former study or it is dependent on the demography of the subjects, need further investigation. Among various causative agents, cigarette smoke (CS) is a major cause of chronic COPD (392). Subacute and chronic exposure of CS augments PTX3 level in the lung tissue of murine model of COPD, particularly in pulmonary veins and venules. This upregulation was found to be dependent on IL-1 pathway (392). Further, a critical role of PTX3 in the regulation of COPD-induced pulmonary inflammation, emphysema and body weight changes was ruled out in this study as no significant difference was observed between CS-exposed PTX3 KO and WT mice.

10.2 Lung Carcinoma: Initially, PTX3 was suggested to be useful only as a marker of lung carcinoma based on studies performed on lung cancer cell lines (393) but later, studies have determined that PTX3 could actually be used as a serum biomarker for the diagnosis as well as prognosis of lung carcinoma. Its suitability is overwhelming due to its ability to differentiate between cancer patients and non-cancer patients who are at higher risk of developing lung cancer (394).

10.3 Acute lung injury (ALI): ALI and Acute Respiratory Distress Syndrome (ARDS) ALI/ARDS is characterized by injury associated with activation of the innate immune system in lungs. PTX3 is widely accepted as a marker of ALI since it is found in patients diagnosed with ALI within 24 hrs (395) and is closely associated with the severity of the disease. A concurrent infiltration of neutrophils, enhanced nitric oxide production, augmented expression and function of tissue factor in lungs was observed in this pathological state (335, 396, 397). Consequently, it was suggested that high level of PTX3 activates local innate immune system and was presumed to serve a protective role against insults to which the lung tissue is exposed. LPS-induced ALI was investigated in PTX3 knock-out (KO) mice and found that PTX3 KO mice were more susceptible to tissue damage due to LPS exposure (398). Along the line of this finding, PTX3 knock-in mice were observed to be better protected from LPS-induced endotoxemia (379). In ALI model, PTX3 level and disease severity are found to be regulated by tissue factor (TF) and TF, in turn, is induced by PTX3 (399). Additional detailing of mechanisms in PTX3 KO mice other than the involvement of TF, which rendered them susceptible to ALI would shed more light on the role of PTX3.

11. PTX3 in asthma

Asthma is a chronic inflammatory disease of the lungs and the airways. Inflammation in asthma is characterized by activation of innate and adaptive arms of immune system. PTX3, a newly discovered acute phase protein that was traditionally known to be an innate inflammatory molecule, has also been found to be associated with the pathology of asthma. In human biopsy samples collected from healthy and asthmatic subjects, our lab found an increased expression of

PTX3 in airway epithelium, infiltrating inflammatory cells and airway smooth muscle bundles of bronchial biopsies obtained from asthmatics compared to their healthy counterparts. Interestingly, we did not observe any difference in PTX3 positivity in the sections of mild, moderate and severe asthmatics. Studies examining the role of potential proteolytic cleavage of PTX3 might provide insight of the comparable PTX3 expression in different severity stages of asthma. Also, Marseglia et al showed that asthmatic condition accompanied with other allergic diseases such as atopic dermatitis further increases PTX3 levels in the blood of these donors. Among pulmonary structural cells, primary human airway smooth muscle cells (HASMCs) produce greater PTX3 at the baseline and also in response to TNF as compared to epithelial cells (ECs). TNF treatment of HASMC seemed to promote PTX3 expression through NF-kb and API-dependent mechanisms. Moreover, JNK and ERK but not P38 signaling pathways were also involved in TNF-induced PTX3 expression in these cells. Like TNF, IL-1β was also found to upregulate PTX3 expression in HASMCs; however, the effect of Th2, Th1 and Th17 cytokines was found negligible. PTX3 treatment of HASMCs induced the production of eoatxin-1/CCL-11. Also, stimulation with PTX3 inhibited FGF-2 mediated migration of HASMCs (400). Based on these observations, Zhang et al suggested a novel role of PTX3 in regulating HASMCs phenotype and functions in asthmatic condition.

Chapter 2: Rationale, Hypothesis, and Aims

Rationale

PTX3, a unique member of the long pentraxins family, plays an indispensable role in regulating our immune system against pathogens involved in several pulmonary pathologies. Multiple mechanisms to recognize pathogens and to coordinate with the activation of humoral and cell-mediated immunity explain evolutionarily conserved of PTX3 from non-vertebrates to complex and highly evolved vertebrates. Several diseases and infection models have been extremely useful in understanding the role of PTX3 in normal as well as in immuno-compromised disease states. In the lungs, inflammation due to infection and lung injury induces elevated production of PTX3 (401). Murine studies have demonstrated that deletion of PTX3 rendered mice susceptible to *P aeruginosa* infection, aspergillosis, influenza, SARS, and LPS induced acute lung injury. Protective role of exogenous PTX3 in aspergillosis, pneumonia and tuberculosis further strengthened the preceding observations (reviewed in (401)). Also, from previous publications from our lab, association of PTX3 with asthma is clearly evident (400). Whether increased levels of PTX3 are just representative of allergic inflammation and its severity or it is also involved in the development of allergic inflammation, is not known. Therefore, in this project, I sought to understand role of PTX3 in mechanisms involved in asthma pathogenesis. In order to achieve my research objectives, I employed murine model of allergen-induced experimental asthmaperformed my experiments on wild-type mice and PTX3 deleted/ knockout/ PTX3-/- mice. Ovalbumin (OVA) was used as an allergen as its ability to induce many features of asthmatic lungs including a robust allergic inflammation, airway hyper responsiveness and tissue remodeling in mice have been well characterized.

The outcome of this project would provide critical insights of the role of PTX3 in allergic inflammation in mice. Having known that PTX3 in mouse and human exhibit shared structure and functions, my conclusions may also be extrapolated to asthma in humans.

Hypothesis

PTX3 plays a protective role in allergen-induced asthma in murine model, the absence of which results in the impaired inflammatory response, aggravating allergic inflammation.

Aims

In order to test my hypothesis, development of OVA-induced inflammation in PTX3+/+ and PTX3-/- mice was determined. As CD4 T cells constitute an important component of allergic inflammation, we also assessed the effect of PTX3 deletion on CD4 T cells in response to OVA sensitization and challenge (Chapter 4). We also compared the phenotype and functions of PTX3+/+ and PTX3-/- DCs in allergic and basal condition and how such difference impacted the fate of CD4 T cells (Chapter 5).

Chapter 3: Methods

Subjects: BALF used in this study was obtained from non-asthmatic healthy and severe asthmatic subjects in accordance with procedures approved by the Human Research Ethics Board of the University of Chicago (Institutional Review Board # 13198A and 15361A). All subjects were seen in a refractory obstructive lung disease clinic by an asthma/COPD expert. Subjects were consented at the time of pre-operative clinic visits. Pulmonary function tests were performed with methacholine challenge prior to their surgery. All surgeries were elective and for non-infectious or non-inflammatory reasons: most of the surgeries were in obese patients and were bariatric in nature. One of the surgeries was for a thyroid nodule. Most of the donors showed no symptoms of co-morbidities, except asthmatic #4 who was diagnosed with kyphoscoliosis. All bronchoscopies were done for research purposes only (no clinical indication existed for these procedures). BALF was concentrated 10 times using 10K cutoff concentrating centrifugal filter units Ultracel® from Millipore.

Animals: Female $PTX3^{-/-}$ and $PTX3^{+/+}$ (129SvEv/Bl/6 background) (5-8 weeks old) were obtained from Dr M Matzuk, Baylor College of Medicine and bred at the University of Manitoba breeding facility. Gene targeting technology in embryonic stem cells was used to generate PTX3 null mice (PTX3-/-). According to Matzuk and group, a genomic clone was isolated from 129/SvEv genomic library to create a targeting vector in order to delete exon 1 and 2. Given that exon 1 of ptx3 gene encodes the initiation methionine codon and signal peptide, deletion of exon 1 resulted in a null mutation. Three AB2.2 ES cell lines

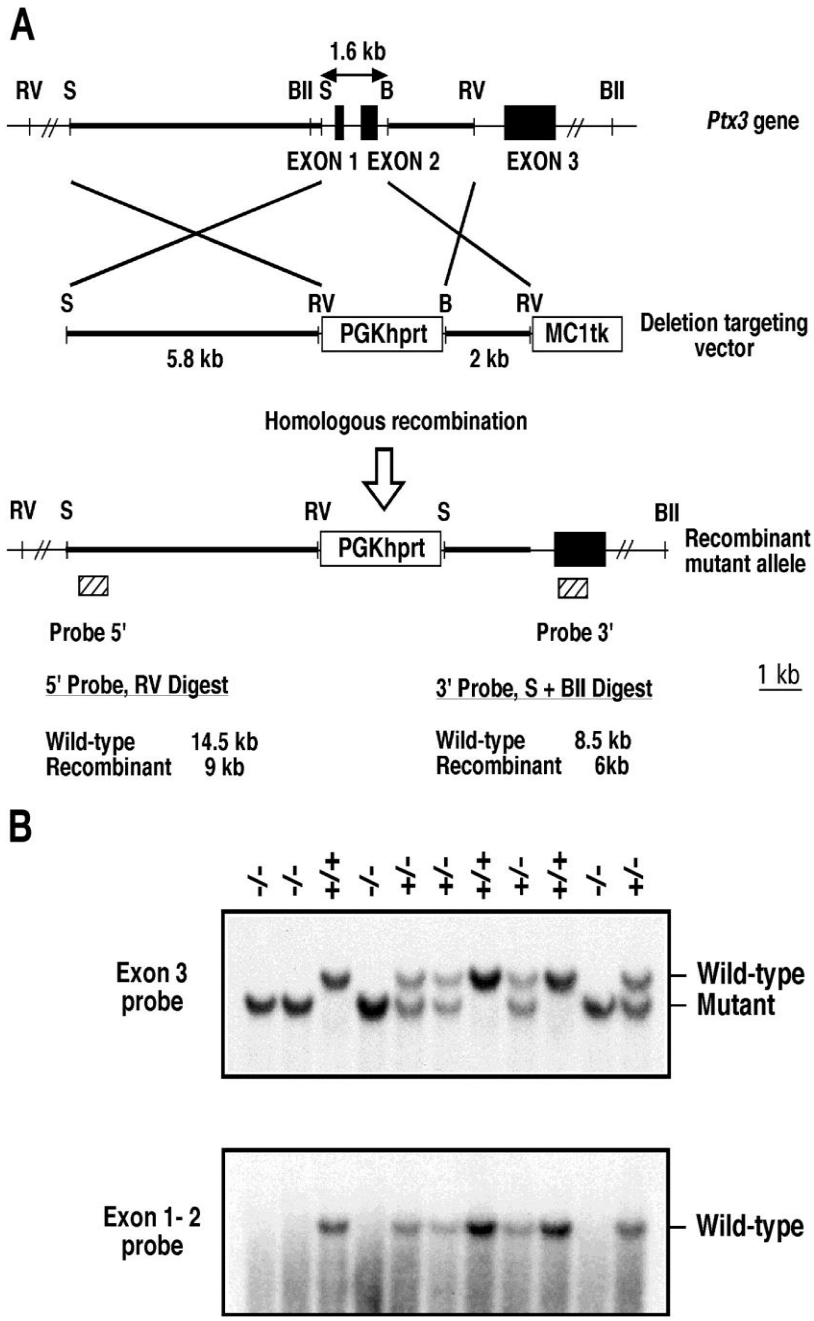

Figure 3.1: *"The Ptx3 replacement-targeting vector was electroporated into AB2.2 ES cells. After selection with hypoxantine, aminopteridine, thimidine, and 1-(2'-deoxy-2'fluoro-β-d-arabinofuranosyl)-5-iodouracil), positive 5' and 3' recombination events were confirmed by Southern blot analysis with 3' external and 5' internal probes. B, Southern blot analysis of tail*

DNA derived from 11 offspring of a litter from a heterozygous mating. Top panel, The probe spanning exon 3 detects an 8.5-kb wild-type band and a 6-kb recombinant band. Bottom panel, Analysis of the same tail DNA with a probe spanning exons 1 and 2 detects only the 8.5-kb band in DNA derived from heterozygous (+/−) and wild-type (+/+) mice, but not in the homozygous (−/−) null offspring. RV, EcoRV; BII, BglII; S, SpeI." (Taken from (402), ***permission for reuse obtained****)*

that contained clones targeting ptx3 locus were used to produce chimeric male mice. These mice were fertile and transmitted the ptx3 null allele to the F1 progeny, which when intercrossed with each other gave rise to F2 progeny 29.2% of F2 progeny were homozygous PTX3 null or PTX3-/-. Deletion of PTX3 was confirmed by southern blotting by Dr Matzuk's lab (figure 3.1). Animals were used according to the guidelines issued by the Canadian Council on Animal Care and University of Manitoba Animal Ethics Board.

OVA Sensitization and Challenge: On day 1, each mouse was sensitized by ip injected of 500ul of a mixture of OVA (Grade V, Sigma Aldrich Canada) and alum (Imject Alum, Pierce Biotechnology). Alum (factory concentration = 40mg/ml) is diluted five times to make 8mg/ml. On the day of ip injection, 250μl of diluted alum and 250μl of 4ug/ml OVA (2μg) are mixed. On day 11, these mice were sensitized with IP injection of OVA/alum mixture. Also, 50μl of OVA (50μg) was administered intra nasally. On day 19 and 20, PTX3-/- and PTX3+/+ mice were challenged with intra nasal administration of 50μg of OVA solution in saline. 48 hrs after last challenge, mice were sacrificed to collect blood, BALF, lung, mediastinal lymph nodes, and

spleen. Establishment of an experimental model of allergic asthma in mice is illustrated in **figure 3.2.**

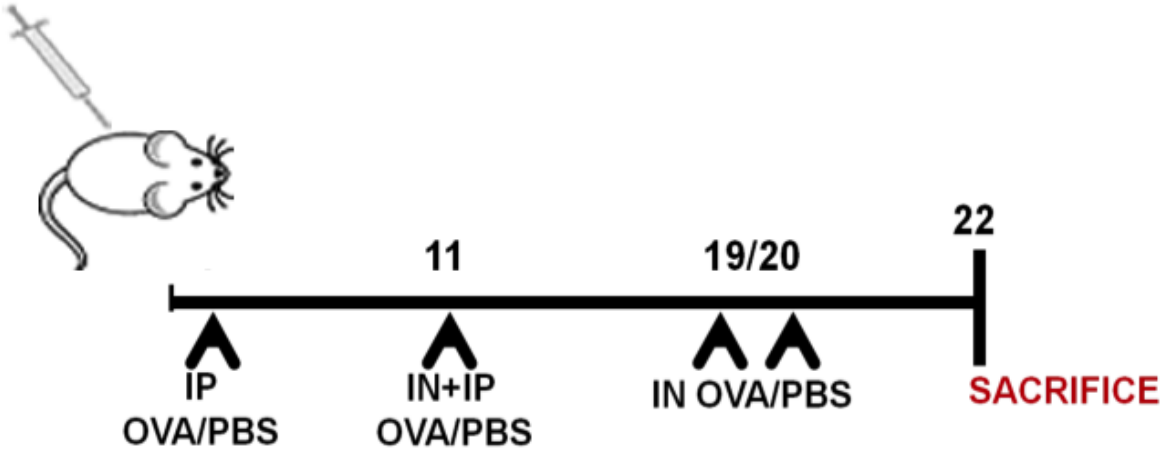

Figure 3.2*: Schematic showing the protocol employed for acute OVA sensitization/ challenge.*

Lung Mechanics: Airway hyperresponsiveness to methacholine was assessed as described previously (403) by using a flexiVent small animal ventilator (Scireq, Montreal, Quebec). Mice were anesthetized intra-peritoneally (sodium pentobarbital 0.1 ml per 10 gram body weight). After establishing anesthesia, mid-cervical tracheotomy was performed by inserting a polyethylene catheter (1.1 × 25 mm), which was kept in place by ligating with surgical silk thread. The catheter was connected to the small animal ventilator and positive end expiratory pressure was maintained at 3 cmH_2O. Mice were subjected to serial aerosol MCh challenge (30 µl, 3–50 mg ml^{-1} MCh in saline), and baseline mechanics was determined using saline-only challenge. Before each challenge with saline or MCh, lung loading history was normalized by inflation to total lung capacity. Respiratory mechanics was assessed using a preset flexiVent Prime-8 low-

frequency forced oscillation protocol to derive respiratory mechanical input impedance (*Z*rs). Airway resistance (R_{aw}) was derived by fitting *Z*rs to the constant phase model.

BALF Collection from mice: Mice were deeply anesthetized with ip injection of sodium pentobarbital followed by surgical opening of the abdomen and the chest. The mice tracheas were cannulated with a 20 gauge catheter and tied in place with a surgical thread. The lungs were washed two times with 1 ml of PBS. We get approximately 700ul fluid from each wash. The BALF was centrifuged at 1000 rpm for 10 minutes and the supernatant was used for ELISA. Pellet was re-suspended in 500ul PBS for counting and used for BAL smears preparation. First BALF cells were counted under the microscope and then loaded onto the cuvette attached to a glass side (superfrost miscroscope glass slife from Fisher Scientific, Ontario, Canada). Cells were then centrifuged for 5 minutes at 1200 rpm to prepare slides. In order to determine different immune cells such as eosinophils, basophils and neutrophils, the BAL slides were air-dried, fixed and stained with a PROTOCOL Hema 3 fixatives and solutions (Fisher Scientific, Ontario Canada). These inflammatory cells were counted based on cellular and nuclear morphology and staining characteristics using hemocytometer.

Histology: For histological analysis, lungs were harvested, fixed in 10% buffered formalin. Tissues were then embedded in paraffin using a tissue processor (Sakura FineTek Inc). Paraffin-embedded tissues were cut into six-micrometer thick sections using a Leica RM2245 microtome (Leica Microsystems Inc, Ontario, Canada). These sectioned were then attached to microscope superfrost plus glass slides. For staining with hematoxylin and eosin (H&E) and periodic acid schiff (PAS), tissue sections were first dewaxed followed by specific staining. For analysis of

inflammation and mucus production, H&E and PAS staining, respectively, of lung tissues was determined under light microscope. Two independent persons in blindfold manner scored the slides.

H&E staining: The dewaxed sections were first stained with hematoxylin (Fisher Scientific, Ontario, Canada) for 15 minutes followed with rinsing with water. These were then destained in 0.5% acid alcohol (5ml HCl in iL 70% alcohol) for few seconds and rinsed in tap water. Slides were then counterstained with eosin (Fisher Scientific, Ontario, Canada) for 5 minutes and rinsed with tap water. Dried slides were then examined under light microscope.

Lung homogenate collection: Mice were sacrificed and their chest was opened. Left lung lobes were collected and homogenized in a glass homogenizer in 2 ml PBS. The homogenate was then centrifuged at 500 rpm and supernatant is collected and stored for cytokine measurement by ELISA.

Ex-vivo spleen and mediastinal lymph node cell culture: *PTX3*$^{+/+}$ and *PTX3*$^{-/-}$ mice were challenged with OVA in alum adjuvant and then spleen and MLN were collected. Single cell suspension of spleenand MLN cells was prepared by grinding them in a 40υm strainer (BD falcon). RBCs were lysed by incubating spleen cells in NH_4Cl solution. Cell suspensions were then centrifuged and resuspendend in complete cell culture media (RPMI supplemented with 10% fetal bovine serum (FBS) and 20uM 2-mercaptoethanol). Cells were cultured in complete RPMI at $5X10^6$ cells/ml in a 48 well plate at 37^0C in a 5% CO_2 atmosphere, treated with OVA (50ug/ml) for 72 hrs and supernatant was then collected after 72 hrs and stored at -80^0C freezer.

Serum collection: Blood was collected and coagulated at 4°C followed by centrifugation for 20 minutes at 7500 rpm. Circulating total and anti-OVA IgE and IgG subclasses were measured in the sera by ELISA. Ninety-six–well plates were coated overnight at 4^{o}C with 50 μL 0.1 mol/L $NaHCO_3$ containing 100 μg OVA/ml. Then plates were blocked for 2 hours at 37°C with 200 μL 3% BSA in PBS. Plates were washed, and 50 μL of 4 series with 10-fold serum dilutions for OVA-specific and 1:20,000 for total IgE and IgG2a in PBS containing 1% BSA was applied overnight at 4°C. The amount of bound antibody was analyzed by using horseradish peroxidase conjugated antibodies against mouse heavy chain classes.

ELISA: Level of PTX3 was determined by ELISA using antibodies from R&D systems. Cytokines in the lung homogenate and supernatant of *in vitro* cultured spleen and MLN cells was assayed using ELISA MAX^{TM} Deluxe sets from Biolegend, San Diego, CA according to the manufacturer's protocol. No modifications were done in the protocol. Suggested sensitivity of kits for cytokines according to manufacturer is: IL-4- 1pg/ml, IFN-γ- 4pg/ml, IL-17-10pg/ml, IL-6- 2pg/ml, IL-12- 4pg/ml, IL-5- 4pg/ml and IL-10- 16pg/ml.

Flow cytometry: Single cells were collected from lungs, spleen, MLN and bone marrow, washed with flow buffer (1X PBS with 2%FBS). Washes cells were then blocked with Fc blocker at 4^{0}C for 10-15 minutes followed by washing with flow buffer. Cells were then incubated in fluorochrome-conjugated specific antibodies solution for approximately 30 miutes at 4^{0}C in dark. Labeled cells were again washed in flow buffer and resupended in 100-200ul of flow buffer and acquired on FACS Canto II (BD Biosciences). For intracellular staining, cells were stimulated with 500ul of cell stimulation cocktail (eBiosciences, San Diego, CA)/ml of media for 4-6 hrs. Cell stimulation cocktail contains PMA (40.5μM), ionomycin (670μM), monensin (1μM) and brefeldin A (5.3μM). PMA and ionomycin induce activation of cells to produce cytokines.

Brefeldin A and monensin inhibit secreted of cytokines from the cells, resulting in their intracellular accumulation. After culturing cells in cell stimulation cocktail, cells were washed in flow buffer and cell surface markers were stained as above. Cells were then fixed in 2% paraformaldehyde in dark on ice for 10-15 minutes, washed and resuspended in 0.1% saponion solution for permeabilization. Permeabilized cells were then stained for intracellular cytokines and washed and acquired in flow cytometer. For staining of trancription factors Foxp3 staining buffer set (ebiosciences Inc) was used according to manufacturer's instructions. Briefly, cells that were stained for extracellular markers were washed in flow buffer, centrifuged at 12000rpm and pellet of labeled cells was collected. Pellet was then resuspended in 1ml Foxp3 fixation/ permeabilization working solution and incubated at 40C for 30-60 minutes. These tubes were then added 2ml 1X permeabilization buffer and centrifuged at 1200 rpm for 5 minutes. Supernatant was discarded and pellet was resuspended in antibody solution, incubated at 4^0C for 30 minutes, washed and acquired in flow cytometer. Detailed list of antibodies used is provided below:

Marker	**Ab clone**	**Company**
CD3e	145-2C11	eBioscience, Inc
CD4	GK1.5	eBioscience, Inc
CD8	53-6.7	eBioscience, Inc
IL-17A	eBio17B7	eBioscience, Inc
IFN-γ	XMG1.2	eBioscience, Inc
CD69	N418	Biolegend, Inc
CD25	PC61	Biolegend, Inc
CD11b	M1/70	eBioscience, Inc

CD11c	N418	eBioscience, Inc
Gr-1	RB6-8C5	Biolegend, Inc
F4/80	BM8	eBioscience, Inc
IL-6	MP5-20F3	eBioscience, Inc
Siglec F	E50-2440	BD Biosciences.
Bcl-2	BCL/10C4	Biolegend, Inc
IL-4	11B11	eBiosciences Inc
IL-23	Fc23cpg	eBiosciences Inc
Lin	mix	Biolegend, Inc
SCA-1	D7	Biolegend, Inc
CD16/32	93	eBiosciences Inc
CD34	RAM34	eBiosciences Inc
cKit	2B8	eBiosciences Inc
Ly6C	HK1.4	eBiosciences Inc
CCR2	SA203G11	Biolegend, Inc
CCR5	HM-CCR5	eBiosciences Inc
CCR6	29-2L17	Biolegend, Inc
CCR7	4B12	eBiosciences Inc
CD40	1C10	eBiosciences Inc
MHC II	M5/114.15.2	eBiosciences Inc
CD80	16-10A1	eBiosciences Inc
CD86	GL-1	eBiosciences Inc
IL-2	JES6-5H4	Biolegend, Inc

CD45	30-F11	eBiosciences Inc
CD44	1M7	eBiosciences Inc
B220	RA3-6B2	eBiosciences Inc
IL-6R	D7715A7	Biolegend, Inc
γδ-TCR	eBioGL3	eBiosciences Inc
CD62L	MEL-14	eBiosciences Inc
CX3CR1	SA011F11	Biolegend, Inc

Survival assay: Single cell suspension of lung, spleen and lymph node cells were cultured for 3 days in complete media. Apoptosis/ survival of CD4 T cells was determined using Annexin V Apoptosis Detection kit FITC from eBioscience Inc. according to manufacturer's instructions. Briefly cells were first stained with specific fluorochrome-conjugated antibodies, washed with flow buffer once and then in 1X annexin V binding buffer. Resuspended cells were then centrifuged at 12000rpm and stained with FITC or APC labeled annexin V. These cells were incubated at room temturature for 10-15 minutes followed by washing in 1X binding buffer. After washing, cells were resuspended in 100-200ul 1X binding buffer to which 1ul of DAPI was added and incubated at room temperature for atleast 5 minutes. These cells were then acquired in flow cytometer. Annexin V positive DAPI negative cells represent early apoptotic cells, Annexin V positive DAPI positive cells are characterized as late apoptotic cells and Annexin V negative DAPI positive cells represent necrotic cells.

In vitro Th17 polarization assay: Naïve CD4 T cells were enriched from murine splenocytes using naïve CD4 T cell negative selection kit (Stem cell technologies) according to manufacturer's instructions. Briefly, 10^8 cells were resuspended in 1ml of recommended medium in a flow tube.

To this solution, 50µl of EasySep™ Mouse CD4+T cell isolation cocktail was added, mixed and incubated at room temperature for 7-8 minutes. Thereafter 50µl of EasySep™ Mouse Memory T cell Depletion Cocktail was added, mixed well and incubated at room temperature for 2-3 minutes followed by addition of 75µl of uniform solution of EasySep™ Streptavidin RapidSpheres™ for 2-3 minutes. Total mixture was then brought to volume 2.5 ml by adding recommended medium and tube was placed in purple EasySep™ Magnet for 2-3 minutes at room temperature. EasySep™ Magnet was then picked and inverted in one continuous motion so as to pour solution in a fresh tube. According to manufacturer, magnetically labeled bound cells are the unwanted cells and unbound cells that would come with poured solution are the wanted naïve CD4 T cells.

Purified naïve CD4 T cells were then cultured for 4--5 days in the presence of IL-6 (100ng/ml, eBioscience, Inc), TGF-b (10ng/ml, R&D), anti-IL-4 (10µg/ml, eBioscience, Inc), anti-IFN-gamma (10µg/ml, eBioscience, Inc), anti-CD3e (2µg/ml, eBioscience, Inc) and ant-CD28 (2µg/ml, eBioscience, Inc) in round bottom 96 well plate. (Adapted from (404)).

CD4 T cell-DC co-culture: Purified naïve PTX3+/+ or OTII CD4 T cells were co-cultured with purified DCs from OVA-exposed PTX3+/+ and PTX3-/- lungs in ratio 10:1. DCs were sorted using FACS Aria as $CD11c^{+}CD11b^{+}$ $Gr\text{-}1^{-}F4/80^{-}Siglec\ F^{-}$. Supernatant from co-cultured cells were collected after 4-5 days and IL-17A level was detected by ELISA and flow cytometry.

Bone marrow derived cell culture: Naïve PTX3+/+ and PTX3-/- mice were sacrificed and tibia and femur bones were collected. In order to extract bone marrow cells, bones were flushed with 10 ml of complete media using 26cc syringe. Thereafter 18cc syringe is used to transfer bone marrow cells to a 50 ml tube that has a cell strainer on the top. Bone is flushed until it turns white.

Collected cells were centrifuged at 1200 rpm and pellet was resuspended in ACK buffer for 5 minutes to lyse red blood cells. ACK was neutralized with complete media, centrifuged and resuspended in fresh complete cell culture media. Finally 500,000 cells were seeded per ml in each well of 24 well plate in the presence of 2ng/ml GM-CSF. Every third day, media was replenished with fresh GM-CSF containing complete media. On day 7, cells were treated with OVA (50ng/ml) or LPS (1mg/ml) for 24 hrs and phenotypic characterization and/or cytokine production was evaluated.

CFSE Staining: RBC free Splenocytes were isolated from PTX3+/+ and PTX3-/- mice as explained above and their cell count was estimated using trypan blue. 3-10 million cells were resuspended in 5 ml serum free media or PBS and 3uM CFSE in 5 ml PBS or serum free media was added. Total 10 ml mixture was incubated in dark at room temperature for 8 minutes. Thereafter 5 ml FBS was added, centrifuges at 1200rpm for 5 minutes and pellet was then resuspended in complete cell culture media. Cells were washed one more time. Seed approximately 200,000 cells/100ul in each well of round bottom 96 well plate. Anti CD3e (1ug/ml) and anti-CD28 (1ug/ml) (ebiosciences Inc) were used as positive control.

mRNA analysis: Total cellular RNA from lungs was obtained using Trizol Reagent (Invitrogen, Carlsbad, Calif). Right lung lobes were collected in 1 ml trizol in RNAse free conditions and RNA was isolated according to manufacturer's instructions. Concentration and purity of isolated RNA was determined by UV absorbance at 260nm and 280nm. cDNA synthesis was done with 2ug RNA using Superscript II Reverse Trancriptase (Invitrogen), random primers, 10mM dNTP and dithiothreitol (DTT). 1 ul cDNA was used to perform quantitative real time PCR on ABI RT-PCR machine (the list of primers is provided below). GAPDH was used as an internal control (Forward

primer: 5'AAC TTT GGC ATT GTG GAA GGA3' and Reverse primer: 5' ACA CAT TGG GGG TAG GAA CA3').

Gene	**Primer sequence**
muc5ac	Reverse: 5' GTT GCA GAG ACC AGG GAA GT3'
	Forward: 5'GCA TGT TGG TAC CCC ACT CA3'
muc5b	Reverse: 5'CAG GTG TAA GGC GCT CAT GC3'
	Forward: 5'GAA ACT GGA GCT GGG CTC TG3'
col1a2	Reverse: 5'CAC ACT GCT CTG ACC AAT CC3'
	Forward: 5'TCT GTC CTA GTC GAT GGC TG3'
col3	Reverse: 5' TTC CAT CAT TGC CTG GTC3'
	Forward: 5'GCA GGA CCC AGA GGA GTA G3'
fibronectin	Reverse: 5'TGG TAG GAG AGT ATG TGG TGGG3'
	Forward: 5' GGG GAG ACA TGG CAA ACC TGT3'
sm-actin	Reverse: 5' TCT ATC GGA TAC TTC AGC GTC A3'
	Forward: 5'CCC AGA CAT CAG GGA GTA ATG GT3'
il-17a	Reverse: 5'ACA CCC ACC AGC ATC TTC TC3'
	Forward: 5'TCC AGA AGG CCC TCA GAC TA3'

kc	Reverse: 5’CTT GGG GAC ACC TTT TAG CAT C3’
	Forward: 5’CAA TGA GCT GCG CTG TCA GTG3’
il-4	Reverse: 5’GCA TGG AGT TTT CCC ATG TT3’
	Forward: 5’AGA TGG ATG TGC CAA ACG TC3’
il-6	Reverse: 5’TAA GCC TCC GAC TTG TGA AGT GGT3’
	Forward: 5’AAC CGC TAT GAA GTT CCT CTC TGC3’
il-10	Reverse: 5’TGG CCT TGT AGA CAC CTT GGT CTT3’
	Forward: 5’TGC ACT ACC AAA GCC ACA AAG CAG3’
ifn-gamma	Reverse: 5’TTC GCC TTG CTG TTG CTG AAG AAG3’
	Forward: 5’CTG CAT CTT GGC TTT GCA GCT CTT3’
il-13	Reverse: 5’AGG AGC TGA GCA ACA TCA CA3’
	Forward: 5’GGA ATC CAG GGC TAC ACA GA3’
il-12	Reverse: 5’TAG CCA GGC AAC TCT CGT TCT TGT3’
	Forward: 5’ACC TGC TGA AGA CCA CAG ATG ACA3’
tgf-beta	Reverse: 5’TGT ACT GTG TGT CCA GGC TCC AAA3’
	Forward: 5’TGA TAC GCC TGA GTG GCT GTC TTT3’
tnf	Reverse: 5’CAG CCT TGT CCC TTG AAG AGA ACC3’
	Forward: 5’TAC TGA ACT TCG GGG TGA TTG GTC C3’

Statistical analysis: Results are shown as mean-/+ SEM, analyzed using one-way ANOVA (Graph Pad prism). For experiments where only two datasets are compared, student's t test or Wilcoxon signed-rank test was performed. Unless otherwise stated, data are collected from atleast three experiments, each experiment contained al least three-five mice in each experimental group. p-value <0.05 was considered significant in all experiments.

Chapter 4: PTX3 Deletion Aggravates Allergic Inflammation through a Th17 -Dominant Phenotype and Enhanced CD4 T cell Survival

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Jyoti Balhara[1], M.Sc, Lianyu Shan[1], M.Sc, Jingbo Zhang[1,*], PhD, Anik Muhuri[1], B.Sc, Andrew J. Halayko[2], PhD, Muhamad S. Almiski[3], MD, Diana Doeing[4], MD, John McConville[4], MD, Martin M Matzuk[5], MD, PhD and Abdelilah S.Gounni[1#], PhD

[1]Department of Immunology, [2]Physiology and [3]Pathology, College of Medicine, Faculty of Health Sciences, University of Manitoba; [4]Department of Medicine, University of Chicago, Chicago, USA; [5]Department of Pathology & Immunology, Baylor College of Medicine, Houston.

Introduction

According to the World Health Organization (WHO), asthma is a serious public health concern with more than 300 million sufferers worldwide. Further, the disease prevalence globally increases by approximately 50% every decade (1). According to Braman *et al* (1), collateral increase in atopic sensitization and other allergic conditions such as eczema and rhinitis are associated with an increase in asthma pervasiveness. Genetic predisposition (2, 3), environmental factors (17) and gene-environmental interactions contribute to the increased asthma prevalence in susceptible subjects.

Asthma is classically defined as a disease orchestrated by the adaptive immune system. However, recent advances suggest considerable crosstalk between the innate and the adaptive immune system in the initiation and propagation of allergic immune response (405). Pattern recognition receptors (PRR) such as toll like receptors are key components of innate immune system, which have been found to play an important role in allergic inflammation. PRRs on epithelial cells and dendritic cells recognize pathogen associated molecular patterns and damage associated molecular patterns and act as adjuvants in directing allergen sensitization (406-408).

Pentraxin 3 (PTX3) is a prototypic soluble pattern recognition receptor, involved in the regulation of inflammation through multiple mechanisms that include interaction with components of complement pathways, Fcg receptors, pathogenic moieties on microbes and regulation of leukocyte migration (401). Previously, lack of PTX3 has been reported to exaggerate inflammation in *P. aeruginosa* infection, aspergilosis, influenza, severe acute respiratory syndrome (SARS) and LPS-induced acute lung injury (ALI) in mice. Protective role of exogenous PTX3 in aspergillosis, pneumonia and tuberculosis further strengthens the preceding observations (reviewed in (401)). Collectively, these studies demonstrate that infection and lung injury increase the production of PTX3 within the lung (401).

Previously published data by our group (400), showed an enhanced PTX3 expression in the lungs of severe allergic asthmatics compared to healthy donors. Structural cells particularly epithelial and airway smooth muscle cells were the main producers of PTX3 *in vivo* and upon proinflammatory cytokine stimulation *in vitro.* However, the role of PTX3 in the development of allergic asthma remains unknown.

In this study, my aim is to evaluate the effect of PTX3 deletion in the development of OVA-induced physiological and immunological metrics. Here we showed that deletion of PTX3 resulted in exaggerated inflammation, AHR, and airway remodeling. We also demonstrate that *PTX3*$^{-/-}$ mice exhibit a Th17 dominant inflammatory response as compared to *PTX3*$^{+/+}$ mice in response to OVA. Altogether my report suggests a critical role of PTX3 in regulating CD4 T cell-mediated inflammation in a murine model of allergen-induced inflammation.

Results

PTX3 level is enhanced in human asthma

PTX3 expression has been shown to be elevated in several pulmonary diseases, including cigarette smoke-induced chronic obstructive pulmonary disease (COPD), LPS-induced ALI and murine SARS infection (401)**.** Previously, we have shown augmented expression of PTX3 in bronchial biopsies obtained from severe allergic asthmatics compared with healthy subjects (400). We further validated it in bronchoalveolar lavage fluid (BALF) of human severe asthmatics and healthy donors. Severe asthmatics exhibit greater concentration of PTX3 in the BALF as compared to healthy donors (240.2±58.6 versus 78.60±47.6 pg/ml) (n= 10, *P<0.01, Figure 4.1).

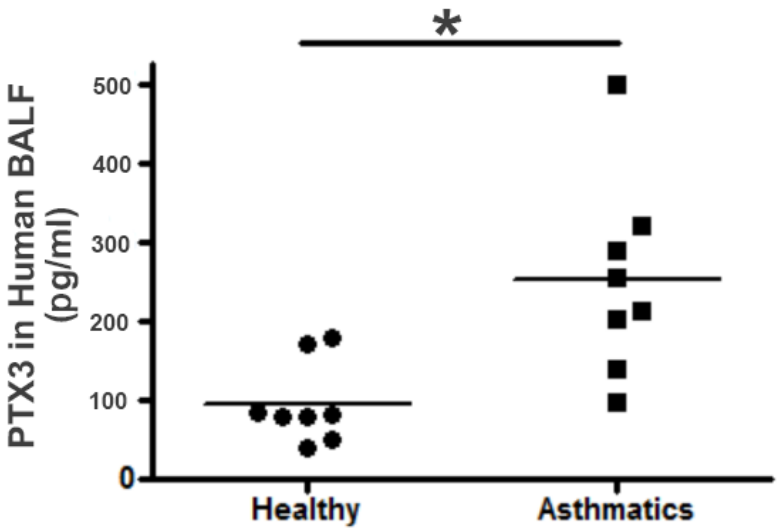

Figure 4.1: *PTX3 concentration is increased in asthmatic condition in humans and mice. PTX3 level in BALF obtained from healthy and severe asthmatics was evaluated by ELISA. *p<0.01*

Concentration of PTX3 increased in OVA-exposed murine lungs

Next we sought to determine the production of PTX3 in the lungs of mice in response to acute OVA exposure. Mice were sensitized with OVA+ alum intra-peritoneally on day 1 and 11 and challenged intra-nasally on day 18 and 19 (Figure 4.2A). OVA sensitized and challenged mice display significant increase in BALF and lung PTX3 concentration as compared to their saline controls (BALF PTX3: 321±89pg/ml vs 9.36±1.06pg/ml; lung PTX3:14.8±1.4ng/ml versus 6.1±0.8ng/ml) (n=8-12, *P<0.01) (Figure 4.2B and C). Interestingly, OVA exposure did not affect the level of PTX3 in the serum (Figure 4.2D), suggesting local production of PTX3 in response to allergen.

Deletion of PTX3 exacerbate allergic inflammation in mice

Given that PTX3 level increases in asthmatic condition and that $PTX3^{-/-}$ mice exhibit enhanced susceptibility to various infections (reviewed in (401)), we sought to examine the consequence of PTX3 deletion on airway inflammatory response in an OVA-induced murine model of experimental asthma. We first determined influx of inflammatory cells into the airway lumen by assessing inflammatory cells in the BALF. Therefore we extracted BALF from OVA–exposed PTX3+/+ and PTX3-/- mice and determined count of inflammatory cells using a haemocytometer. According to manual counting BALF cell cytospin slides under light microscope, there was a significant infiltration of inflammatory cells into the airways of $PTX3^{+/+}$ and $PTX3^{-/-}$ mice post-OVA challenge, however, $PTX3^{-/-}$ mice exhibited greater number of total inflammatory cells (Figure 4.3A) (n=12, *P<0.01).

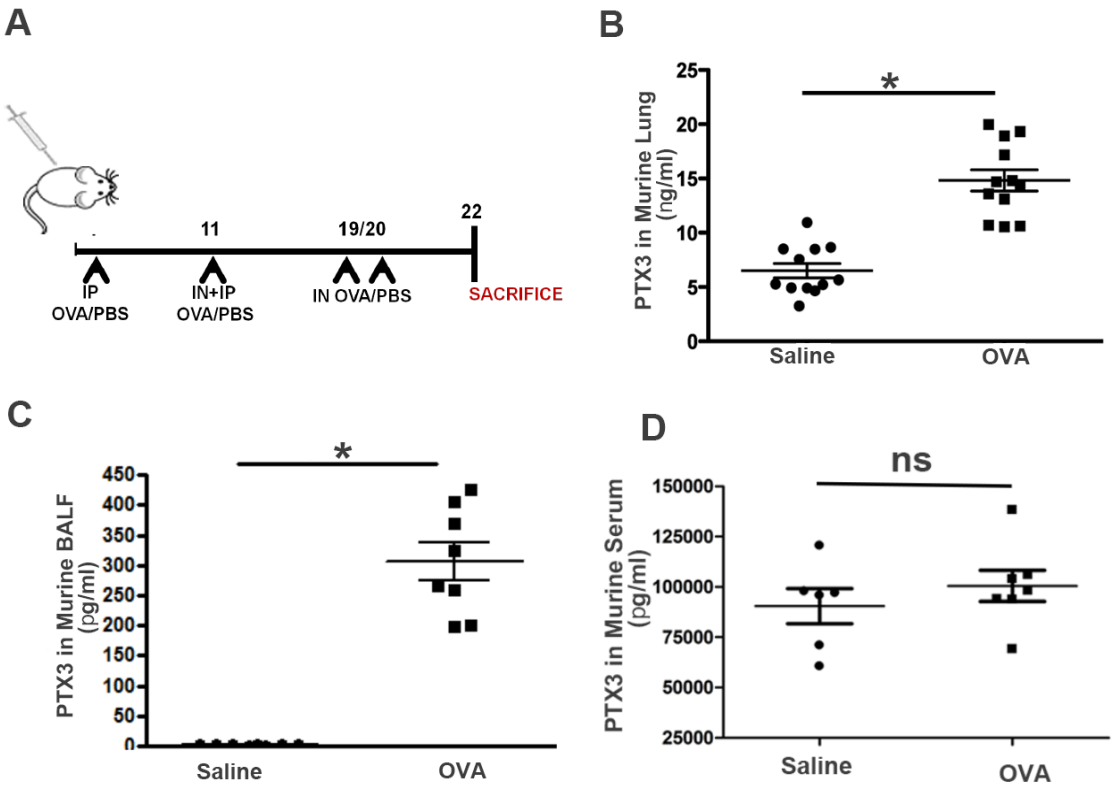

Figure 4.2: *Production of PTX3 is increased upon OVA exposure. (A) Schematic showing the protocol employed for acute OVA sensitization/ challenge in mice. Concentration of PTX3 measured in the lungs (B), BALF (C) and serum (D) of PTX3*$^{+/+}$ *mice in saline and OVA exposed condition. *p<0.01.*

Although both genotypes showed an eosinophil-dominant response upon OVA challenge, *PTX3*$^{-/-}$ exhibited greater BALF eosinophil count (Figure 4.3B) as compared to PTX3+/+ controls. Similarly, we detected enhanced lung tissue eosinophilia (Siglec F^{high}/ Gr-1 $^{low/neg}$) (Figure 4. 4 C and D) by flow cytometry as compared to their *PTX3*$^{+/+}$ counterparts.

We also detected greater neutrophil count in the airways (BALF) (Figure 3B) and lungs (CD11b^{+}/ Gr-1^{+}) (Figure 4.4E and F) in *PTX3*$^{-/-}$ mice compared to PTX3$^{+/+}$ counterpart (409)**.** Given that allergic inflammation is accompanied with neutrophil dominant early stage followed by eosinophil-dominant late stage, we questioned whether increased neutrophil numbers 48 hrs post challenge (late stage) was indicative of enhanced neutrophilia at early stage. Therefore we assessed the status of neutrophils 12hrs post OVA-challenge. As expected, PTX3-/- mice exhibited a significantly enhanced neutrophilia as compared to PTX3+/+ mice (Figure 4.4G and H). Enhanced infiltration of inflammatory cells surrounding airways and blood vessels in *PTX3*$^{-/-}$ mice as compared to *PTX3*$^{+/+}$ mice was further supported by an examination of H&E stained lung sections (Figure 4.5). Taken together, my data indicate that lack of PTX3 results in an enhanced recruitment of granulocytes, particularly eosinophils and neutrophils.

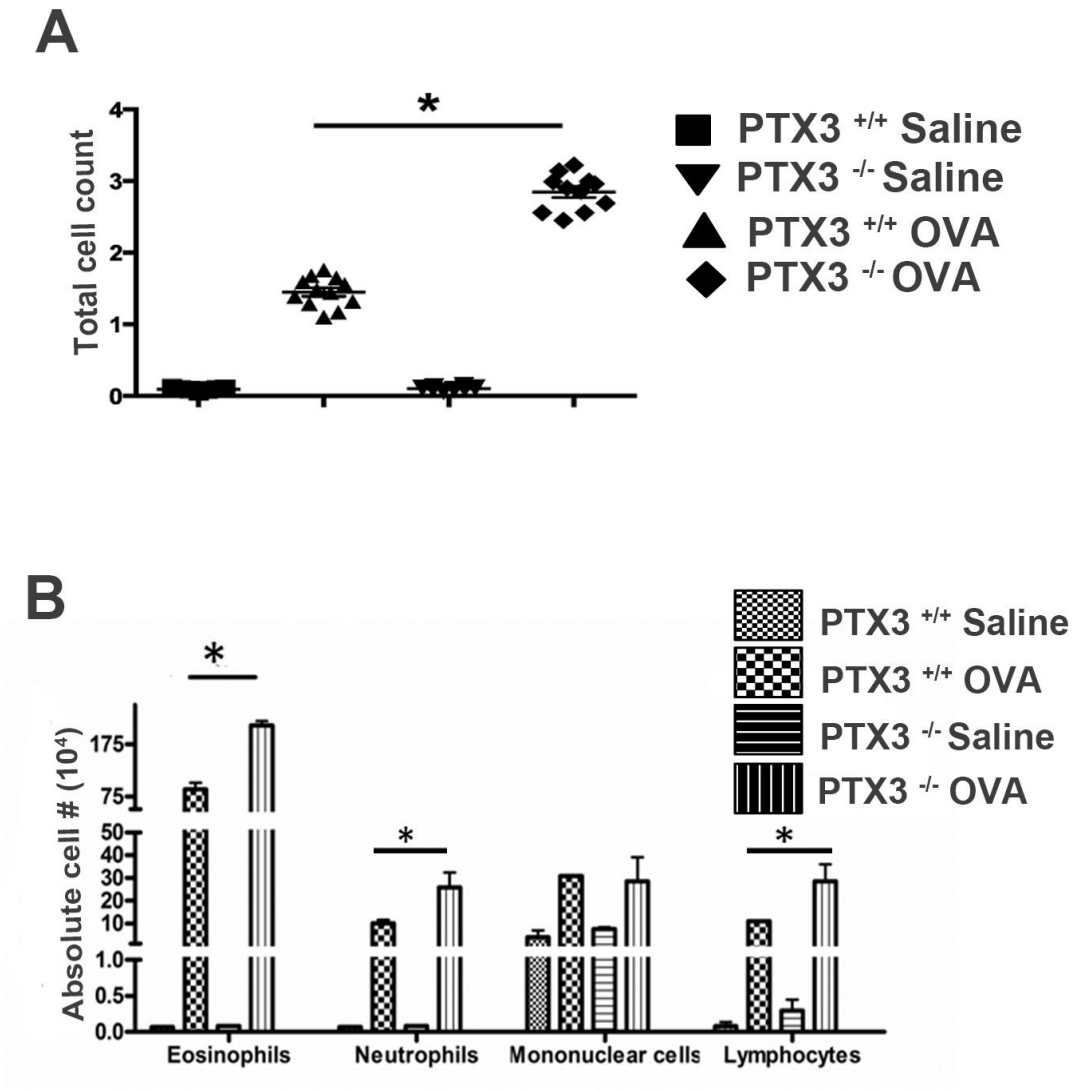

Figure 4.3: *Disruption of PTX3 worsens airway and lung inflammation. Total (10^6) (A) and differential cel (10^4)l (B) count in BALF of OVA-sensitized/ challenged and saline $PTX3^{+/+}$ and $PTX3^{-/-}$ mice.*

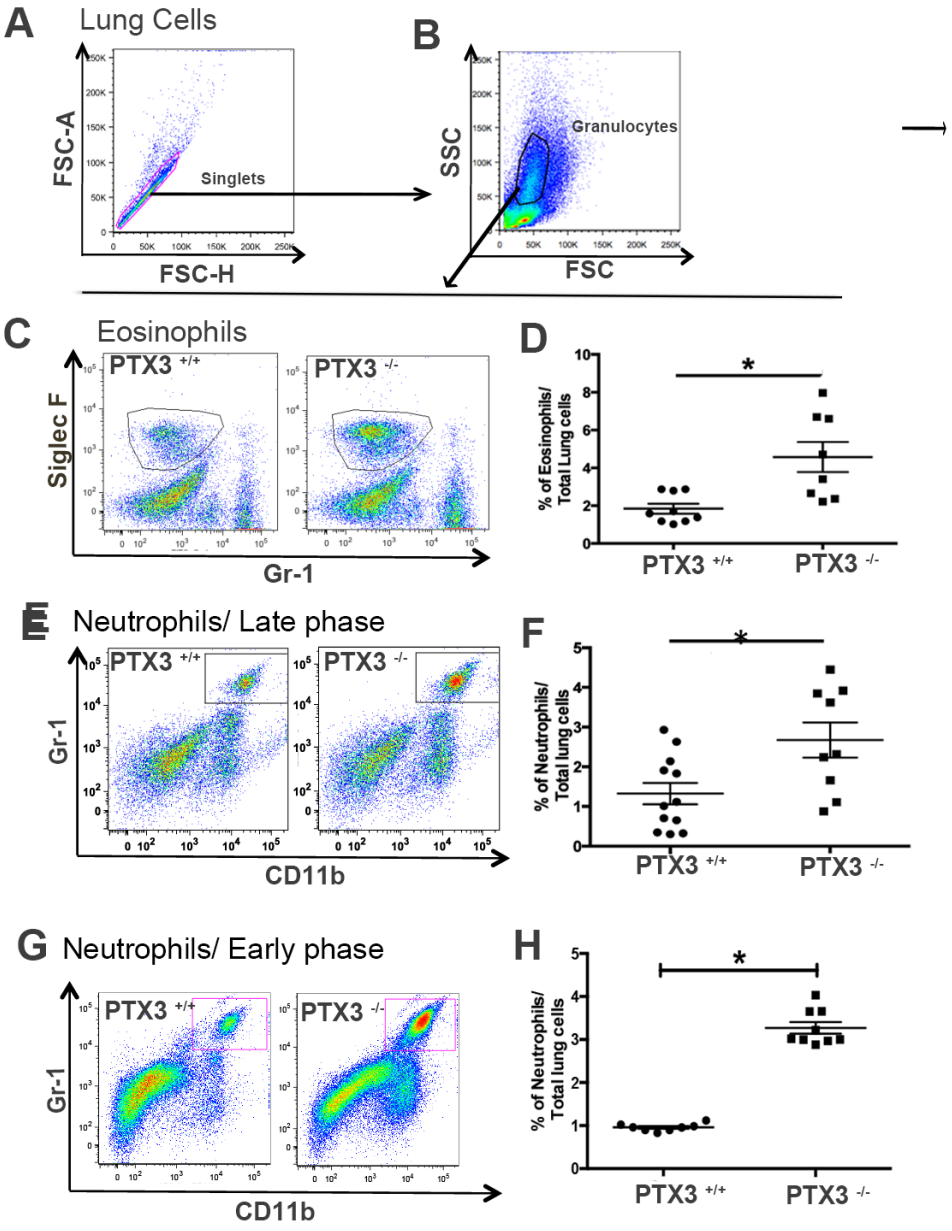

Figure 4.4*: PTX3-/- mice showed increased granulocytic inflammation in the airways upon OVA exposure. Flow cytometric analysis of singlets (A) and granulocytes (B): eosinophils (C and D) and neutrophils at early (E and F) and (G and H) in the lungs of mice from both the strains 48 hrs post OVA challenge, n= 8-9/ group, *p<0.01.*

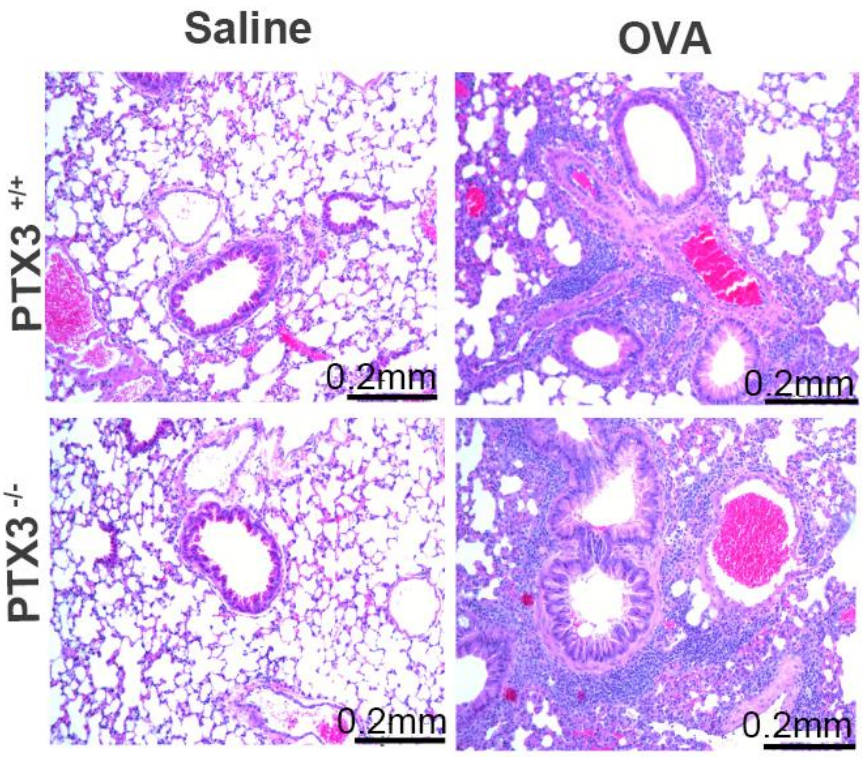

Figure 4.5: *Histological examination of lung inflammation (200X).*

PTX3-/- mice demonstrated higher levels of IgE, IgG2a and IgG2b

OVA sensitization and subsequent challenge is known to affect immunoglobulin concentration in blood (410, 411). Indeed serum levels of total and OVA-specific immunoglobulins including IgE, IgG1, IgG2a and IgG2b were substantially increased in OVA-exposed mice as compared to their saline controls. Heightened levels of IgE and IgG1 suggest Th2 dominant response. Interestingly we observed increased level of total and OVA-specific IgE (Figure 4.6A and B) in OVA-challenged PTX3-/- mice, however, levels of total or OVA-specific IgG1 (Figure 4.6C and D) were comparable in both genotypes. We also observed a significant increase in the levels of total IgG2a (Figure 4.6E), a Th1 regulated immunoglobulin in PTX3-/- mice upon OVA exposure as compared to their PTX3+/+ counterparts. However, levels of total IgG2b was greater in OVA–exposed PTX3+/+ mice in contrast to their PTX3-/- controls (Figure 4.6G). OVA-specific IgG2a and IgG2b were found to be comparable in both strains in allergic condition (Figure 4.6F and H). Altogether it seems that OVA exposure induced a mixed inflammatory response to allergen in PTX3+/+ and PTX3-/- mice, however, PTX3-/- mice showed exaggerated secretion of IgE and IgG2a.

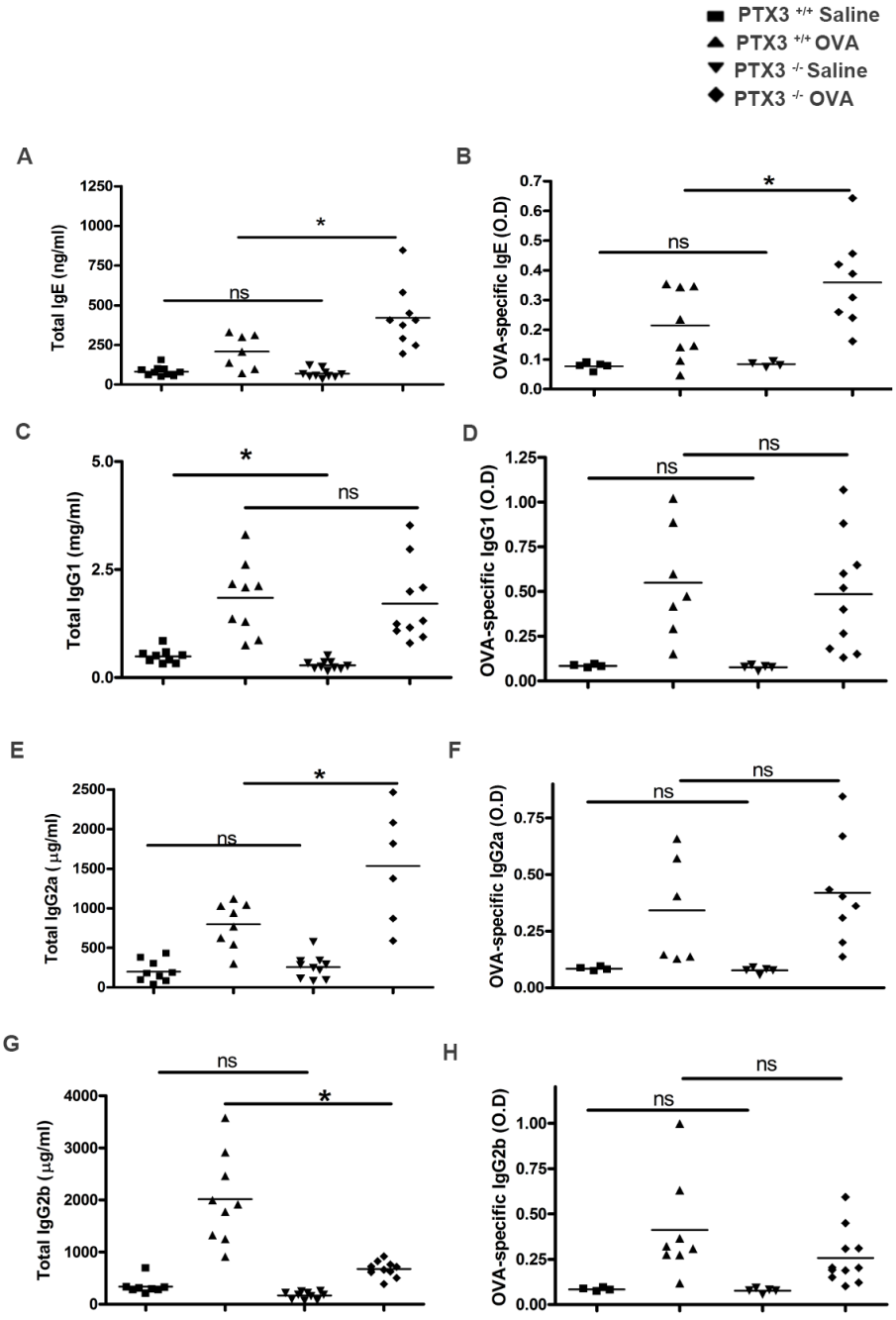

Figure 4.6: *OVA exposure resulted in increased prodution of total IgE, IgG2a and OVA-specific IgE in PTX3-/- mice. Level of total and OVA-specific IgE (A and B), IgG1 (C and D) IgG2a (E and F) and IgG2b (G and H) was assessed in serum of saline and OVA exposed PTX3$^{+/+}$ and PTX3$^{-/-}$ mice by ELISA, n= 7-10/ group, *p<0.01.*

Deletion of PTX3 resulted in enhanced OVA-induced airway hyperresponsiveness and tissue elastance

As a measure of altered lung mechanics as a result of allergen exposure, we determined the response of *PTX3*$^{-/-}$ and *PTX3*$^{+/+}$ mice to methacholine (MCh) post allergen (OVA) exposure. Particularly we measured airway resistance that is a physiological parameter of airway resistance to airflow during inspiration and expiration. Given that resistance of the respiratory tract depends on the diameter of airways, it is indicative of airway constriction. OVA challenge resulted in an amplified airway resistance in PTX3$^{-/-}$ mice (n=12 *P<0.01) (Figure 4.7A) as compared to *PTX3*$^{+/+}$ mice. The greatest difference was observed at higher doses of MCh (50mg/ml) (Figure 4.7B). However, the response of PTX3+/+ and PTX3-/- mice was found to be comparable in naïve state. Similarly, we observed increasing pattern in parenchymal maximal response (tissue resistance) to MCh in PTX3-/- mice post-challenge compared to their wildtype counterparts (Figure 4.7C). At 50mg/ml methacholine, although there is an increasing trend in tissue resistance, we did not observe a significant difference (Figure 4.7D). Given that PTX3-/- mice showed increased responsiveness to MCh, especially at high doses, it suggests increased brionchoconstriction in PTX3-/- mice as compared to PTX3+/+ mice. However, sensitivity to MCh remained unaffected upon PTX3 deletion.

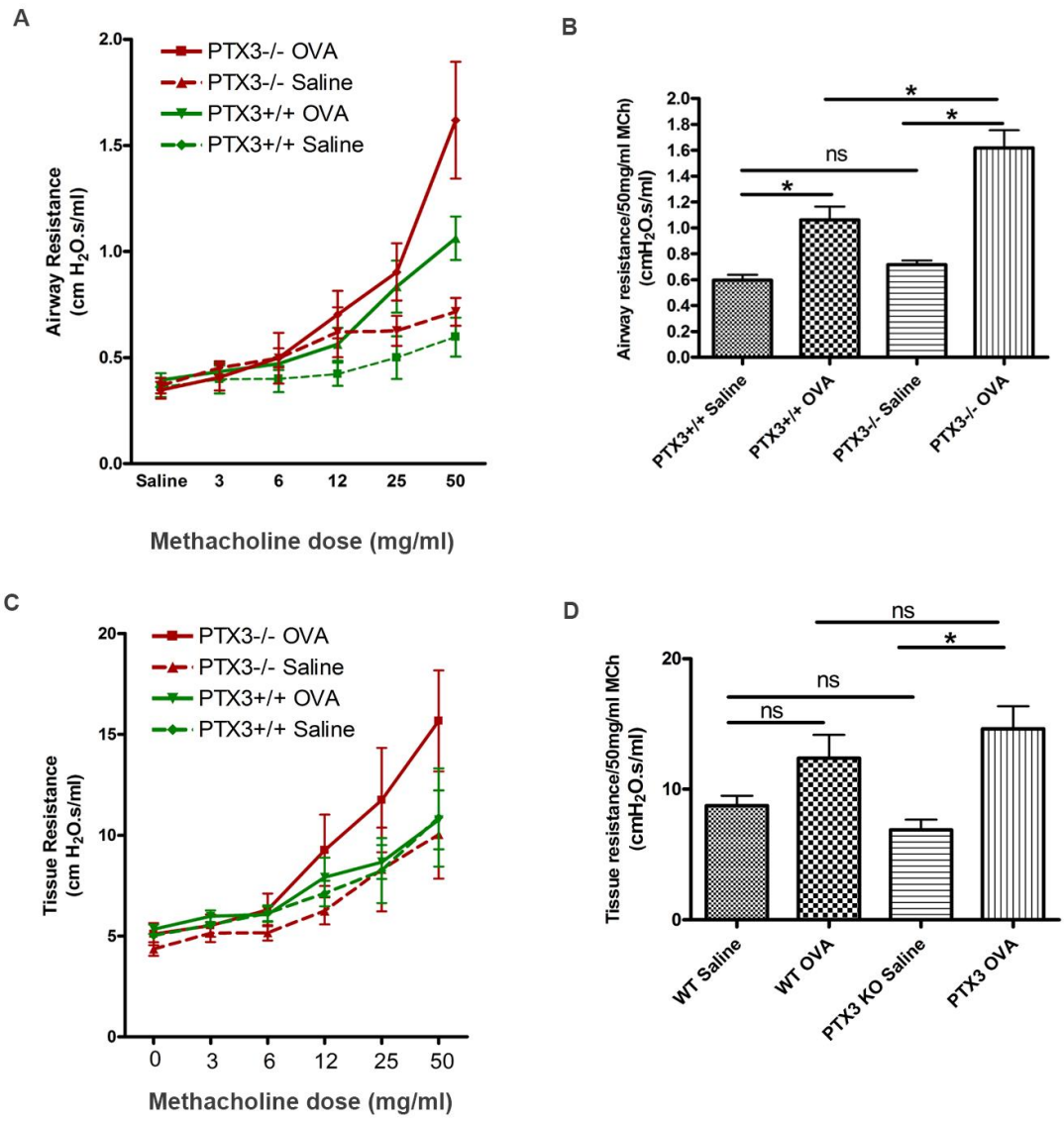

Figure 4.7: *Deletion of PTX3 aggravates OVA-induced airway and tissue hypereactivity. (A) Airway and (C) tissue resistance in OVA-sensitized/ challenged PTX3$^{+/+}$ and PTX3$^{-/-}$ mice as determined using flexiVENT Small Animal Ventilator. B and D show resistance at 50mg/ml methacholine dose.*

PTX3$^{-/-}$ mice displayed an enhanced airway remodeling

We observed evidence of enhanced goblet cell hyperplasia as determined by PAS staining (Figure 4.8A and B) in *PTX3*$^{-/-}$ mice in contrast to *PTX3*$^{+/+}$ mice. Also, PTX3-/- mice showed increased mRNA level of mucus genes such as *muc5ac* (Figure 4.8C) and *muc5b (*Figure 4.8D*).* We also determined the expression of other molecules that are involved in the development of airway remodeling upon allergen exposure in murine models. mRNA level of collagen genes such as *col1 (*Figure 4.9A*)* and *col3* (Figure 4.9B) was increased in PTX3-/- mice as compared to PTX3+/+ mice upon OVA exposure. mRNA expression of fibronectin gene (Figure 4.9C) and smooth muscle actin gene (*sm-actin) (*Figure 4.9D*)* were not affected by PTX3 deletion. These results collectively indicate that PTX3 deletion result in increased OVA-induced mucus production and collagen genes expression.

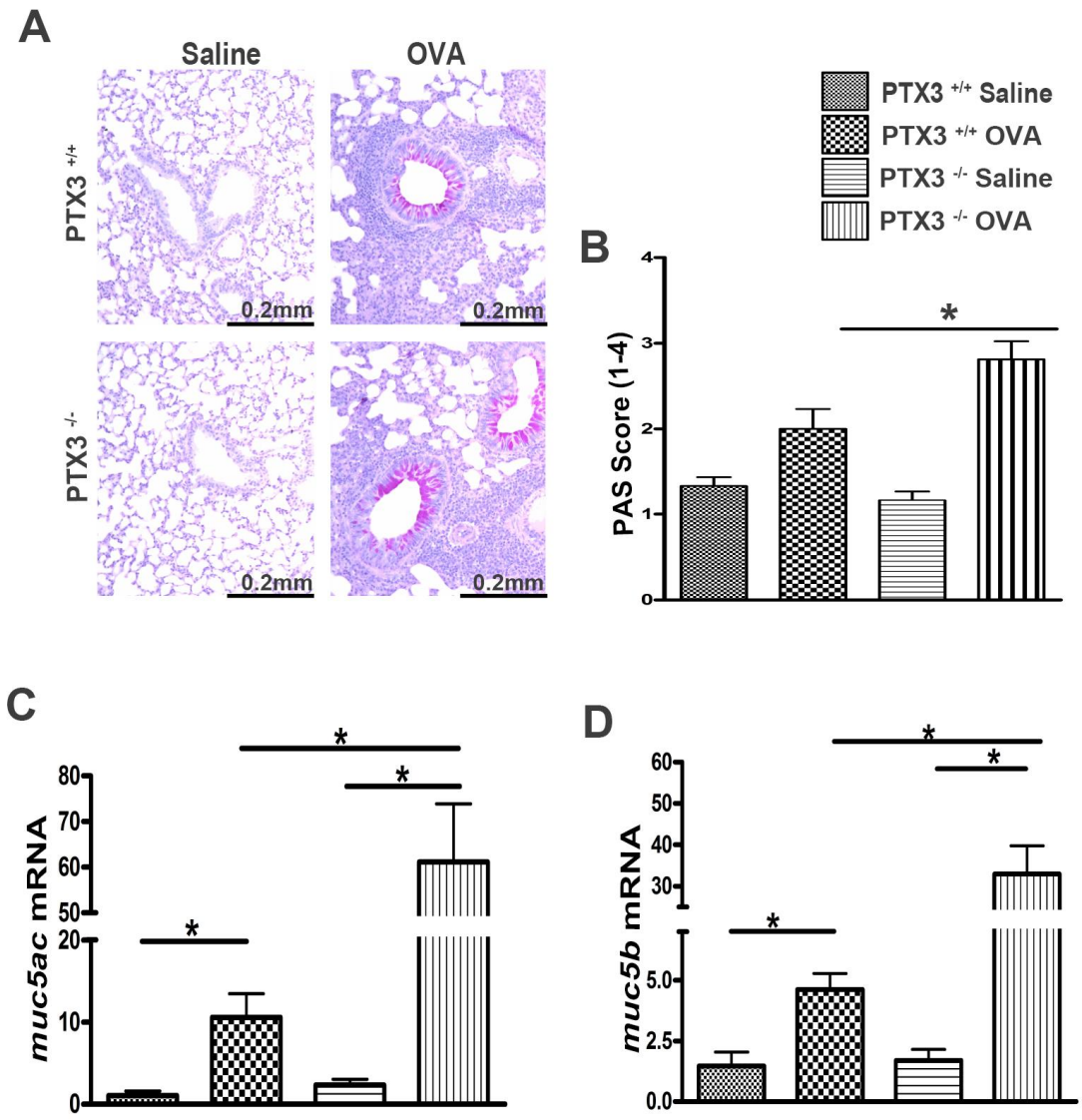

Figure 4.8: *Deletion of PTX3 aggravates OVA-induced mucus gene expression. Mucus production in the lungs (B and C) was determined by PAS staining (200X magnification). mRNA expression of mucus genes muc5ac (D) and muc5b (E) was assessed by real time PCR. n= 12/ group, *p<0.01.*

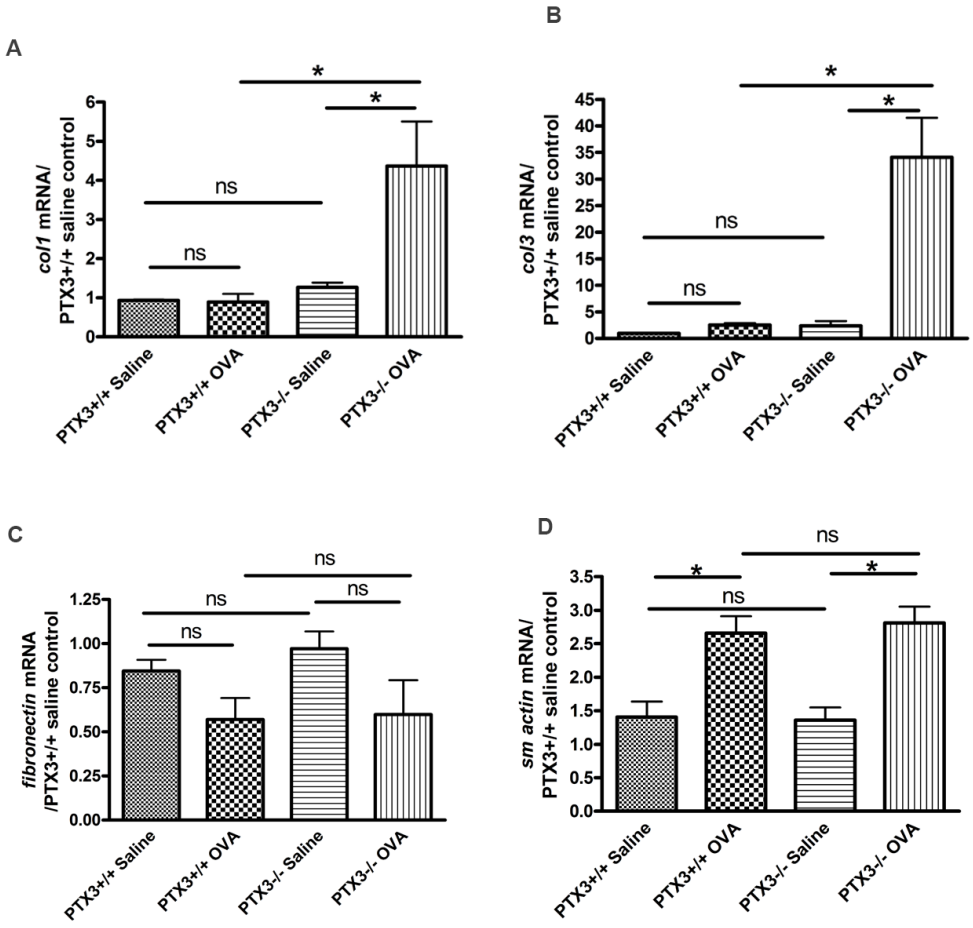

Figure 4.9: *mRNA expression of molecules involved in airway remodeling: (A) collagen 1, (B) collagen 3, (C) Fibronectin and (D) smooth musle actin was assessed by real time PCR. n= 12/ group, *p<0.01.*

PTX3$^{-/-}$ mice exhibit enhanced IL-17A levels in the lungs and mediastinal lymph node

Evaluation of cytokine production in the lungs and mediastinal lymph node (MLN) is an essential parameter used to assess allergic inflammation (412, 413). Therefore, we next explored the levels of inflammatory cytokines in lung homogenate and in the supernatants collected from recall response in MLN. OVA exposure induced Th2 response as determined by IL-4 (Figure 4.10A) and IL-5 (Figure 4.12A) levels in the lungs of both strains of mice as compared to their saline-exposed counterparts. An absolute concentration of IL-4 (*PTX3*$^{-/-}$*:* 25.1±2.5 vs *PTX3*$^{+/+}$*:* 52.9±3.9 pg/ml) and IL-5 (*PTX3*$^{-/-}$*:* 122.8±47 vs *PTX3*$^{+/+}$*:* 211±13.9 pg/ml) was lesser in *PTX3*$^{-/-}$ mice as compared with *PTX3*$^{+/+}$ mice upon OVA challenge. However, magnitude of IL-4 induction (Figure 4.10D) but not IL-5 (Figure 4.12D) was greater in PTX3$^{-/-}$ mice upon OVA challenge in comparison with their *PTX3*$^{+/+}$ littermates (Figure 4.10D and 12D showed fold change in the concentration of IL-4 and IL-5 resp, upon OVA exposure in *PTX3*$^{+/+}$ and *PTX3*$^{-/-}$ mice). mRNA expression of IL-4 was also greater in the lungs of OVA-exposed PTX3-/- mice as compared to their wild type counterparts (Figure 4.11A).

OVA+alum sensitization and challenge regimen preferentially generate a Th2-skewed response over Th1 or Th17 response (414, 415). As expected, *PTX3*$^{+/+}$ OVA-challenged mice did not show considerable change in the protein and the mRNA level of IFN-γ (Figure 4.10B and 4.11B) and IL-17A (Figure 4.10C and 4.11C) in the lungs as compared to their saline controls (IFN-γ- OVA: 212.7±7.8 vs Saline: 199.1±10.1 pg/ml, IL-17A OVA: 190.2±15.5 vs Saline: 172.4±12.6 pg/ml). Lung homogenate from *PTX3*$^{-/-}$ mice on the other hand showed significant increase in the concentration of both cytokines upon OVA exposure as compared to their saline counterparts (IFN-γ- OVA: 377.0±38.6 pg/ml vs Saline: not

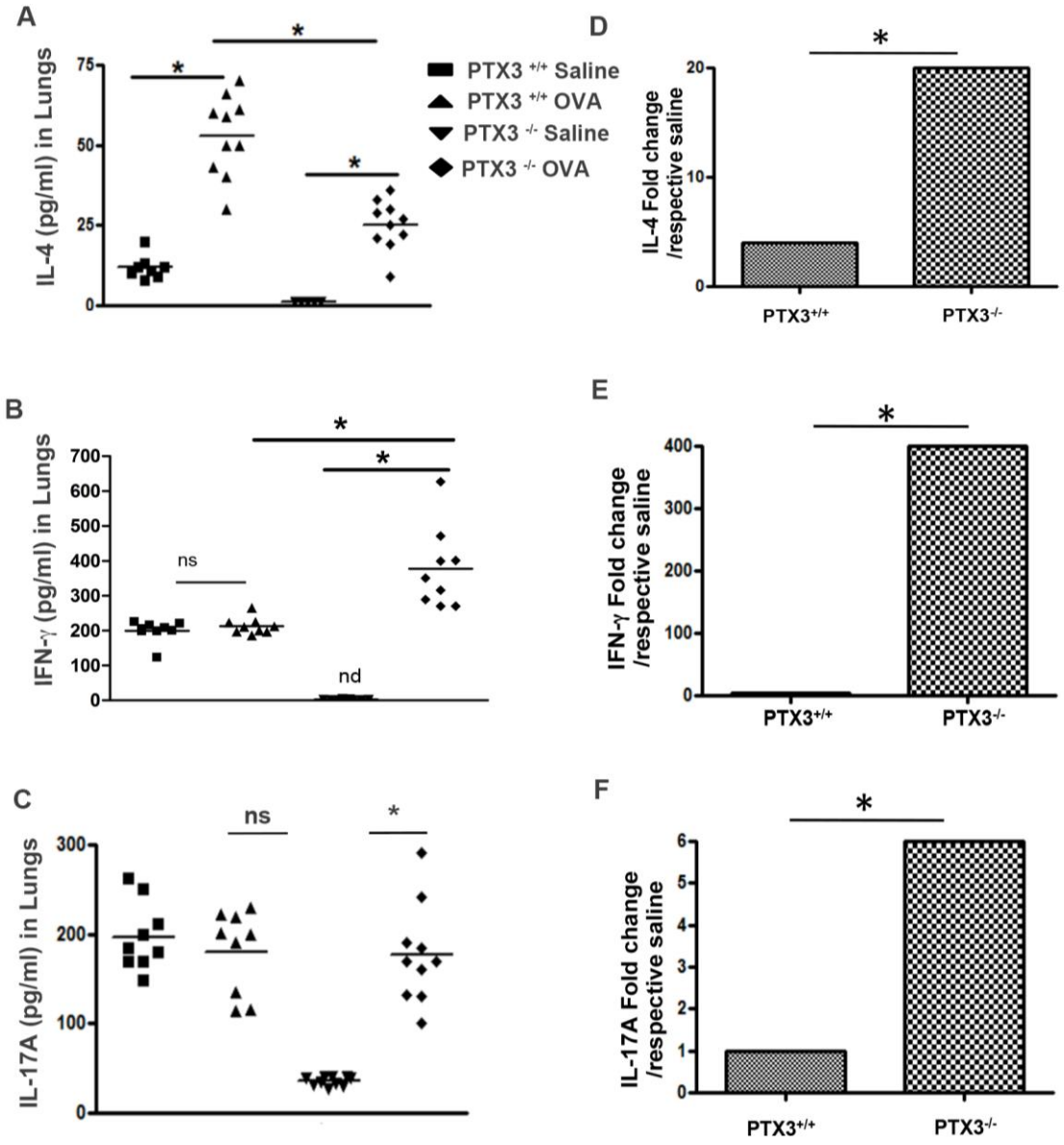

Figure 4.10: *Absence of PTX3 induces enhanced IL-17A-dominant response. Level of proinflammatory cytokines in the lungs (A, B and C) was determined by ELISA (8-10 mice /group). *p<0.01.* Fold increase in the level of cytokines (D-E) in OVA exposed condition compared to their respective saline controls in *PTX3*$^{+/+}$ and *PTX3*$^{-/-}$ mice.

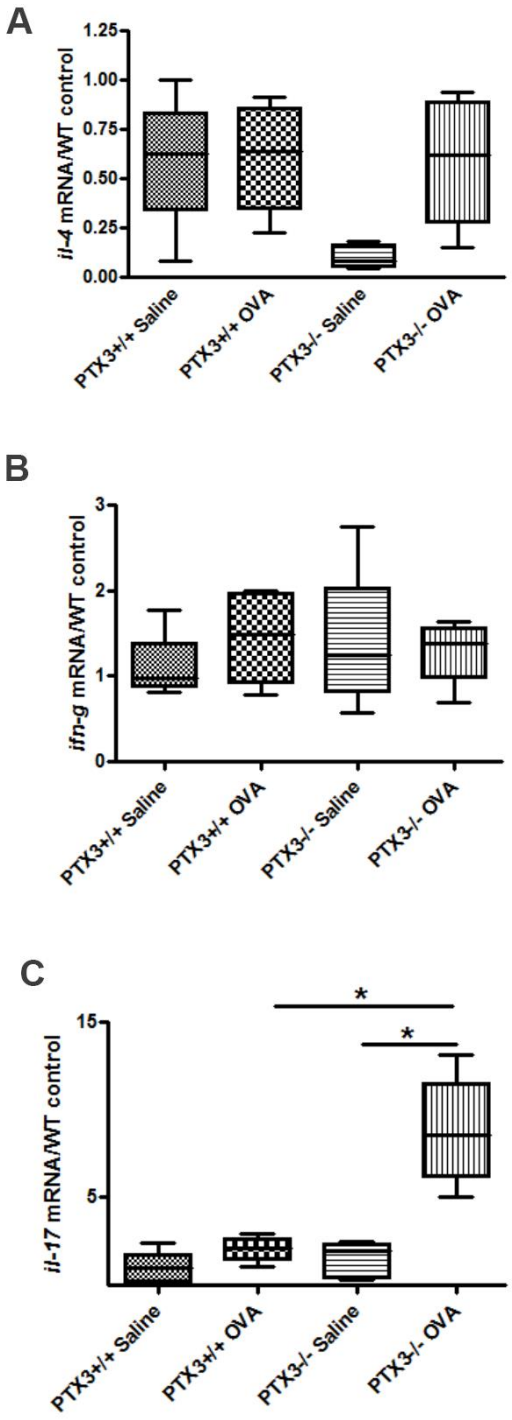

Figure 4.11: *PTX3 deleted mice exhibited increased expression of IL-17a mRNA in the lungs in OVA-exposed condition. Lungs were isolated from OVA-exposed PTX3+/+ and PTX3-/- mice and used for isolating RNA. mRNA level of proinflammatory cytokines IL-4, IFN-γ and IL-17A was determined by quantitative real time PCR in the lungs of $PTX3^{+/+}$ and $PTX3^{-/-}$ mice in saline and OVA-exposed condition. n=8-12/ group, *p<0.01*

detected; IL-17A- OVA: 176.8±1.8 vs Saline: 35.6±1.6 pg/ml) (Figure 4.10B, C, E and F and 15A-B). Concomitantly, an increase in the induction of IL-6 in the lungs was also observed in PTX3$^{-/-}$ mice upon OVA exposure (Figure 4.12C and F), no such increase was apparent in IL-12 levels (Figure 4.12B and E). Although IFN-g mRNA (Figure 4.11B) expression was not affected by PTX3 deletion, OVA-exposed PTX3-/- mice showed a significant increase in mRNA level of IL-17A (Figure 4.11C) in the lungs.

Recall response in MLN also demonstrated induction of IL-4, IFN-g and IL-17A responses in both strains (Figure 4.13 A-C). However, *PTX3*$^{-/-}$ mice exhibited an IL-17A prevalent response compared to PTX3$^{+/+}$ littermates (*PTX3*$^{-/-}$*:* 2367±184.2 vs *PTX3*$^{+/+}$*:* 966.7±68.9 pg/ml) (Figure 4.13C). However, the absolute IFN-g (Figure 4.13B) response was similar in both genotypes upon OVA challenge (*PTX3*$^{-/-}$*:* 1902±44.8 vs *PTX3*$^{+/+}$: 2052±105.4 pg/ml). IL-4 (Figure 4.13A) production was lower in PTX3$^{-/-}$ mice compared to their PTX3$^{+/+}$ littermates (*PTX3*$^{-/-}$*:* 50±11.5 vs *PTX3*$^{+/+}$: 78±21.3 pg/ml). Altogether it suggests generation of a mixed proinflammatory response with an apparent dominance of Th17 phenotype in *PTX3*$^{-/-}$ mice in contrast to their wild type counterparts upon allergen exposure.

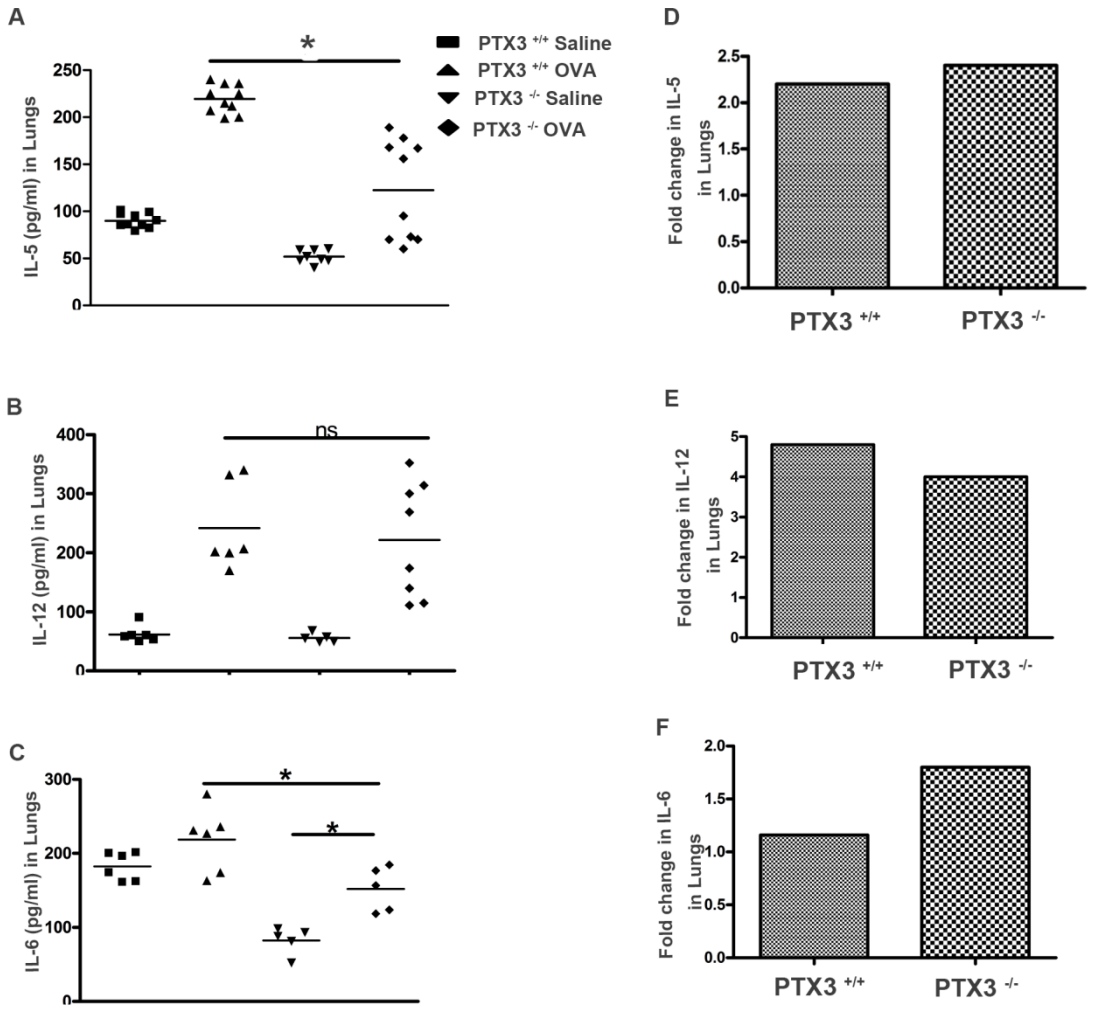

Figure 4.12: *Concentration of IL-5 (A), IL-12 (B) and IL-6 (C) in lungs of PTX3$^{+/+}$ and PTX3$^{-/-}$ mice as determined by ELISA, n=5-10/ group, *p<0.01. Fold increase in the level of cytokines (D-E) in OVA exposed condition compared to their respective saline controls in PTX3$^{+/+}$ and PTX3$^{-/-}$ mice.*

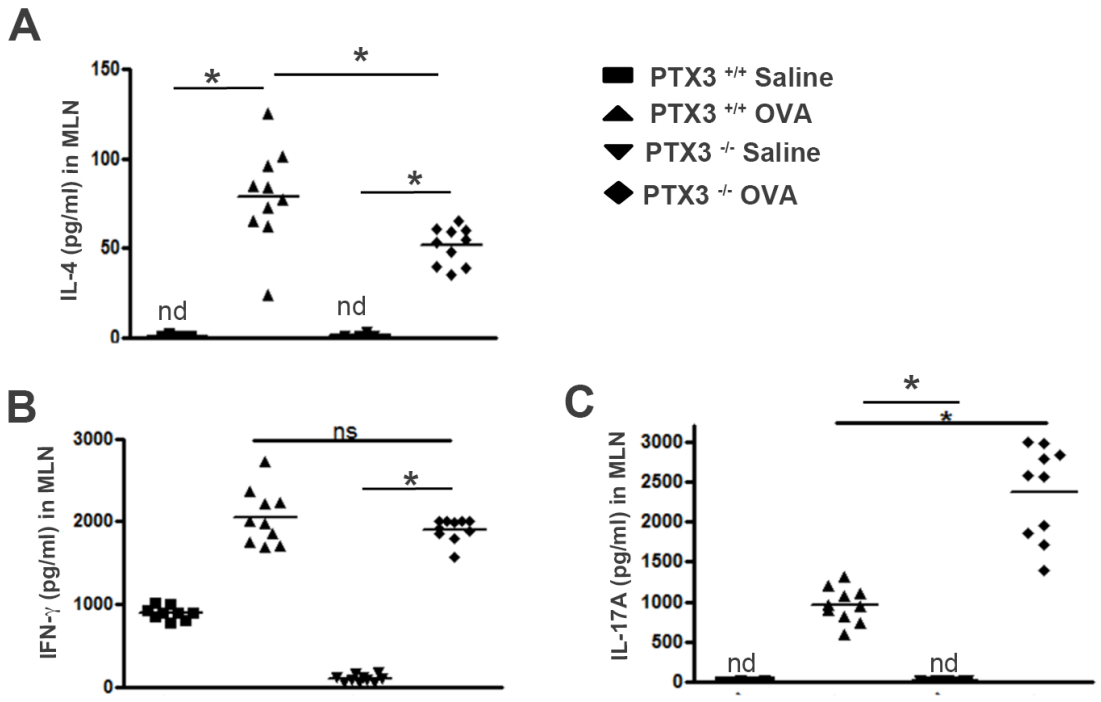

Figure 4.13: OVA sensitized/ challenged PTX3-/- mice showed reduced protein level of IL-4 but increased level of IL-17A in MLN. *Level of proinflammatory cytokines IL-4 (A), IFN-g (B) and IL-17A (C) was determined by ELISA (8-10 mice /group). *p<0.01.*

PTX3$^{-/-}$ CD4 T cells display enhanced activation and Th17-dominant phenotype

Given that CD4 T cells play a critical role in the production of proinflammatory cytokines in the lungs upon allergen exposure (416, 417) (418), I first explored activation status of CD4 T cells (Figure 4.14A) in *PTX3*$^{+/+}$ and *PTX3*$^{-/-}$ mice upon OVA exposure. *PTX3*$^{-/-}$ lung CD4 T cells showed an enhanced activated phenotype as determined by CD69 (Figure 4.14B and C) and CD25 (Figure 4.14D and E) expression (419, 420) as compared to their wild-type counterparts. Also OVA exposure induced enhanced generation of effector CD4 T cells in *PTX3*$^{-/-}$ mice as determined by CD62L^{neg}CD44$^{int/high}$ staining (Figure 4.14F and G).

Flow cytometry analysis of cytokine producing CD4 T cells showed that deletion of PTX3 allowed a significant expansion of IL-17A producing CD4 T cells in lungs in OVA-induced inflammatory condition (*PTX3*$^{+/+}$*:* 0.096±0.012%, *PTX3*$^{-/-}$: 0.27±.024 of total lung cells) (Figure 4.15A and B). Similar preponderance of Th17 phenotype was observed in the MLN of *PTX3*$^{-/-}$ mice (Figure 4.15C and D). However, no significant difference was detected in the expansion of IL-4 (Figure 4.16 A and B) and IFN-g (Figure 4.16C and D) producing lung CD4 T cells in both genotypes.

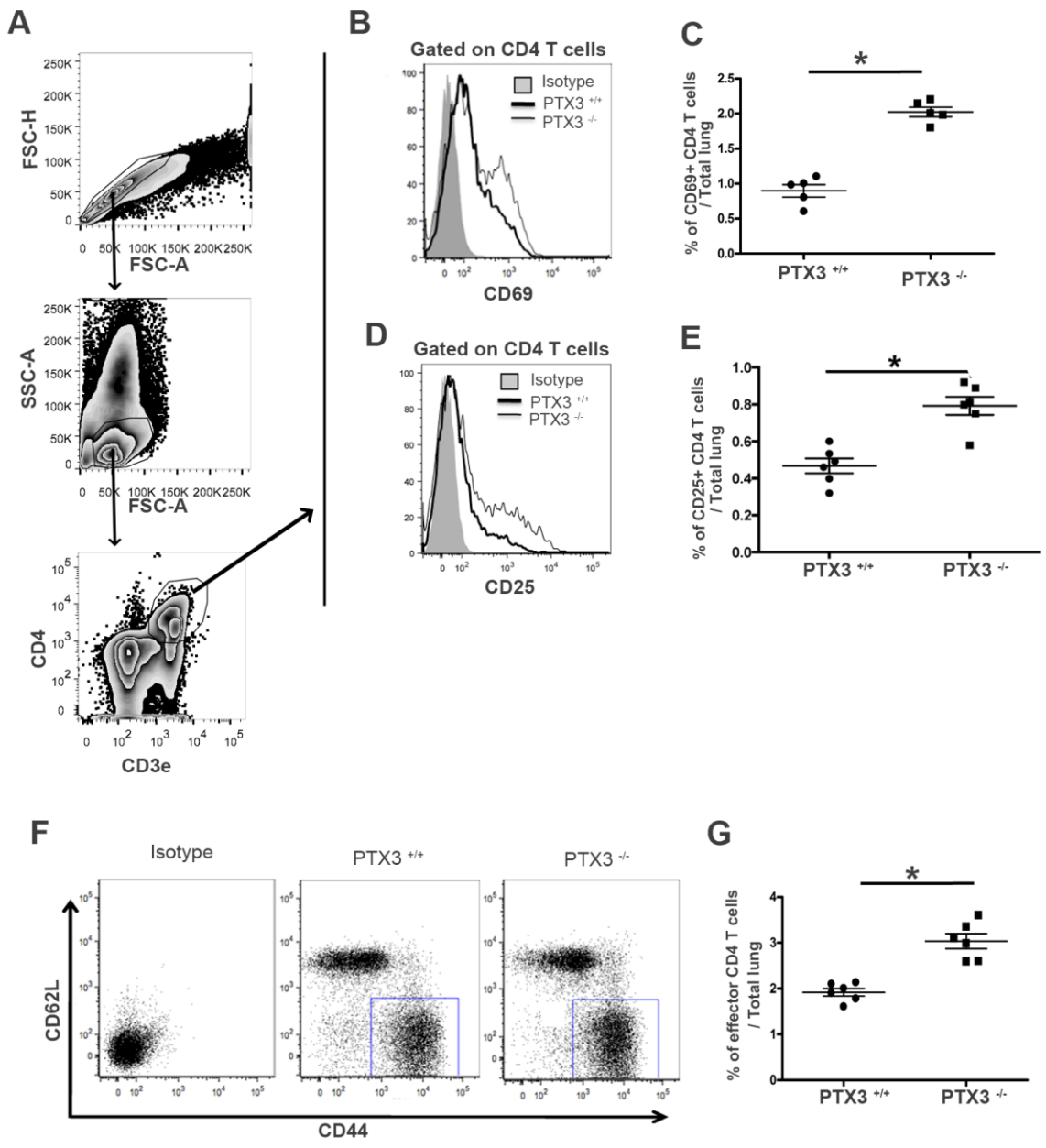

Figure 4.14: *PTX3-/- lung CD4 T cells showed enhanced activation in OVA-exposed condition. Lung CD4 T cells (A) were studied for the expression of CD69 (B and C) and CD25 (D and E). Characterization of effector lung CD4 T cells (F and G) (5-6 mice/ group). *p<0.01.*

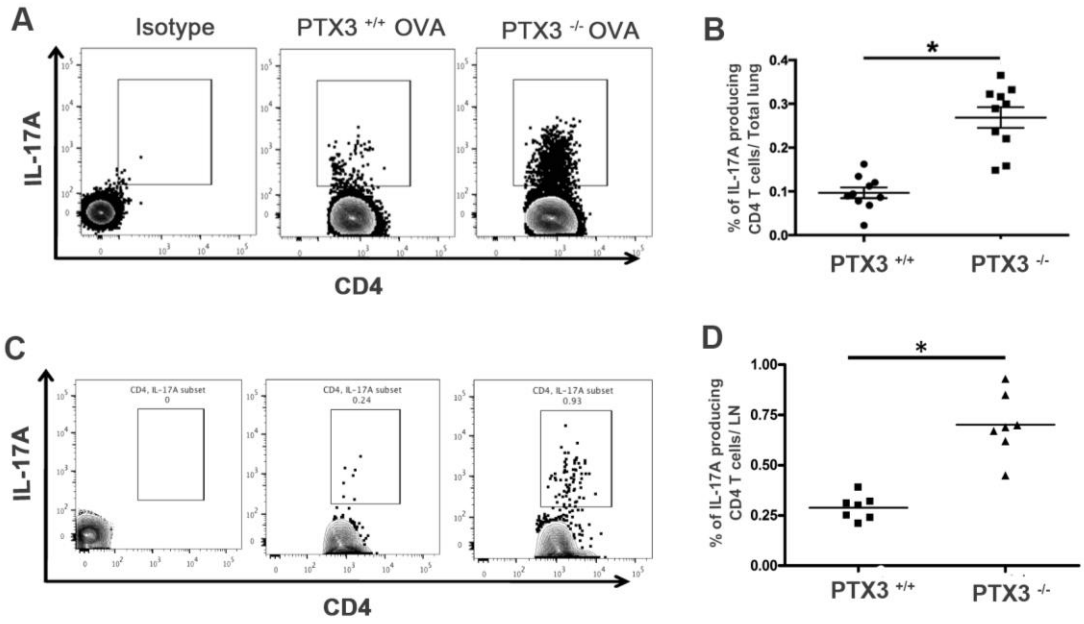

Figure 4.15: *Flow cytometric analysis of IL-17A production by lung and MLN CD4 T cells. **E**xpression profile of IL-17A by lungs (A and B) and draining lymph nodes (C and D) CD4 T cells in OVA-exposed PTX3$^{+/+}$ and PTX3$^{-/-}$ mice was assessed by flow cytometry. (10 mice/ group) *p<0.01.*

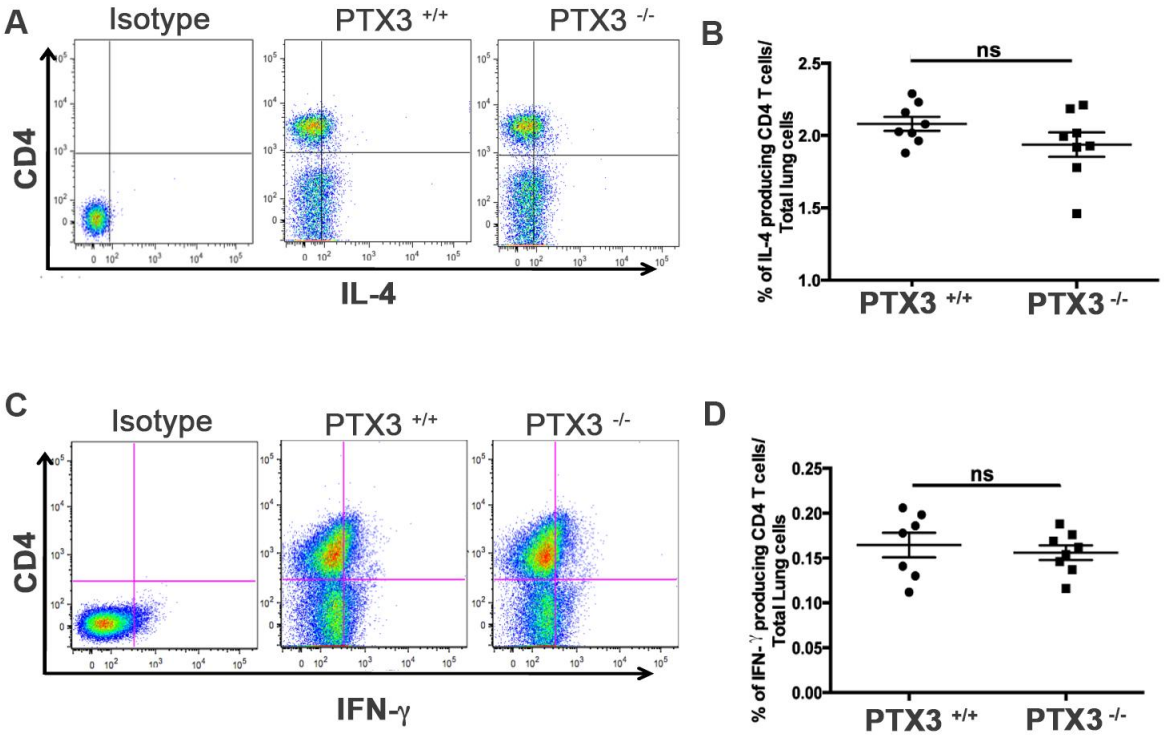

Figure 4.16*: Flow cytometric analysis of IL-4 and IFN-γ production by lung CD4 T cells.* ***E****xpression profile of IL-4 (A and B) and IFN-g (C and D) by lung CD4 T cells in OVA-exposed* $PTX3^{+/+}$ *and* $PTX3^{-/-}$ *mice was assessed by flow cytometry. (n=8-10 mice/ group).*

IL-17A producing CD4 T cells are uniquely characterized by a transcriptional program, which is regulated by activation of STAT3-dependent pathways (421). I then investigated the status of STAT3 phosphorylation in the lungs of $PTX3^{+/+}$ and $PTX3^{-/-}$ in response to OVA. In contrast to saline treated groups, phosphorylation of STAT3 was increased in the lungs of OVA-exposed $PTX3^{-/-}$ mice as compared to $PTX3^{+/+}$ mice (Figure 4.17).

Next I asked whether deletion of PTX3 favors the differentiation of CD4 T cells towards Th17 phenotype. Enriched naïve $PTX3^{-/-}$ spleen CD4 T cells (Figure 4.18A) exhibited enhanced Th17 polarization when cultured in Th17 polarization conditions as compared to their $PTX3^{+/+}$ counterparts (Figure 4.18B and C). Given that IL-6, a critical component of Th17 polarization cocktail, induced IL-17 production through a STAT3-dependent mechanism, I next assessed STAT3 phosphorylation in $PTX3^{-/-}$ and $PTX3^{+/+}$ naïve CD4 T cells upon IL-6 stimulation (234, 422). Parallel to our previous observation, I observed increased phosphorylation of STAT3 in naïve $PTX3^{-/-}$ CD4 T cells upon IL-6 stimulation as compared to $PTX3^{+/+}$ CD4 T cells (Figure 4.19). Together, these results suggest that deletion of PTX3 favors the development of Th17 phenotype, possibly contributing to an IL-17A dominant inflammatory response in the lungs and MLN of $PTX3^{-/-}$ mice upon OVA exposure.

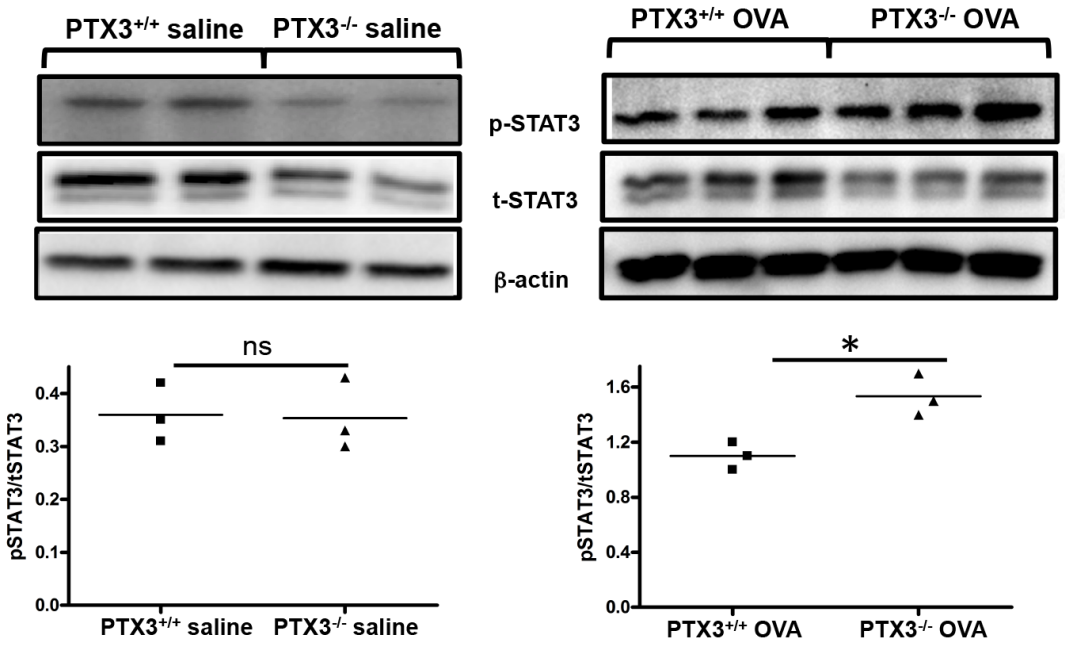

Figure 4.17: *OVA-exposed PTX3-/- CD4 T cells showed increased STAT3 phosphorylation. Phosphorylation of STAT3 was assessed by western blotting in lungs of naïve and OVA-exposed* $PTX3^{+/+}$ *and* $PTX3^{-/-}$ *mice, n=2-3, for each experiment, cells were pooled from 3 different mice.*

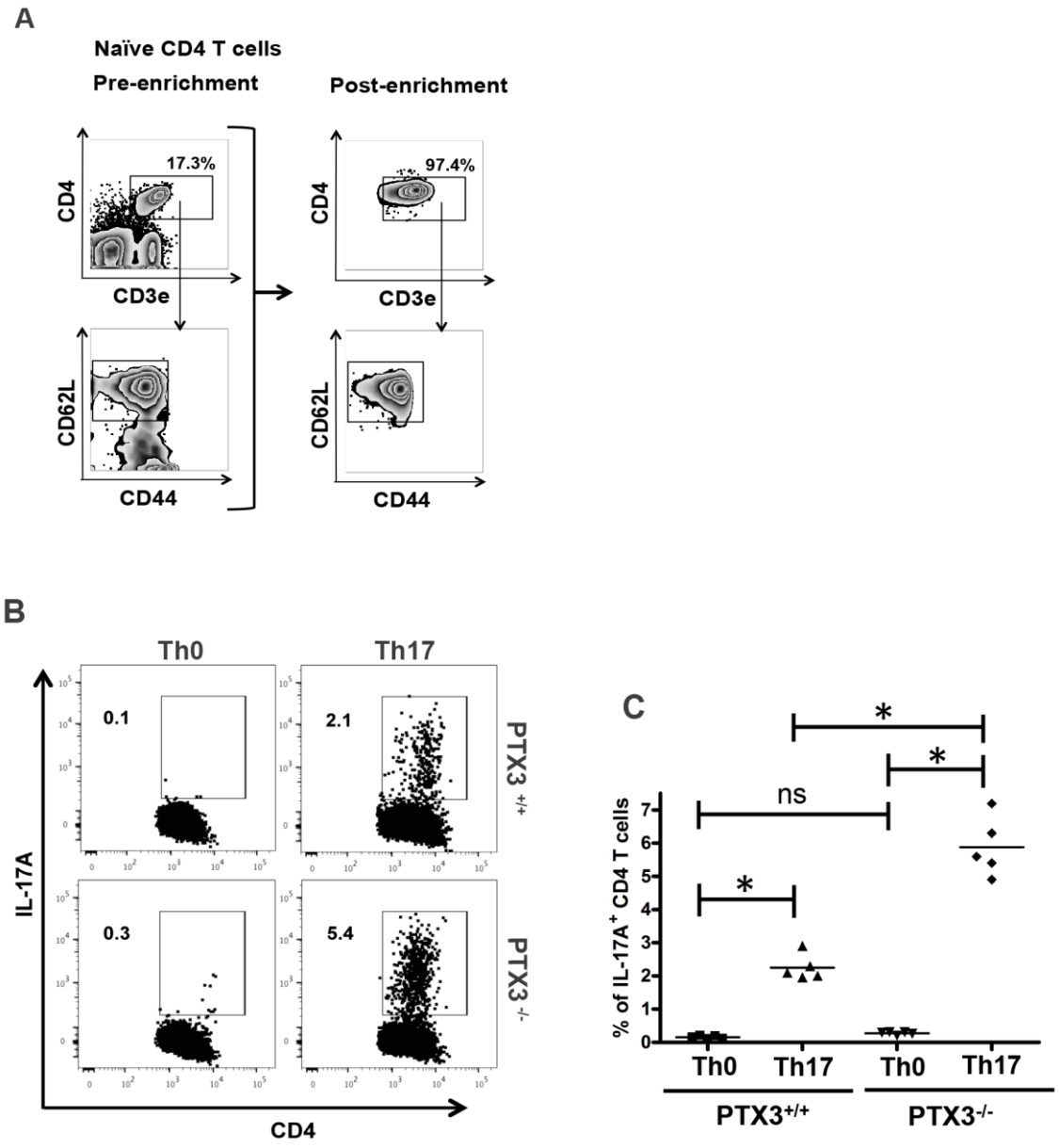

Figure 4.18: *PTX3-/- naive CD4 T cells undergo increased Th17 polarization. (A) Naïve CD4 T cells were enriched from splenocytes and were cultured in the presence of Th17 polarization cocktail for 5 days. (B and C) IL-17A producing CD4 T cells were assessed by flow cytometry. Quantification and statistical analysis of FACS data is shown as graphs. n=5-6/group, *p<0.01.*

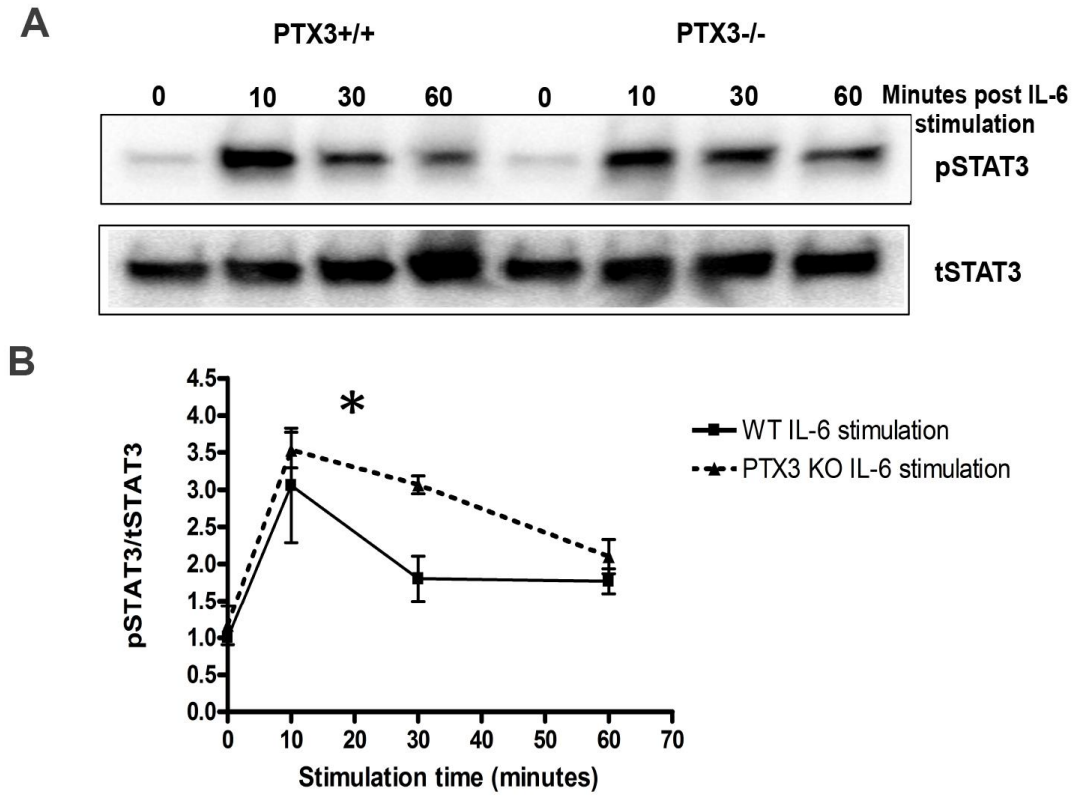

Figure 4.19: *PTX3-/- CD4 T cells showed increased STAT3 phosphorylation in response to IL-6. CD4 T cells were stimulated with 20ng/ml IL-6 and phosphorylation of STAT3 was detected by western blotting. Graph shows average from three independent experiments, *p<0.01.*

PTX3 deleted CD4 T cells exhibit enhanced survival

IL-17A through STAT3 promotes cell survival of various cells including T cells by upregulating prosurvival molecules such as Bcl-2 (423-425). Therefore, next I questioned whether IL-17A-dominant phenotype as observed in *PTX3*$^{-/-}$ mice results in altered survival of CD4 T cells. Lung cells were isolated from OVA exposed *PTX3*$^{+/+}$ and *PTX3*$^{-/-}$ mice, cultured for three days in complete media and assessed for CD4 T survival. OVA-exposed *PTX3*$^{-/-}$ CD4 T cells displayed enhanced survival (Figure 4.20) as assessed by annexin V and DAPI staining.

Naïve *PTX3*$^{-/-}$ CD4 T cells also showed an increased survival (Figure 4.21), however, such a survival benefit was more pronounced in OVA-challenged condition. Together it suggests that PTX3 deficiency is likely to confer an added survival advantage to CD4 T cells in OVA-exposed mice.

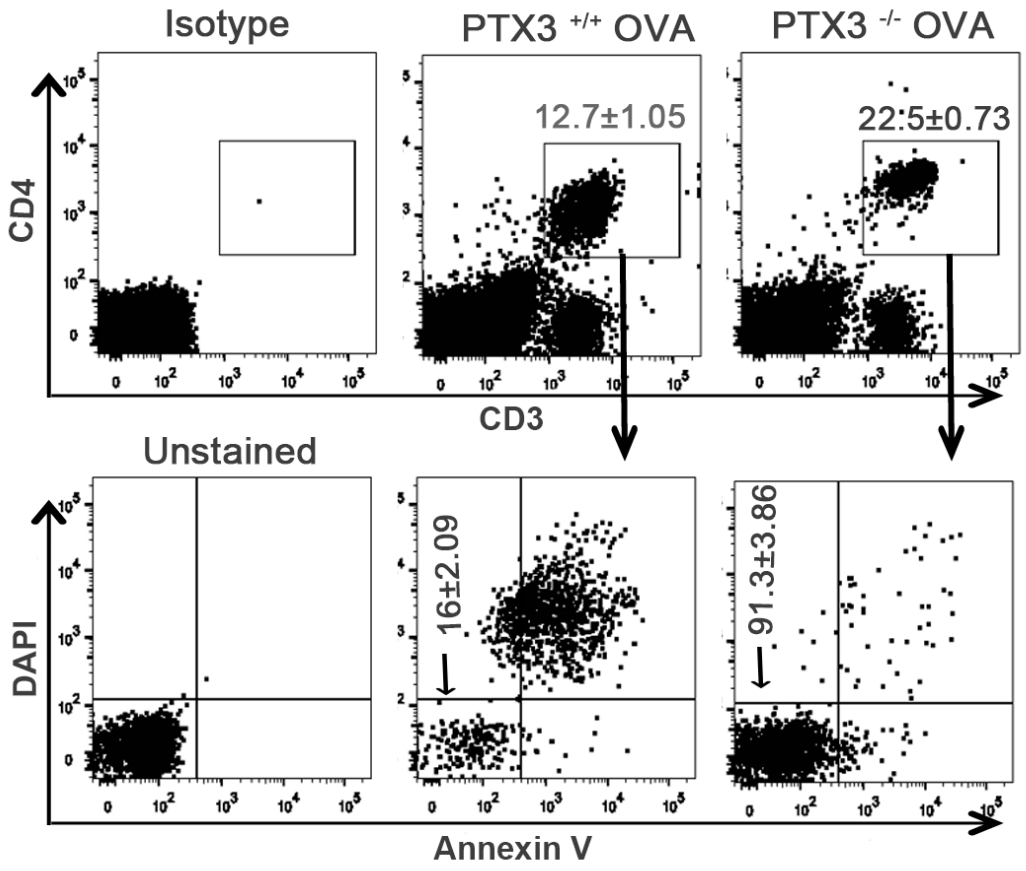

Figure 4.20: *PTX3$^{-/-}$ CD4 T cells show enhanced survival. (A) Survival of lung CD4 T cells (CD3e+CD4+) was assessed by annexin V and DAPI staining. % of gated cells is presented as mean+/- SEM, n=8-10/ group.*

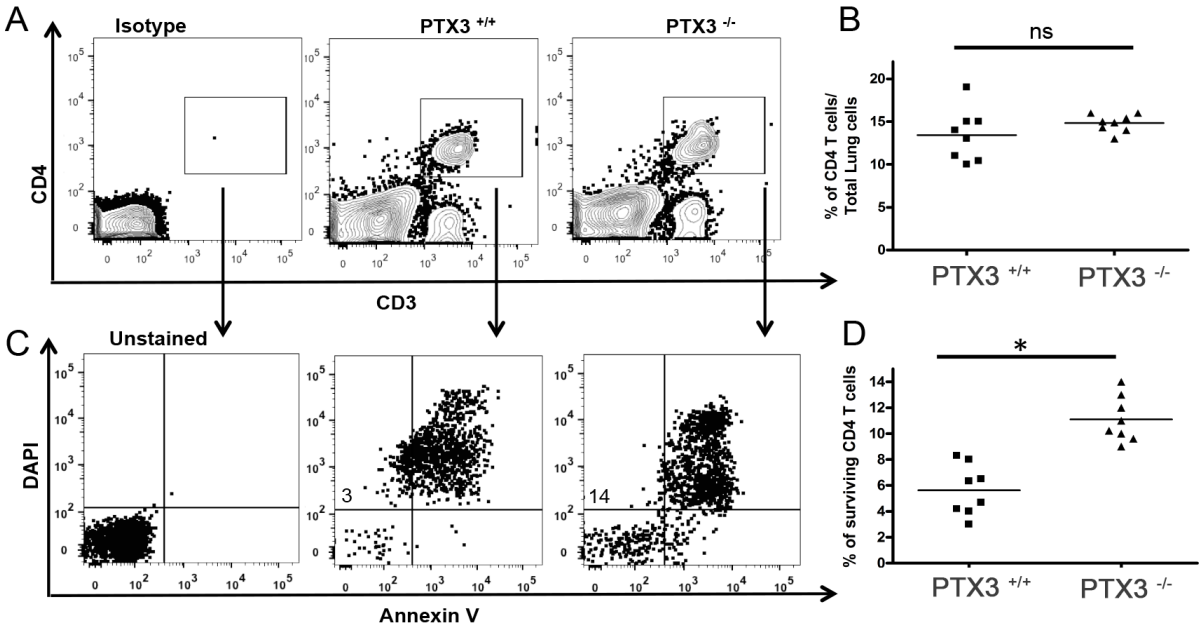

Figure 4.21: *Survival of naïve PTX3+/+ and PTX3-/- CD4 T cells. (A and B) Naïve PTX3$^{+/+}$ and PTX3$^{-/-}$ mice lung CD4 T cells were cultured for 3 days and survival of CD4 T cells was assessed by annexin V and DAPI staining (C and D). n=8/group, *p<0.01.*

CD4 T cells from OVA-exposed PTX3$^{-/-}$ mice exhibit enhanced expression of Bcl-2 and reduced production of IL-2

Bcl-2 is a prototypic member of a large Bcl-2 family, which regulates T cell apoptosis and helps in maintaining immune system homeostasis (426, 427). Therefore, next I examined whether the absence of PTX3 affects the expression of Bcl-2 in the lung CD4 T cells. Although basal expression of Bcl-2 is comparable between PTX3$^{-/-}$ and PTX3$^{+/+}$ CD4 T cells (Figure 4.22A and B), OVA exposure induced greater expression of pro-survival protein, Bcl-2 in pulmonary CD4 T cells (Figure 4.22 C-D) from *PTX3*$^{-/-}$ mice as compared with *PTX3*$^{+/+}$ mice, which is in parallel with enhanced survival.

A previous study showed an association between IL-17A and enhanced survival of T cell by downregulating IL-2 production (232). Subsequently, I examined the production of IL-2 by pulmonary CD4 T cells from OVA-exposed *PTX3*$^{+/+}$ and *PTX3*$^{-/-}$ mice. Consistent with enhanced Th17 phenotype and increased survival, OVA exposed *PTX3*$^{-/-}$ lung (Figure 4.23A and B) and MLN (Figure 4.23C and D) CD4 T cells exhibited reduced IL-2 production. Similarly, PTX3$^{-/-}$ CD4 T cells exhibited reduced IL-2 production in the presence of Th17 polarizations conditions as compared with PTX3+/+ CD4 T cells (Figure 4.24). Naïve PTX3$^{-/-}$ and PTX3$^{+/+}$ CD4 T cells did not show substantial difference in IL-2 production (Figure 4.24). Collectively, my data showed enhanced Bcl-2 expression and reduced IL-2 production by lung CD4 T cells in PTX3$^{-/-}$ mice, supporting their enhanced survival upon OVA exposure.

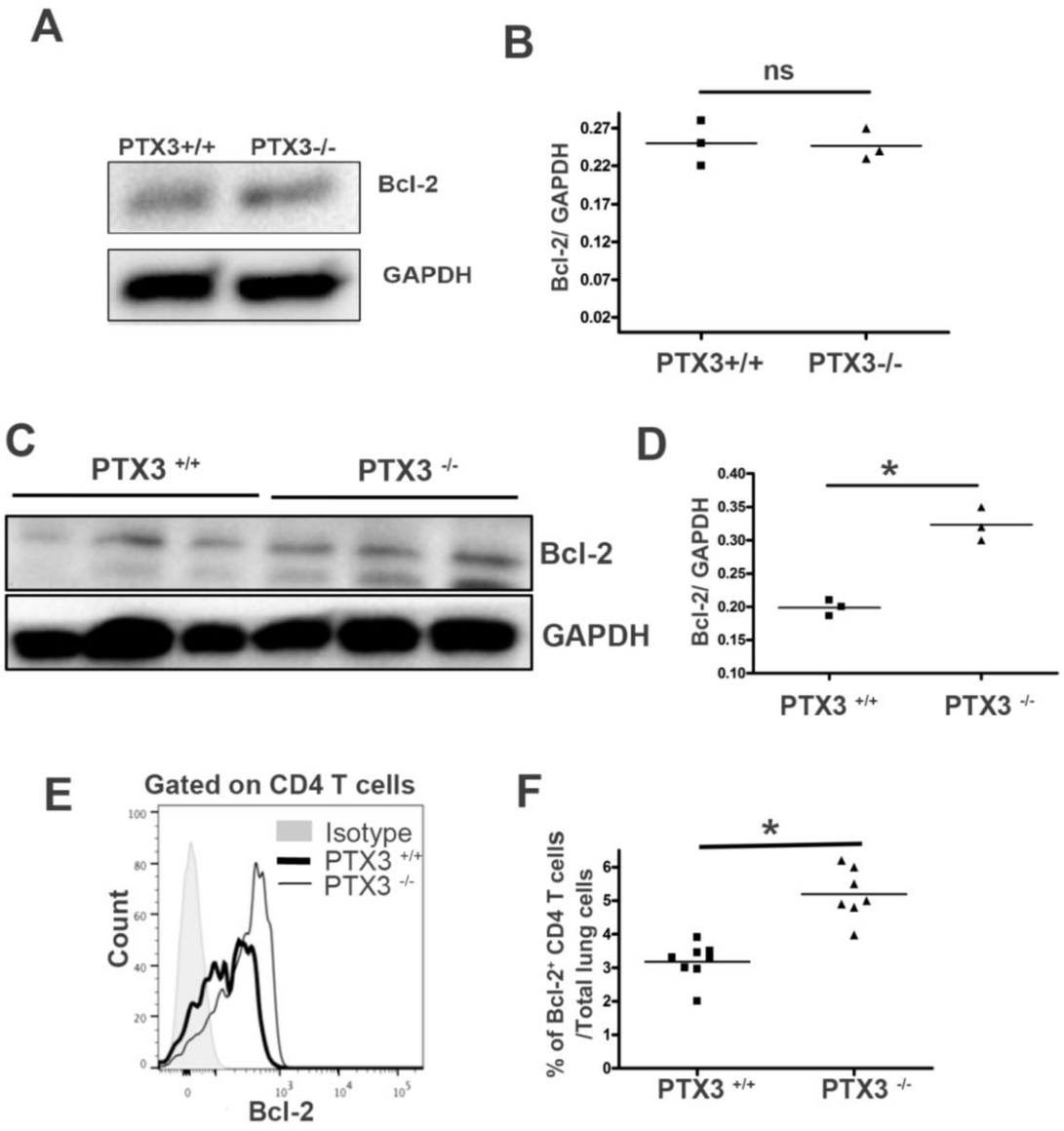

Figure 4.22: *PTX3-/- CD4 T cells showed increased Bcl-2 expression. Expression of Bcl-2 by naïve (A and B) and OVA-exposed (C and D) lung CD4 T cells was determined by western blotting, cells were pooled from atleast 3 mice in each group. OVA exposed lung CD4 T ce4lls were also examined for Bcl-2 expression by flow cytometry (E and F). Quantification and statistical analysis of FACS data is shown as graphs. n=5-10/ group, *p<0.01.*

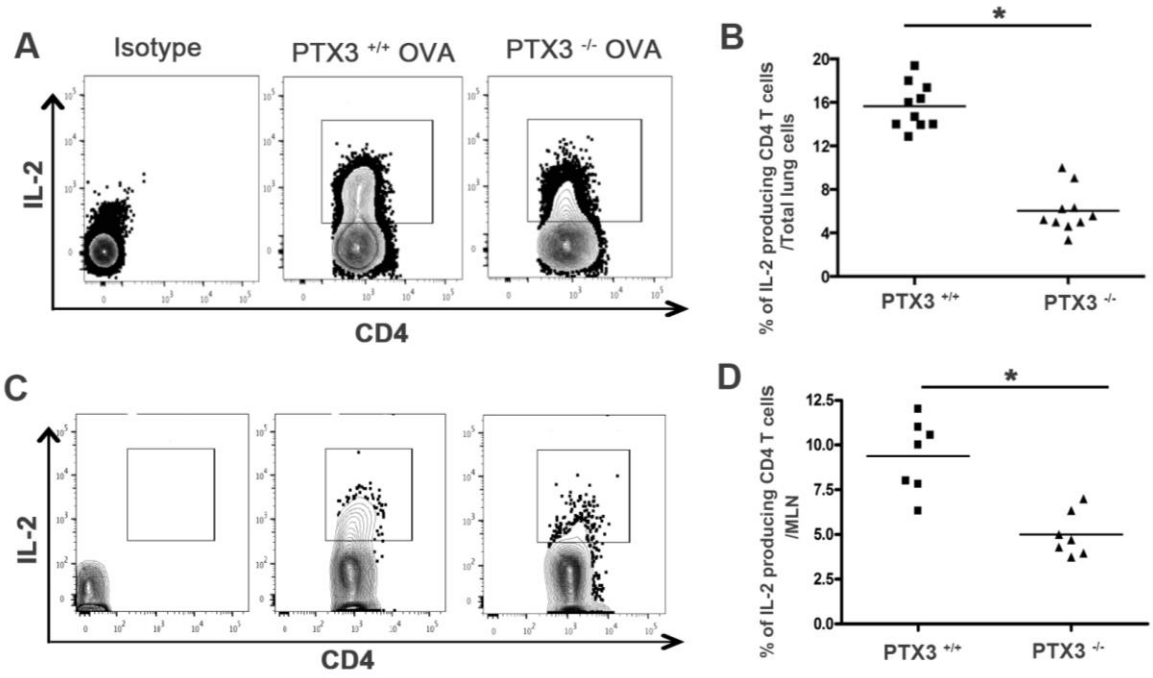

Figure 4.23: *Reduced production of IL-2 by PTX3-/- CD4 T cells. IL-2 production by OVA-exposed lung (A and B) and MLN (C and D) CD4 T cells was determined by flow cytometry. Quantification and statistical analysis of FACS data is shown as graphs. n=5-10/ group, *p<0.01.*

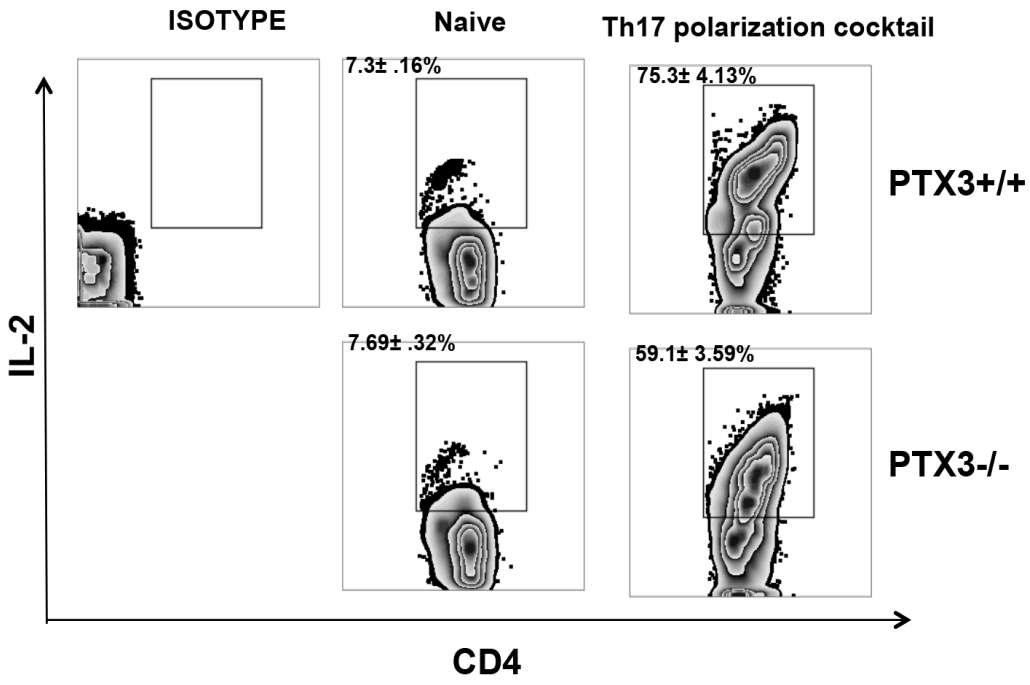

Figure 4.24: *Production of IL-2 by naïve and Th17 polarized PTX3+/+ and PTX3-/- CD4 T cells. Enriched naïve CD4 T cells were cultured in the presence of Th17 polarization cocktail for 5 days and production of IL-2 by PTX3$^{+/+}$ and PTX3$^{-/-}$ CD4 T cells was assessed by flow cytometry. n=5-6/group.*

PTX3-/- mice exhibited increased level of IL-17A producing γδ T cells and innate lymphoid cells type 3

Next I examined other IL-17A producing inflammatory cell types such as γδT cells and ILC3, which might be involved in an amplified production of IL-17A in PTX3-/- mice in response to OVA as compared to their wild type counterparts. IL-17A and IFN-g producing γδT cells that constitute a rare population of T cells [9,10] were observed to be greater in number in the lungs of PTX3-/- mice than PTX3+/+ mice upon OVA exposure (Figure 4.25A-E). This observation was parallel with increase in IL-17A and IFN-γ production by these cells in PTX3-/- mice.

Recently, pulmonary type 3 innate lymphoid cells (ILC3), constituting an extremely small population in the lungs, have been linked to IL-17A production and resulting AHR [14], In allergic PTX3+/+ mice, these cells are approximately 0.1% of total pulmonary cellular pool, which increases more than twice in the PTX3-/- mice under similar conditions (Figure 4.26). Together, these results seem to suggest the contribution of γδ T cells and ILC3 in inducing an IL-17A-dominant response in the lungs of allergic PTX3 KO mice.

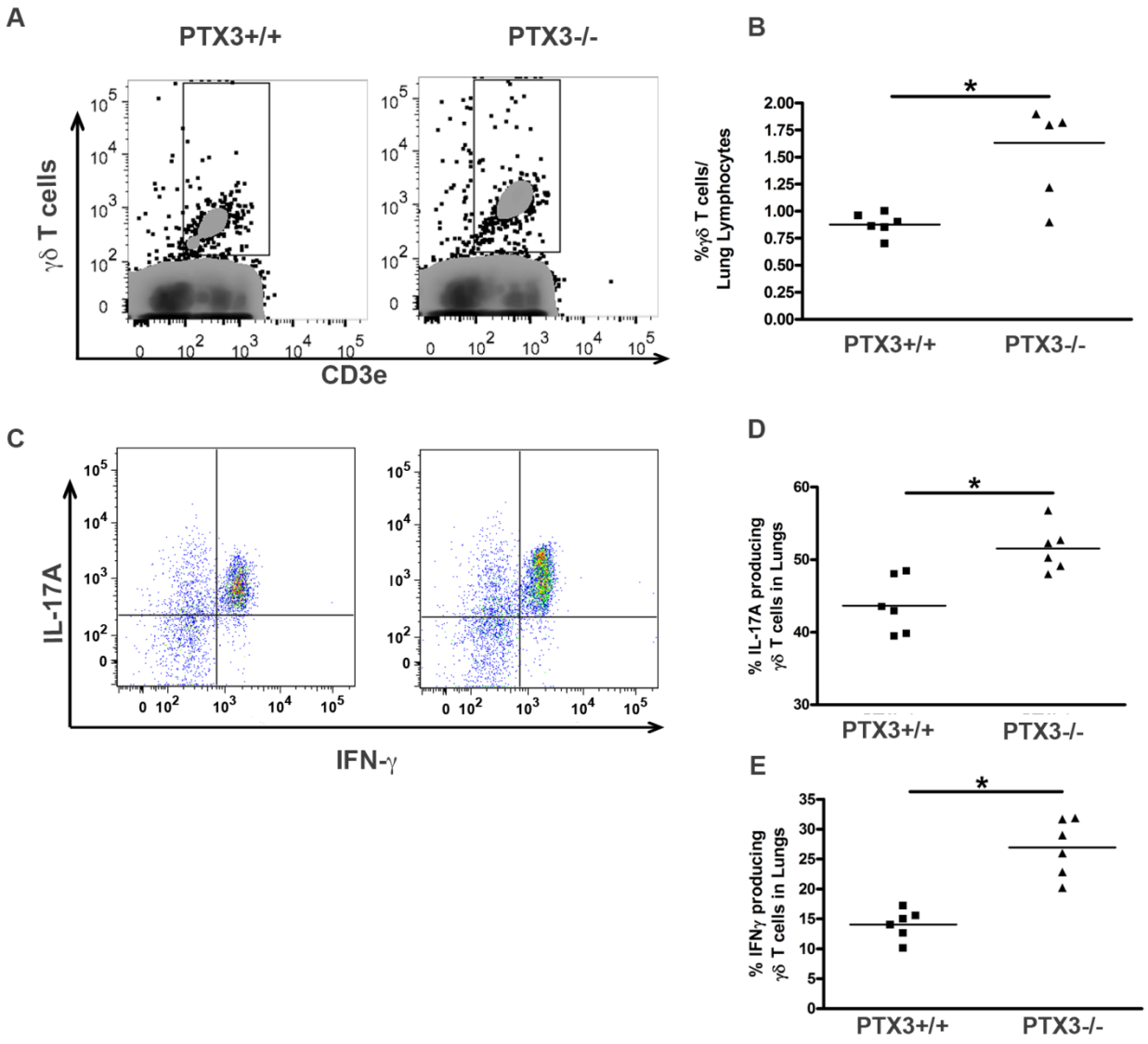

Figure 4.25: *Increased count of γδ-T cells and IL-17A production by them in PTX3-/- mice upon OVA sensitization/ challenge. (A and B) OVA exposed Lung cells were stained for γδ-T cells in PTX3+/+ and PTX3-/- mice. Expression profile of IL-17A and IFN-g by lungs (C-E) γδ T cells in OVA-exposed PTX3$^{+/+}$ and PTX3$^{-/-}$ mice was assessed by flow cytometry. (6 mice/ group) *p<0.01.*

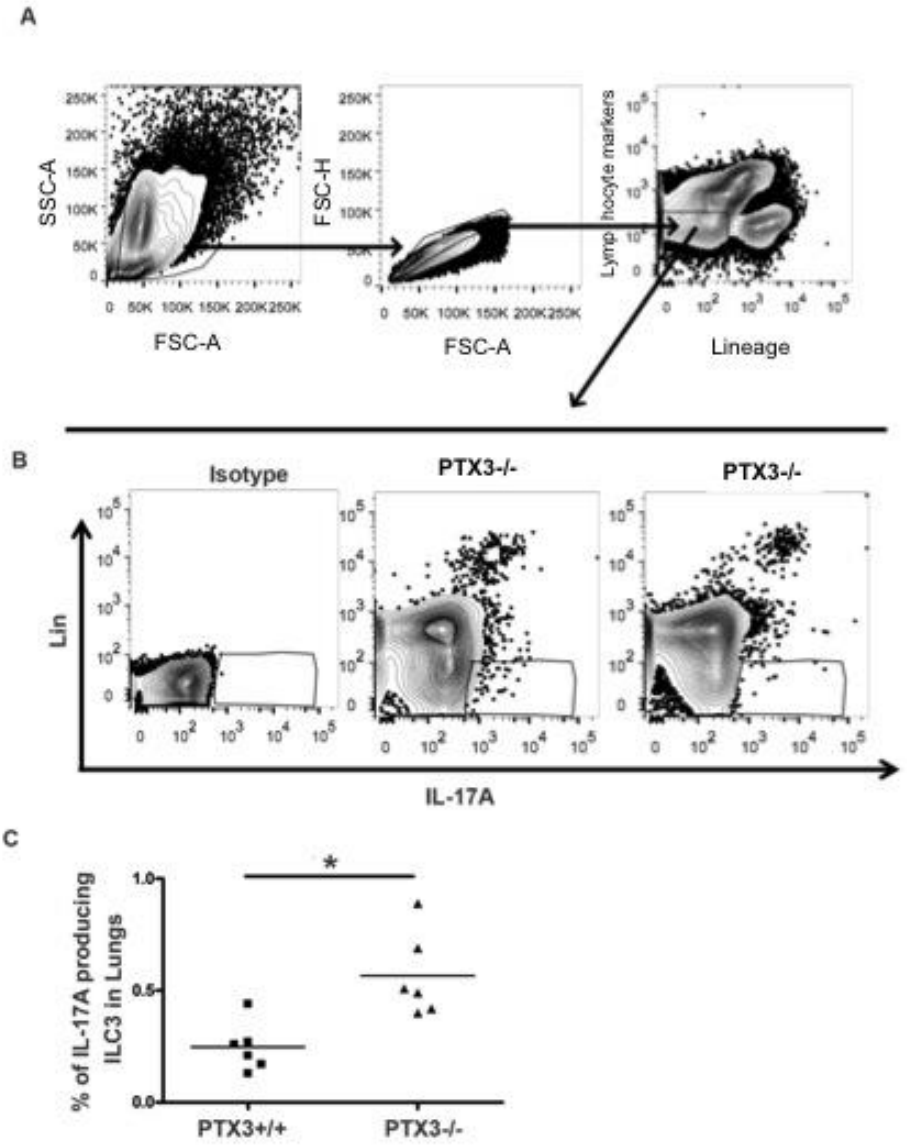

Figure 4.26: *Increase in IL-17A producing ILC3 in OVA-exposed PTX3 deleted mice. OVA exposed Lung cells were stained for IL-17A producing innate lymphoid type 3 cells (ILC3) in PTX3+/+ and PTX3-/- mice as assessed by flow cytometry. (6 mice/ group) *p<0.01.*

Chapter 5: Deletion of PTX3 promoted enhanced accumulation and inflammatory function of $CD11c^{+}CD11b^{+}$ DCs in a murine model of allergic inflammation

Introduction

Allergic asthma is a chronic disease of the airways that is characterized by maladaptive T helper immune response. Several previously published reports have elaborated the crucial role of DCs in the regulation of specific helper T cells phenotype and functions in the lungs in response to allergen. Upon allergen/ antigen exposure, bone marrow release CCR2high monocytes that circulate through the blood, reach to the pulmonary tissue and contributes to the generation of inflammatory monocytes derived CD11c+CD11b+ DCs (98, 146) (166, 167). Airway DCs constitute a network with extended dendrites in the epithelium to capture inhaled antigen or allergen (111, 152-156) (157). DCs are the most potent antigens presenting cells which uptake aeroallergen and migrate to T cell zone in draining lymph nodes (145, 164). During this process, immature DCs undergo maturation that is characterized by upregulation of cell surface expression of MHCII, CD40, CD86 and CD80 (122) (124). DCs process aeroallergens and present the allergen peptides to the naïve CD4 T cells that initiates differentiation of naïve CD4 T cells to specific Th cells (145, 164). Upon repeated exposures of allergen, differentiated Th cells return to the effector site in the lungs and initiate inflammation. During this stage, phenotypically mature DCs that have been shown to co-localize with effector T cells in inflammatory areas, have also been suggested to regulate helper T cell-mediated responses (148, 149). Deletion of DCs at this stage resulted in ablation of Th2-dependent inflammatory responses in mice (146, 147).

Pentraxin 3 is a member of a long pentraxin family of pattern recognition receptors that plays a critical role in tuning inflammation in several pulmonary diseases (401). Several reports have shown production

of PTX3 by structural and inflammatory cells in mice and in humans. Dendritic cells, especially myeloid DCs but not plasmacytoid DCs, have been shown to be among major producer of PTX3. Stimulation with TLR ligands, CD40L, IL-10 and IL-1β induce PTX3 production by DCs (322, 337). PTX3 binds to late apoptotic T cells and inhibits their internalization by human dendritic cells (428). Exogenous administration of PTX3 during early stage of aspergillosis in p47phox-/- mice restored anti-aspergillus resistance. In DCs, PTX3 treatment downregulated IL-23 production that resulted in restricted expansion of IL-23 dependent IL-17A producing γδ T cells (429-432). Whether PTX3 plays a similar role in regulating dendritic cells in allergic inflammation is not known yet.

In the previous chapter, I have shown that deletion of PTX3 resulted in an IL-17A dominant inflammation in mice in response to OVA sensitization. PTX3-/- CD4 T cells and γδ T cells exhibited enhanced IL-17A production. Having known that dendritic cells dictate the differentiation of Th17 CD4 T cells through the production of Th17 polarizing cytokines including IL-6 and IL-23, I sought to understand the effect the PTX3 deletion on the ability of CD11c+CD11b+ DCs in driving IL-17A dominant OVA-induced allergic inflammation in mice. Through my data, I have identified that OVA exposure induced increased infiltration of CD11c+CD11b+ DCs in the lungs in PTX3-/- mice as compared to PTX3+/+ mice. PTX3-/- bone marrow showed increased CMP population that corresponded with increased Ly6C high CCR2high monocytes and CD11c+CD11b+ DCs in the lungs. PTX3-/- pulmonary CD11c+CD11b+ DCs exhibited enhanced expression of maturation markers in OVA-exposed PTX3-/- mice as compared to their wild type controls. These cells also exhibited increased OVA uptake and processing. Furthermore OVA-exposed PTX3-/- mice showed greater IL-6 and IL-23 producing DCs in the lungs and MLN as compared to PTX3+/+ mice. Also, in this chapter, I characterized bone marrow

derived DCs from PTX3+/+ and PTX3-/- mice and found that the phenotype of PTX3-/- BMDCs differs from lung DCs.

Results:

PTX3$^{-/-}$ mice exhibited enhanced accumulation of CD11c^{+}CD11b^{+} DCs in the lungs upon OVA exposure

Recently I showed that deletion of PTX3 resulted in enhanced pulmonary inflammation upon OVA exposure. Given that DCs play a critical role in orchestrating the innate and adaptive immune responses to allergen (433), I hypothesized that PTX3-/- DCs play a key role in mounting a heightened inflammatory response in PTX3$^{-/-}$ mice. Therefore, I evaluated the effect of OVA exposure on lung DCs from PTX3+/+ and PTX3-/- mice *in vivo.* Upon OVA exposure, PTX3$^{-/-}$ mice showed enhanced accumulation of CD11c^{+} DCs in the lungs as compared to PTX3$^{+/+}$ mice (Figure 5.1A-C).

CD11c DCs are further characterized as proinflammatory CD11b$^{+/high}$ pulmonary DCs, which transport antigen from lungs to the lymphoid organs (434, 435). In PTX3$^{-/-}$ mice, I observed a concomitant increase in lung CD11c^{+}CD11b^{+} DCs upon OVA exposure (Figure 5.1D and E).

Next I determined plausible mechanism, supporting increased abundance of lung DCs in PTX3$^{-/-}$ mice. PTX3$^{-/-}$ CD4 T cells showed enhanced survival as compared to PTX3$^{+/+}$ CD4 T cells (figure 4.20 and 4.21). Whether similar phenomenon existed for DCs in PTX3$^{-/-}$ mice, thus contributing in their enhanced abundance in the lungs is not known. Therefore I compared survival of PTX3$^{-/-}$ DCs and PTX3$^{+/+}$ DCs by staining cells with annexin V and DAPI. OVA–exposed PTX3$^{+/+}$ and PTX3$^{-/-}$ lung DCs showed comparable survival (Figure 5.2).

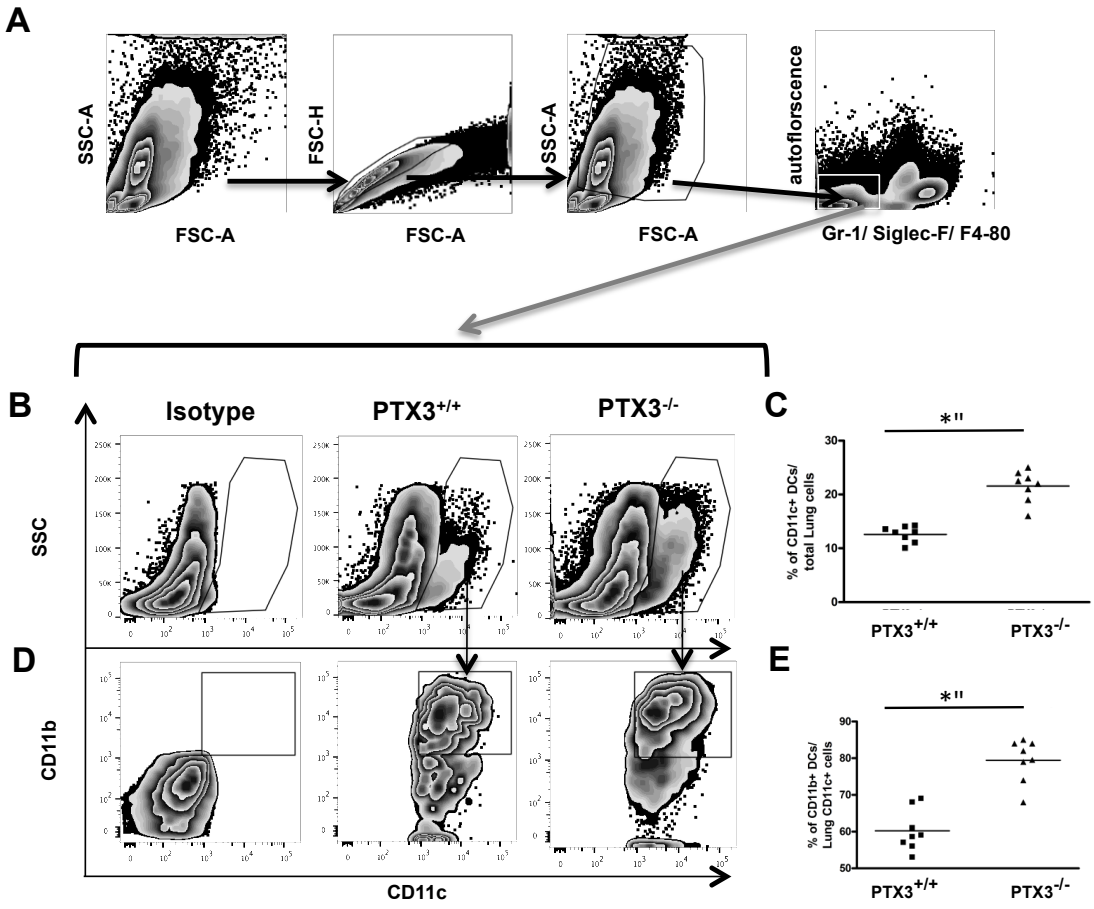

Figure 5.1: *PTX3 deletion results in increased accumulation of CD11c+CD11b+ DCs in the lungs in response to OVA sensitization and challenge. (A) Lung Cells were gated for autoflorescence- Gr-1-Siglec F-F4/80- CD11c+ cells (B and C) that were then gated for CD11b+ cells (D and E). Quantification and statistical analysis of FACS data is shown as graphs. n=6-8/group, *p<0.01.*

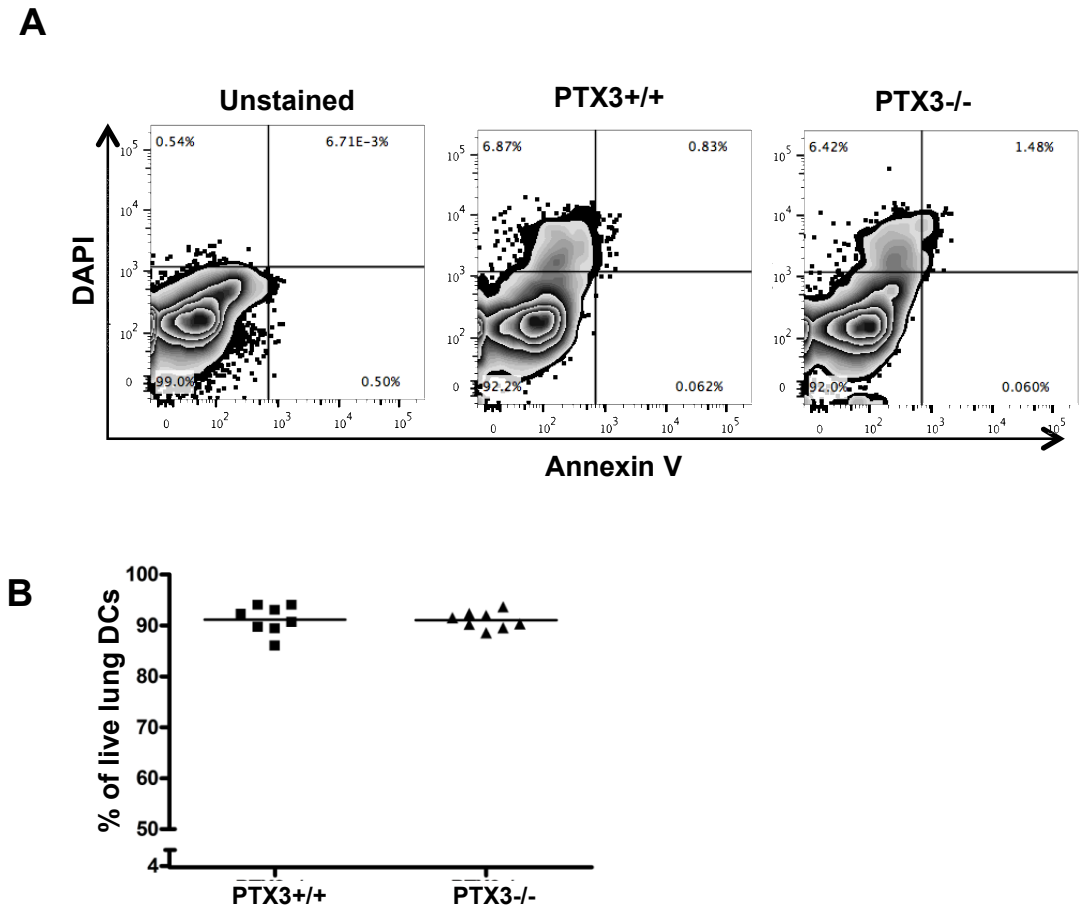

Figure 5.2: *PTX3+/+ and PTX3$^{-/-}$ lung DCS show comparable survival. (A) Survival of lung DCs (CD11c+CD11b+) was assessed by annexin V and DAPI staining. % of gated cells is presented as mean+/- SEM in graph (B), n=8-10/ group.*

PTX3$^{-/-}$ mice showed enhanced CMP generation in bone marrow

According to previously published reports, it is evident that common myeloid progenitors (CMP) in the bone marrow efficiently give rise to Ly-6C^{high}CD11b^{+} inflammatory monocytes (436). Inflammatory monocytes emigrate from the bone marrow, circulate through the blood and rapidly differentiate into CD11c^{+}CD11b^{+} DCs in the peripheral tissue under inflammatory conditions (437). Therefore next I aimed to understand whether augmented pulmonary accumulation of inflammatory DCs in PTX3$^{-/-}$ mice was due to enhanced generation of CMPs, increased recruitment of Ly-6C^{high}CD11b^{+} monocytes and differentiation of the latter into inflammatory DCs in the lungs. PTX3$^{+/+}$ and PTX3$^{-/-}$ mice were challenged with 100μg/ml OVA. First I assessed the frequency of CD11b+CD11c+ DCs in the lungs 12 hrs and 24 hrs post-OVA challenge. As observed in 3 weeks OVA sensitization and challenge, short-term OVA challenges also resulted in increased accumulation of inflammatory DCs in PTX3-/- mice as compared to PTX3+/+ mice (Figure 5.3A and B).

Thereafter I employed a retrospective approach to determine how OVA challenge affected monocytic lineage progenitors in bone marrow in the presence and absence of PTX3. In PTX3$^{+/+}$ mice, CMP count in the bone marrow was unaffected by OVA challenge. However, there was significant increase in CMP count upon OVA exposure in PTX3$^{-/-}$ mice (Figure 5.4). Also, CMP population in PTX3$^{-/-}$ mice was greater than PTX3$^{+/+}$ mice in OVA-exposed

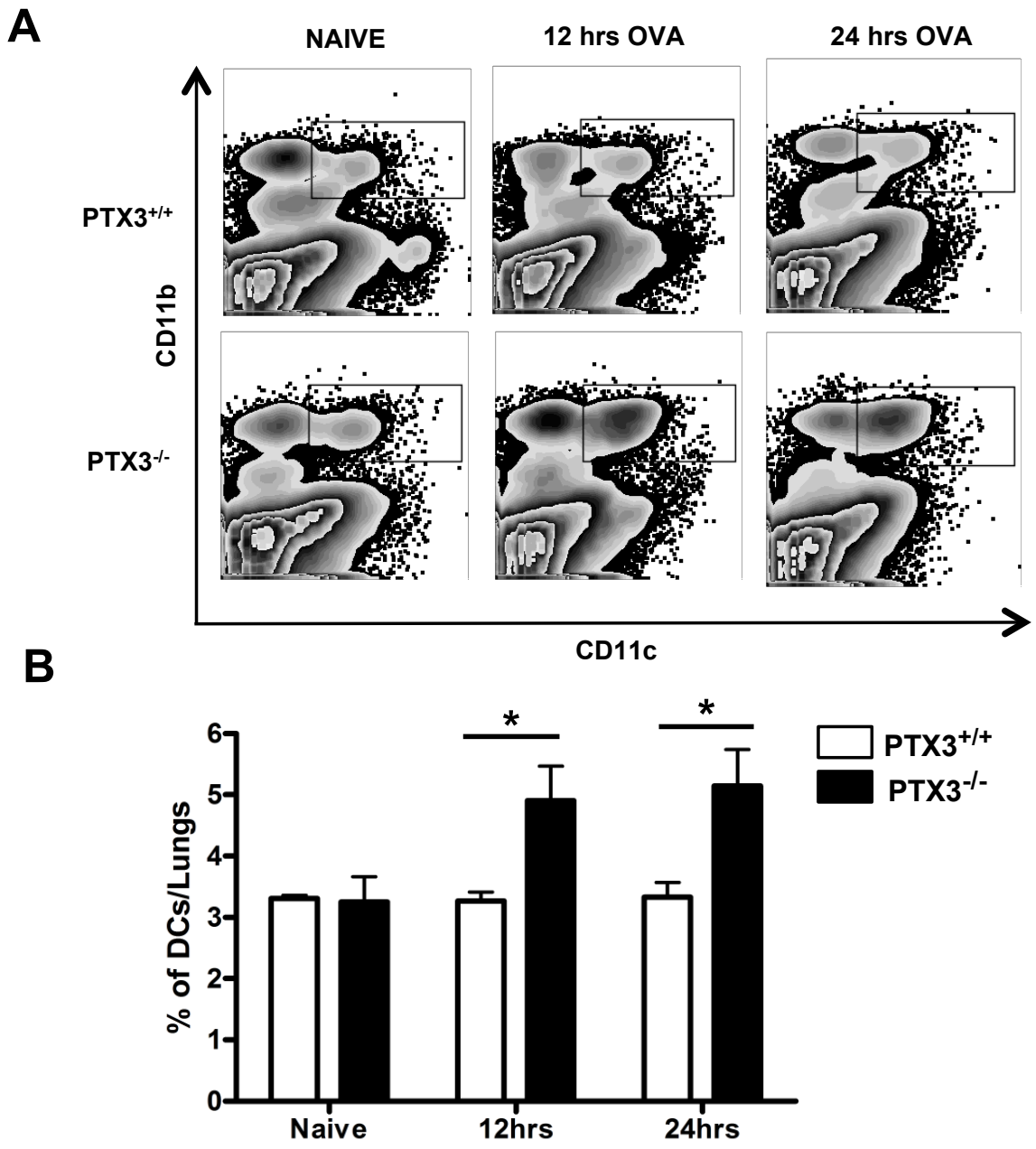

***Figure 5.3**: PTX3-/- mice showed increased accumulation of DCs in lungs. (A) Accumulation of lung DCs 12 hrs and 24 hrs post i.n OVA exposure was compared with naïve lung DCs. (B) Quantification and statistical analysis of Flow cytometry data is shown as graphs. n=6-8/group, *p<0.01.*

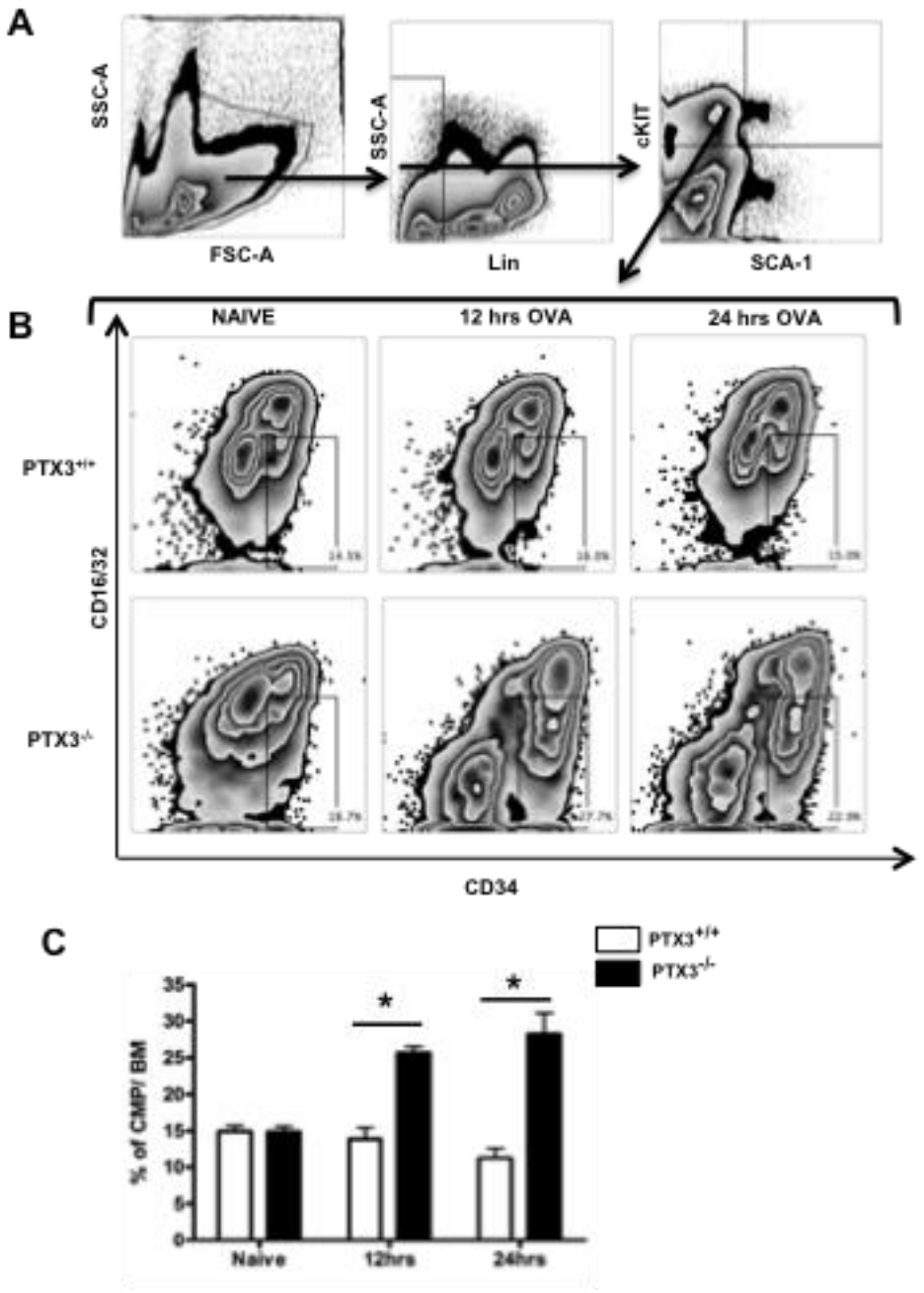

Figure 5.4: *OVA exposure results in increased CMP progenitors in PTX3-/- mice. (A) Lin-cKit+Sca-1- cells were gated for CMP. (B) CMP cells from naïve, 12 hrs and 24 hrs post i.n OVA exposed PTX3-/- mice were compared with CMP cells from PTX3+/+ mice. (C) Quantification and statistical analysis of Flow cytometry data is shown as graphs. n=6/ group, *p<0.01.*

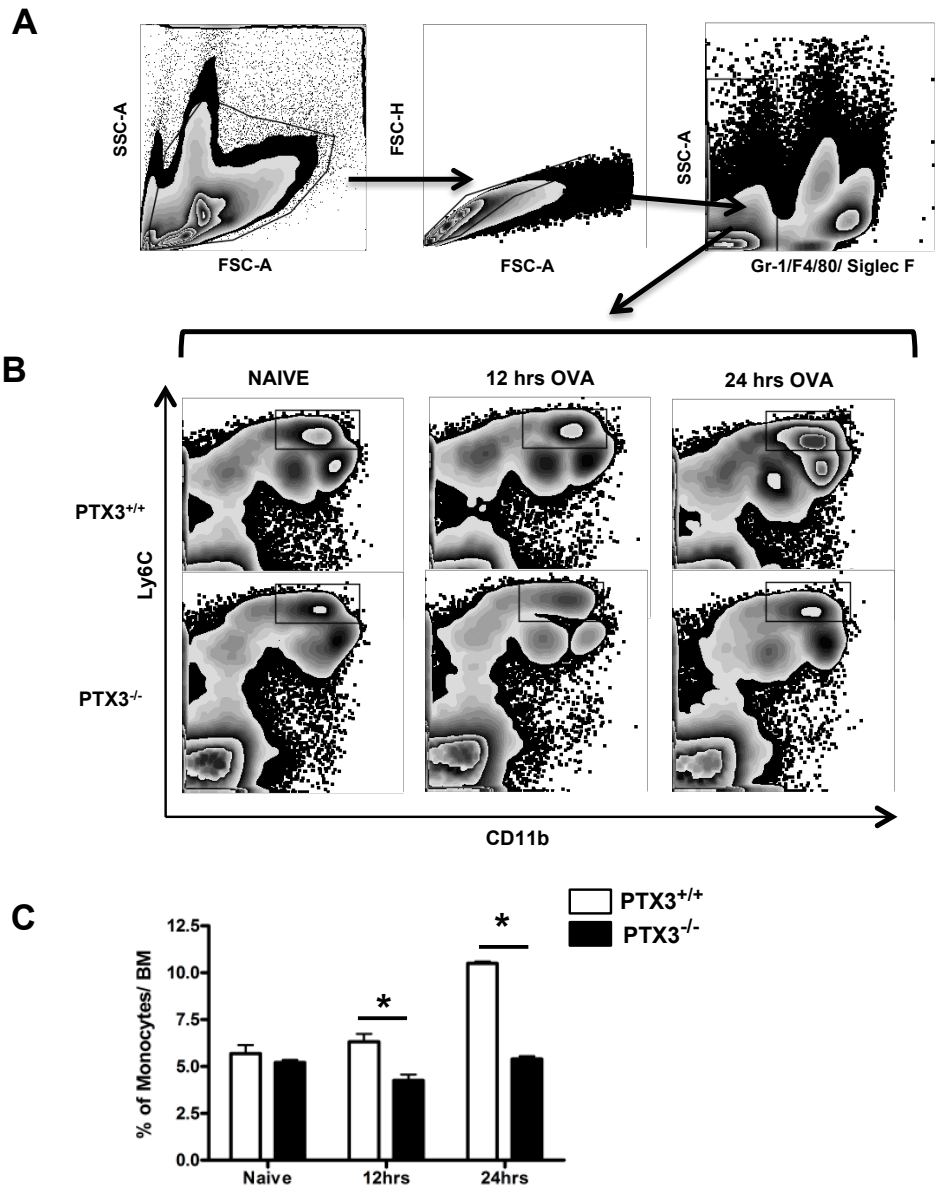

Figure 5.5: *OVA exposure results in increased egress of monocytes from the bone marrow in PTX3-/- mice. (A) shows gating strategy for Ly6C+ CD11b+ monocytes in bone marrow. (B) BM monocytes from naïve, 12 hrs and 24 hrs post i.n OVA exposed PTX3-/- mice were compared with monocytes from PTX3+/+ mice. (C) Quantification and statistical analysis of Flow cytometry data is shown as graphs. n=6/ group, *p<0.01.*

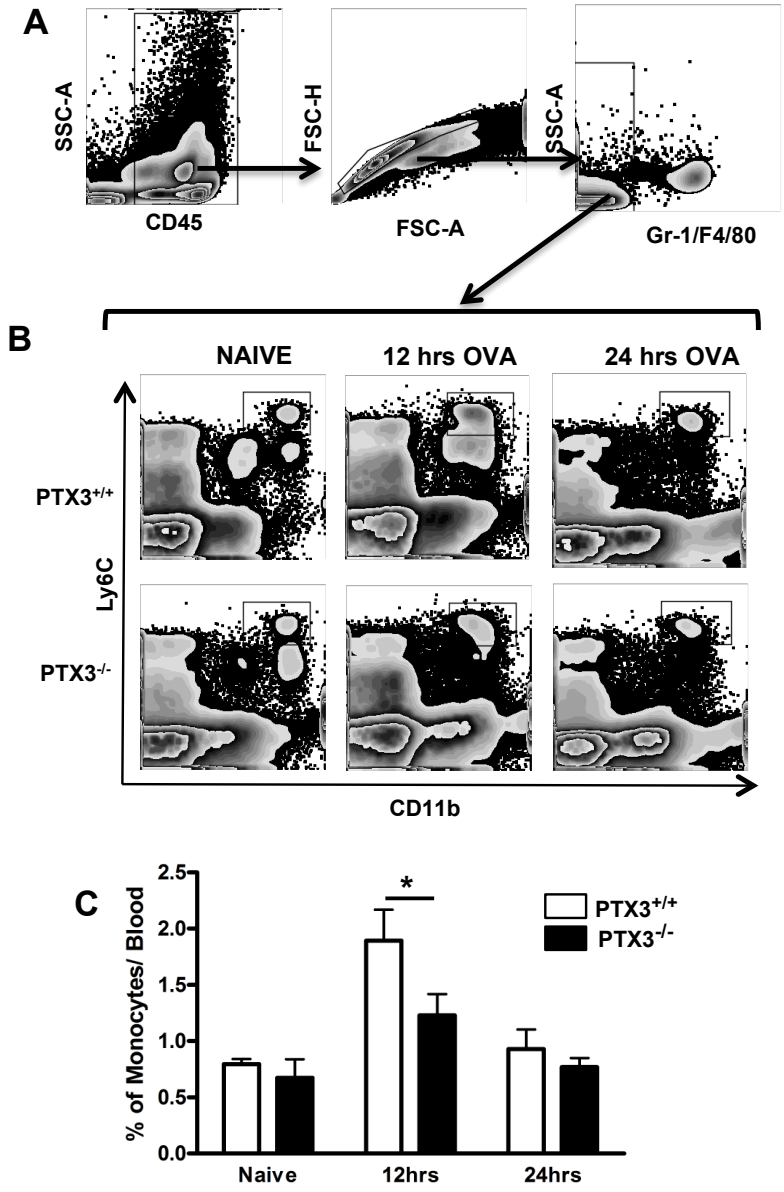

Figure 5.6: *PTX3-/- mice showed increased egress of monocytes from the blood upon OVA exposure. (A) shows gating strategy for Ly6C+ CD11b+ monocytes in blood. (B) Blood monocytes from naïve, 12 hrs and 24 hrs post i.n OVA exposed PTX3-/- mice were compared with monocytes from PTX3+/+ mice. (C) Quantification and statistical analysis of Flow cytometry data is shown as graphs. n=6/ group, *p<0.01.*

condition (Figure 5.4), plausibly suggesting that PTX3-/- mice were more sensitive to antigen exposure, therefore responded more robustly to OVA challenge resulting in enhanced generation of CMPs so as to support enhanced accumulation of DCs in the lungs. I also observed augmented reduction of BM monocytes in $PTX3^{-/-}$ mice (Figure 5.5). In Blood, although monocytes count increased 12 hrs post OVA challenge in both genotypes, their count was reduced in $PTX3^{-/-}$ mice as compared to $PTX3^{+/+}$ mice (Figure 5.6), possibly indicating increased exit of these cells from BM and blood in $PTX3^{-/-}$ mice as compared to $PTX3^{+/+}$ mice.

Concurrent with this, I found increased monocytes in the lungs of PTX3-/- mice upon OVA challenge in contrast to $PTX3^{+/+}$ mice (Figure 5.7), serving as a possible mechanism to facilitate enhanced accumulation of DCs in the lungs. Altogether I observed enhanced generation of DC progenitors in bone marrow, increased egress of monocytes from the bone marrow to peripheral blood and from blood to the lungs in PTX3 deleted mice, possibly resulting in increased accumulation of inflammatory DCs in $PTX3^{-/-}$ mice.

PTX3-/- lung DCs exhibited increased expression of CCR2, CC5 and CCR6

Previously *Norata et al (438),* showed upregulation of CCR2 upon PTX3 deletion in the aorta of ApoE-/- mice. Ly-$6C^{high}CD11b^{+}$ inflammatory monocytes express high level of CCR2 as compared to Ly-$6C^{low/int}CD11b^{+}$ patrolling monocytes (439). CCR2 plays key role in directing DC precursors to egress from bone marrow to blood, enabling their migration to the tissue sites upon antigen exposure (440). It has also been shown to be associated with increased differentiation of Ly-$6C^{high}CD11b^{+}$ monocytes into pulmonary $CD11c^{+}CD11b^{+}$ DCs (441). Therefore next I sought to understand the regulation of CCR2 expression in OVA- challenged mice upon PTX3 deletion. Also, I was interested in determining the role of regulation of

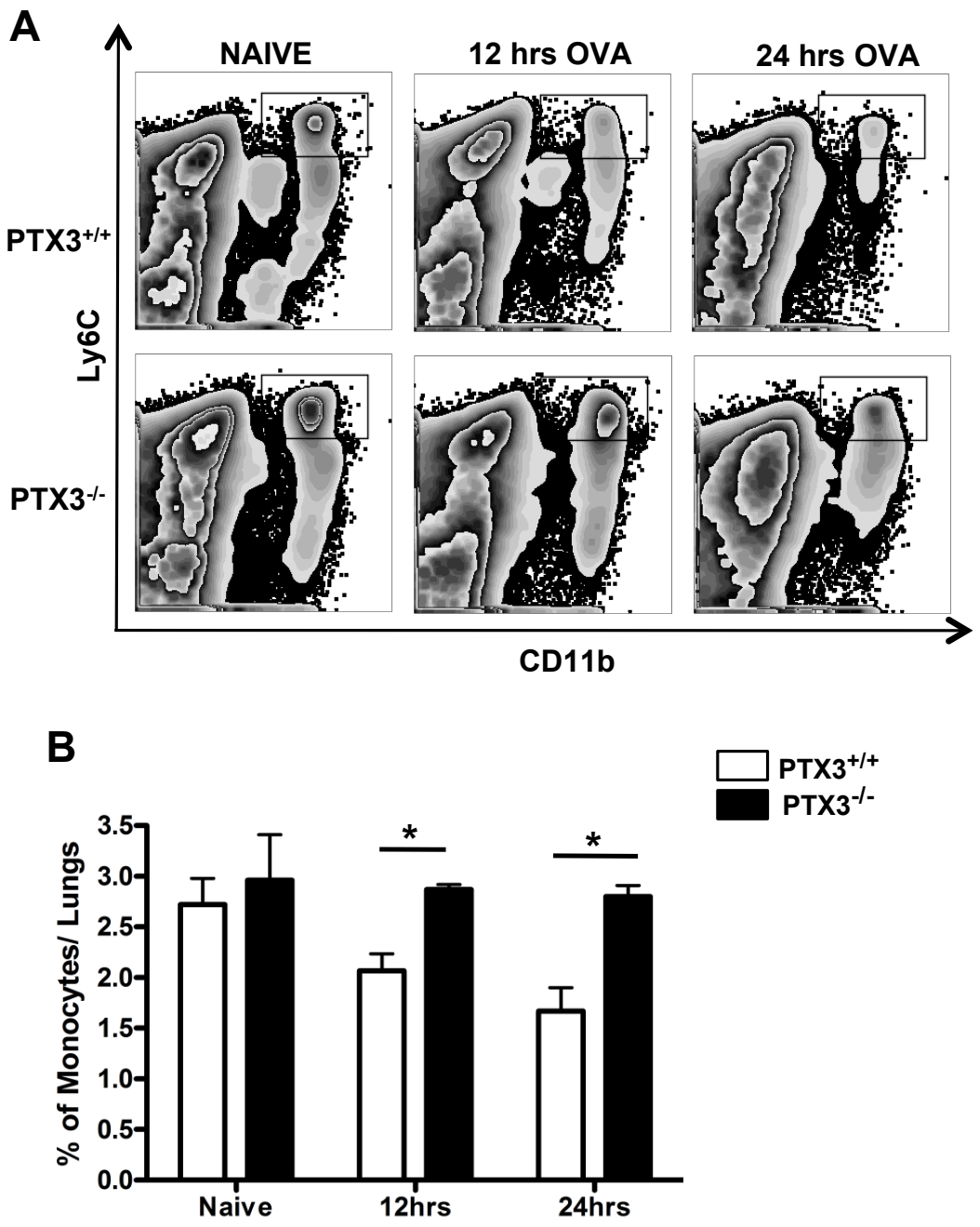

Figure 5.7: *PTX3-/- mice showed increased accumulation of monocytes in the lungs upon OVA exposure. (A) Lung monocytes from naïve, 12 hrs and 24 hrs post i.n OVA exposed PTX3-/- mice were compared with monocytes from PTX3+/+ mice. (C) Quantification and statistical analysis of Flow cytometry data is shown as graphs. n=6/ group, *p<0.01.*

CCR2 expression in increased inflammatory DC accumulation in the lungs upon OVA exposure. Although BM (Figure 5.8A and B) and blood (Figure 5.8 C and D) monocytes showed an increasing trend in CCR2 surface expression in PTX3-/- and PTX3+/+ mice at the naïve and OVA-exposed condition, there was no significant difference in the expression of CCR2 in both genotypes. Similar observation was made in lung monocytes from PTX3-/- and PTX3+/+ mice (Figure 5.8E and F). Interestingly, lung DCs showed increased expression at naïve state that was further enhanced 12 hrs post OVA exposure in $PTX3^{-/-}$ mice than $PTX3^{+/+}$ mice (Figure 5.8G and H). 24 hrs after OVA challenge, although surface expression of CCR2 was increased in both genotypes as compared to lung DCs at naïve state and 12 hrs post OVA challenge, there was not substantial difference between PTX3-/- and PTX3+/+ DCs at 24 hrs (Figure 5.8E and F).

Norata et al (438) also demonstrated upregulation of CX3CR1 and CCR5 in the vascular wall of PTX3 deleted apoE-/- mice as compared to wildtype controls. CX3CR1 is a chemokine receptor that reduces motility of inflammatory monocytes in the bone marrow, thereby regulating their retention. CX3CR1 is involved in monocytes patrolling in the blood vessel lumen (442). Also, it has been found to direct inflammatory monocytes to the site of inflammation. In my study, expression of CX3CR1 was comparable in monocytes from BM (Figure 5.9A and B), lung (Figure 5.9E and F) and lung DCs (Figure 5.9G and H) in PTX3-/- and PTX3+/+ mice at naïve state and upon OVA exposure. However, PTX3-/- blood monocytes showed increase in CX3CR1 expression 12 hrs post OVA exposure (Figure 5.9C and D) with comparable expression at 24 hrs.

CCR5 is expressed by immature DCs that facilitate migration of later to tissue sites. CCR6 is also expressed by immature DCs and performs similar function as CCR5. I evaluated expression of both chemokine receptors on lung DCs and found that the expression of CCR5 (Figure 5.10G and H) and CCR6 ((Figure 5.11G and H)) was greater on PTX3-/- DCs at

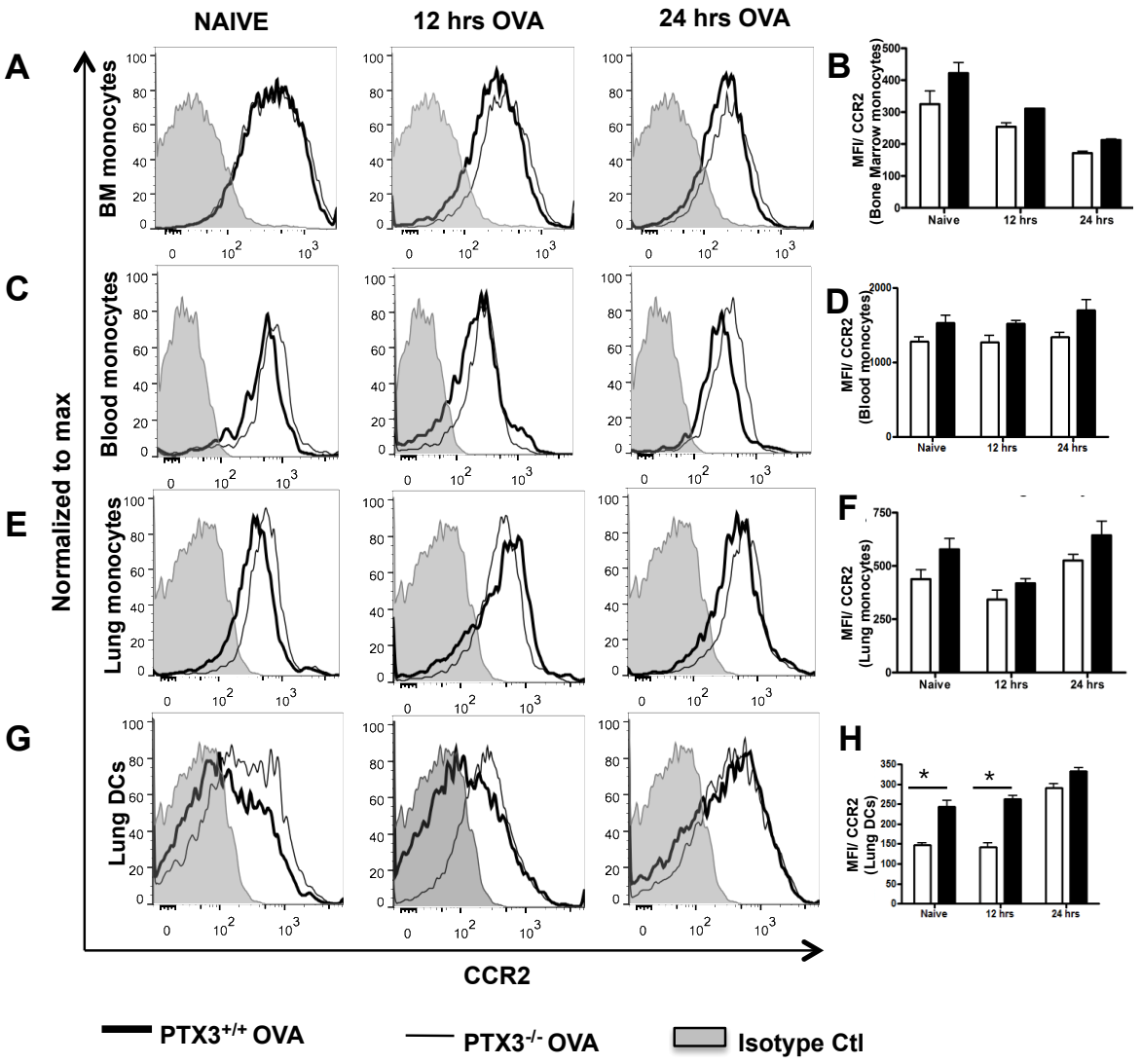

Figure 5.8: *PTX3 deletion affect CCR2 expression on lungs but not on monocytes. Expression of CCR2 was assessed by flow cytometry on monocytes from bone marrow (A and B), blood (C and D), lungs (E and F) and DCs from the lungs (G and H). (B, D, F and H) White bars represent PTX3+/+ and black bars represent PTX3-/-. Quantification and statistical analysis of Flow cytometry data is shown as graphs. n=6/ group, *p<0.01.*

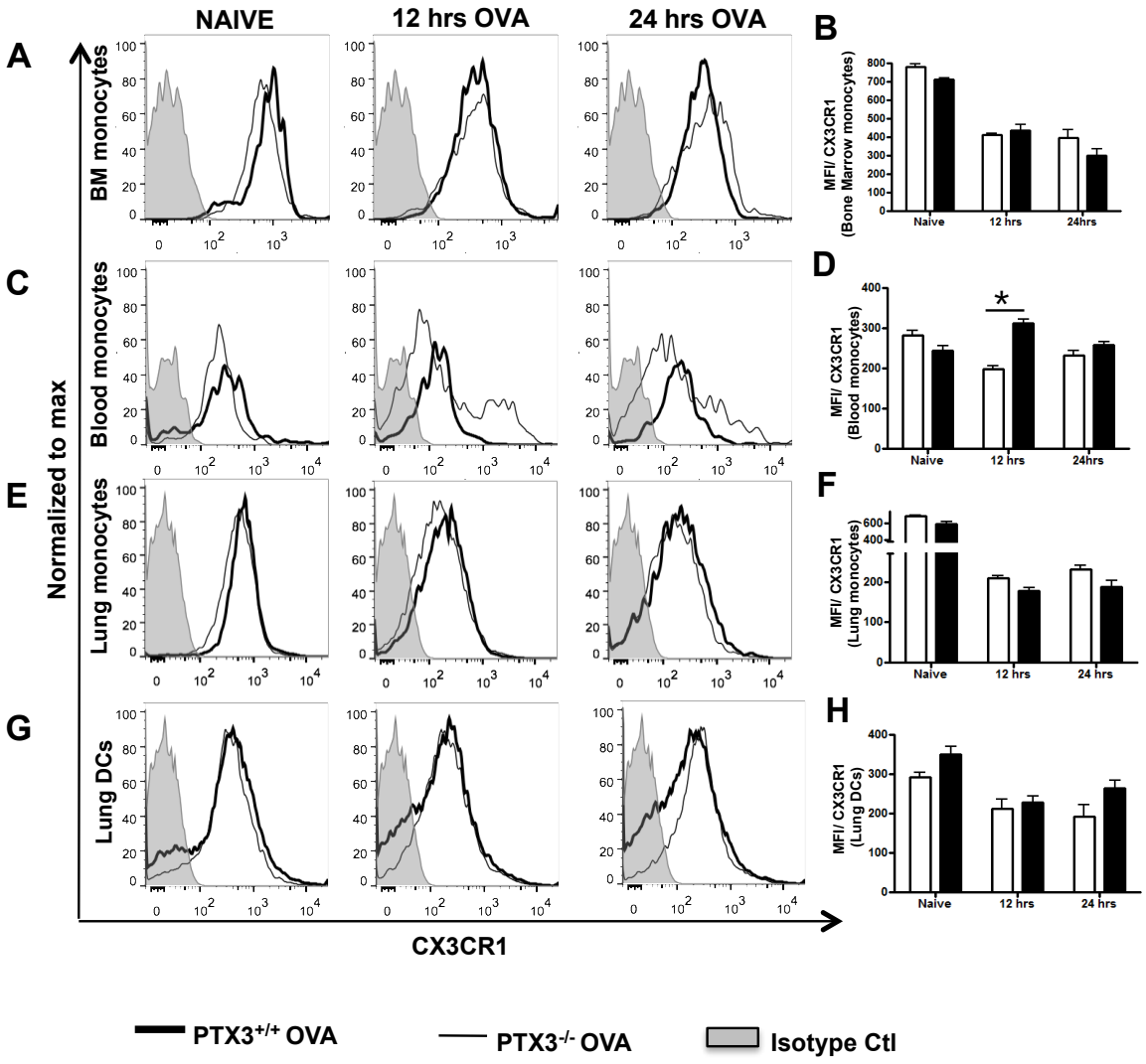

Figure 5.9: *PTX3 deletion does not affect CX3CR1 expression on monocytes. Expression of CX3CR1 was assessed by flow cytometry on monocytes from bone marrow (A and B), blood (C and D), lungs (E and F) and DCs from the lungs (G and H). (B, D, F and H) White bars represent PTX3+/+ and black bars represent PTX3-/-. Quantification and statistical analysis of Flow cytometry data is shown as graph. n=6/ group, *p<0.01.*

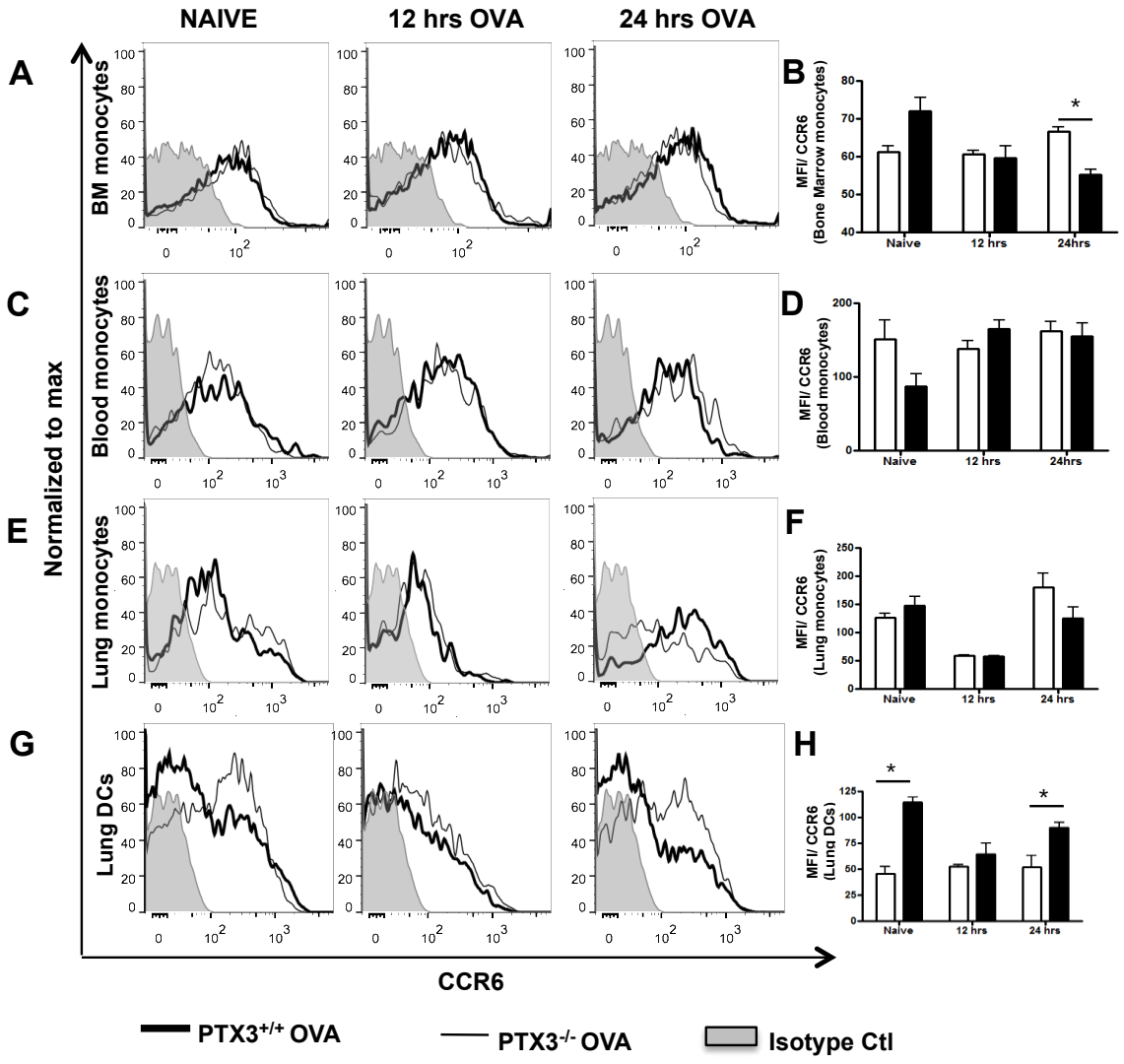

Figure 5.10: *PTX3 deletion does not affect CCR6 expression on monocytes. Expression of CCR6 was assessed by flow cytometry on monocytes from bone marrow (A and B), blood (C and D), lungs (E and F) and DCs from the lungs (G and H). (B, D, F and H) White bars represent PTX3+/+ and black bars represent PTX3-/-. Quantification and statistical analysis of Flow cytometry data is shown as graphs. n=6/ group, *p<0.01.*

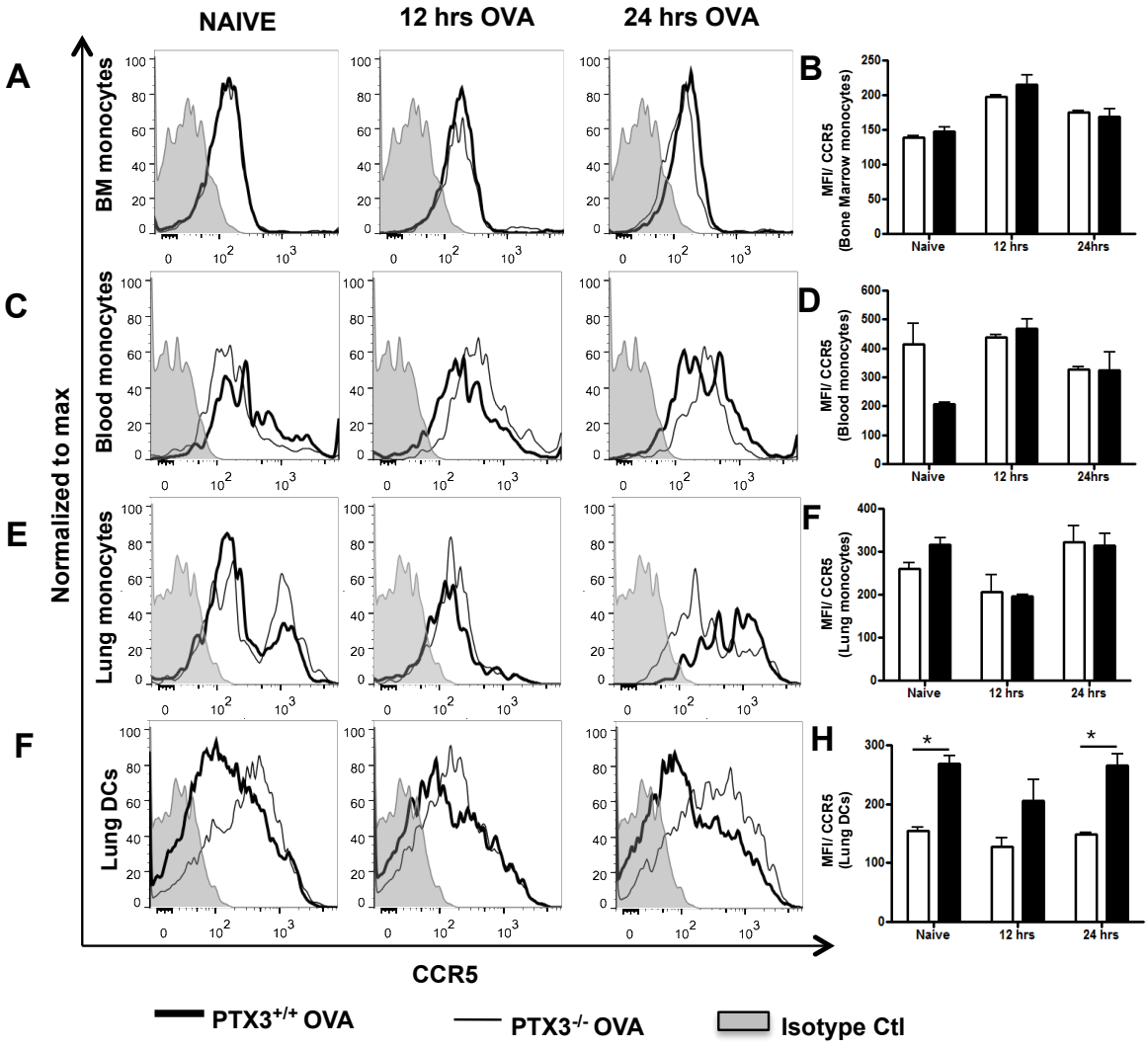

Figure 5.11: *PTX3 deletion does not affect CCR5 expression on monocytes. Expression of CCR5 was assessed by flow cytometry on monocytes from bone marrow (A and B), blood (C and D), lungs (E and F) and DCs from the lungs (G and H). (B, D, F and H) White bars represent PTX3+/+ and black bars represent PTX3-/-. Quantification and statistical analysis of Flow cytometry data is shown as graphs. n=6/ group, *p<0.01.*

naïve state as compared to PTX3+/+ DCs. 12 hrs post OVA challenge, the expression of these receptors was comparable in both genotypes. However, 24 hrs post OVA challenge, lung DCs exhibited increased surface expression of both the receptors as compared to PTX3+/+ lung DCs. No apparent difference in their expression was found on monocytes from BM, blood and the lungs (Figure 5.10A-F and Figure 5.11A-F). Altogether my data suggest increased expression of CCR2, CCR5 and CCR6 on lung DCs and CX3CR1 on blood monocytes upon PTX3 deletion, plausibly suggesting their involvement in enhanced monocytes derived DCs accumulation in PTX3-/- mice as compared to PTX+/+ mice upon OVA challenge.

PTX3$^{-/-}$ lung DCs showed reduced MHCII but enhanced CD86 surface expression

In OVA-exposed PTX3-/- mice, CD4 T cells exhibited enhanced activation and IL-17A dominant cytokine production. Given that DCs process and present antigens to T cells and regulate T cell-dependent inflammatory response through expression of costimulatory molecules such as MHCII, CD80, CD86 and CD40, I next sought to understand whether PTX3 deletion resulted in differential maturation status of DCs, which may affect phenotype and functions of CD4 T cells in an experimental asthma model. Expression of costimulatory markers (Figure 5.12A) was comparable in naïve condition in PTX3$^{+/+}$ and PTX3$^{-/-}$ mice. First, I examined expression of costimulatory molecules on the surface of CD11c^{+}CD11b^{+} DCs in PTX3$^{-/-}$ and PTX3$^{+/+}$ condition after short-term OVA challenges. Lung DCs isolated 12 hrs and 24 hrs post OVA challenge showed an increase in the expression of CD80 (Figure 5.12B, D) and CD86 (Figure 5.12B and 13C). MHC II (Figure 5.12B and C; 5.13A) and CD40 (Figure 5.12 B and C; 13B) surface expression were similar in DCs isolated from OVA challenged (12 hrs and 24 hrs) PTX3+/+ and PTX3-/- mice. 48 hrs post OVA challenge (according to OVA

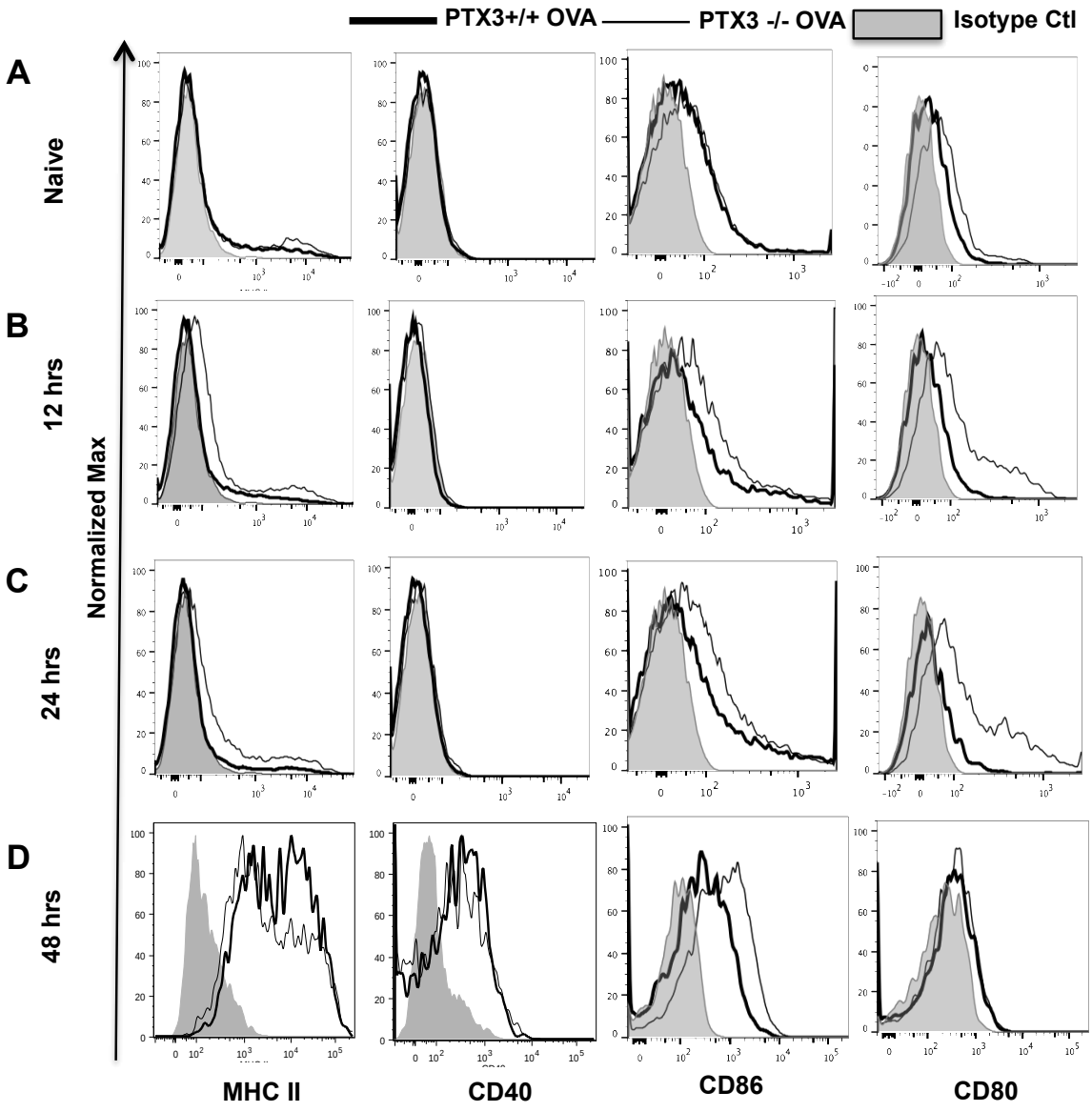

Figure 5.12: *PTX3 deletion results in reduced surface expression of MHCII but enhanced surface expression of CD80 and CD86 in response to OVA. White bars represent PTX3+/+ and black bars represent PTX3-/-. n=6/ group*

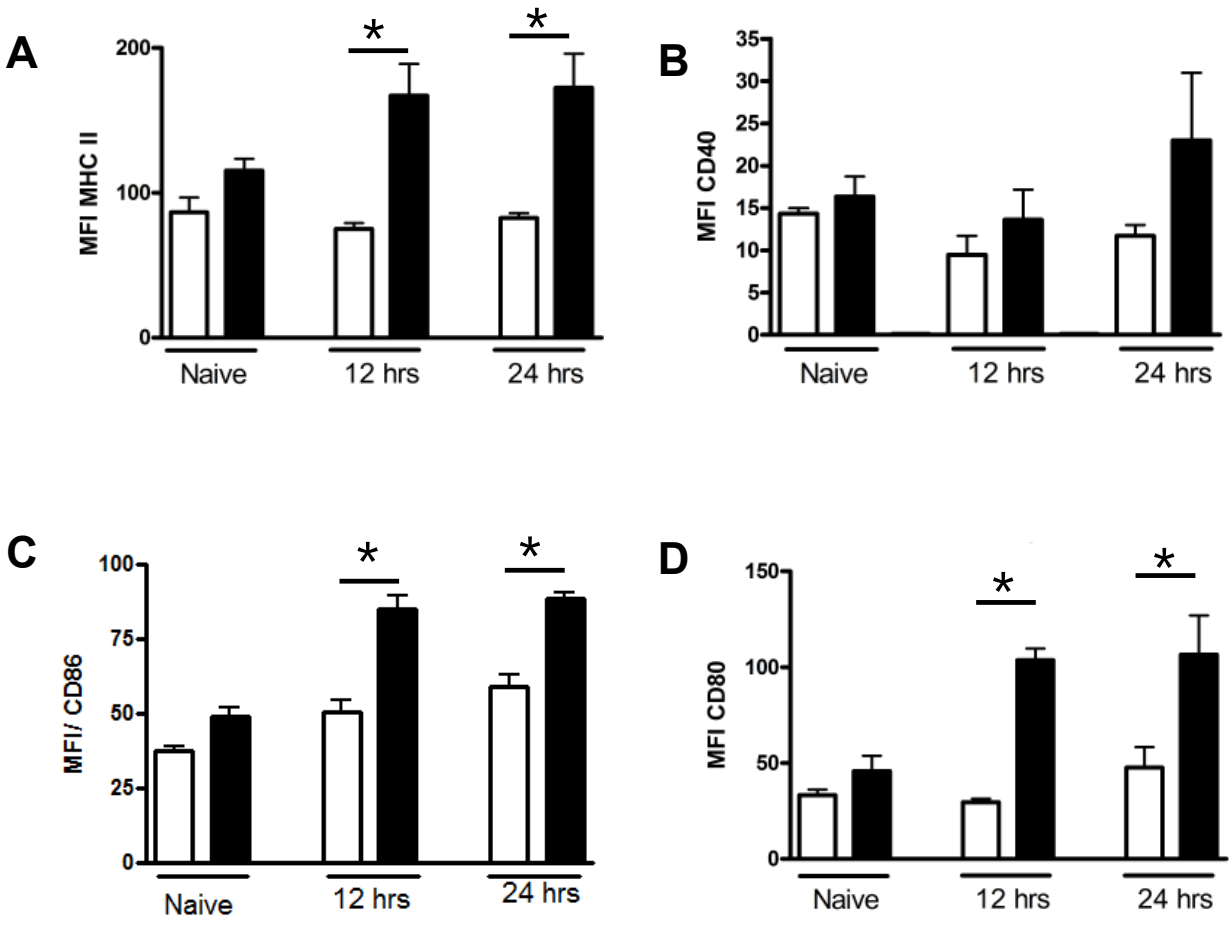

Figure 5.13: *Quantification and statistical analysis of the expression of MHCII (A), CD40 (B), CD86 (C) and CD80 (D) at naïve state, 12 hrs and 24 hrs after single i.n OVA challenge.White bars represent PTX3+/+ and black bars represent PTX3-/- . n=6/ group, *p<0.01.*

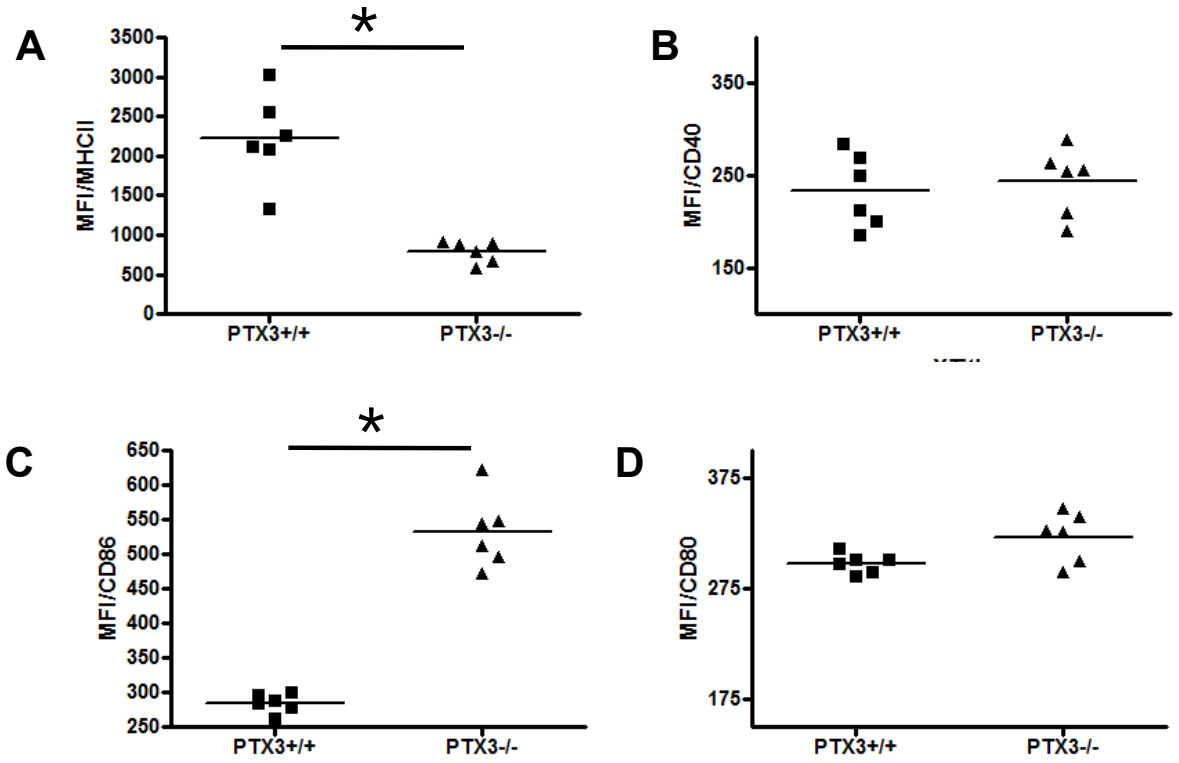

Figure 5.14: *(D) Quantification and statistical analysis of the expression of MHCII (A), CD40 (B), CD86 (C) and CD80 (D) determined 48 hrs after last challenge according to scheme shown in figure 1a. . n=6/ group, *p<0.01.*

sensitization/challenge regimen as shown in figure 1A (Chapter 4)), $PTX3^{-/-}$ lung DCs showed lower surface expression of MHC II (Figure 5.12D, 14A) but enhanced expression of CD86 (Figure 5.12D and 14C). Surface expression of CD40 (Figure 5.12D and 14B) and CD80 (Figure 5.12D and 13D) remained similar in both genotypes at this time point. Altogether it suggests that PTX3 deletion results in downregulation of MHCII but increase in the expression of CD80 and CD86 upon OVA exposure.

$PTX3^{+/+}$ lung $CD11c^{+}CD11b^{+}$ DCs exhibit enhanced OVA uptake and processing as compared to $PTX3^{+/+}$ DCs

DCs take up allergen/antigen, process them into immunogenic peptides for subsequent presentation to T cells (443) . In order to study whether PTX3 deletion affected OVA uptake and presentation by lung DCs, we used DQ-OVA *in vivo*. DQ-OVA is digested proteolytically to release photostable and pH-insensitive green florescent peptides by antigen presenting cells (444). Number of DCs, which processed OVA, was higher in $PTX3^{-/-}$ mice lungs as compared to $PTX3^{+/+}$ mice (Figure 5.15A, D and E). Similar to total lung DCs, subset of $CD11c^{+}CD11b^{+}$ DCs that were actively processing OVA showed reduced MHC II (Figure 5.15B, F and G) and increased CD86 (Figure 5.15C, H and I) surface expression in $PTX3^{-/-}$ mice.

Lung DCs transport allergen to mediastinal lymph nodes and present allergen peptides to T cells to generate a specific effector T cell response (443). Therefore I then explored the migration of DQ-OVA containing lung DCs to MLN. $PTX3^{-/-}$ mice showed greater infiltration of processed DQ-OVA containing DCs into the MLN (Figure 5.16A). DCs in MLN also showed reduced MHCII (Figure 5.16B), however, CD40, CD86, and CD80 expression remained similar (Figure 5.16C-E).

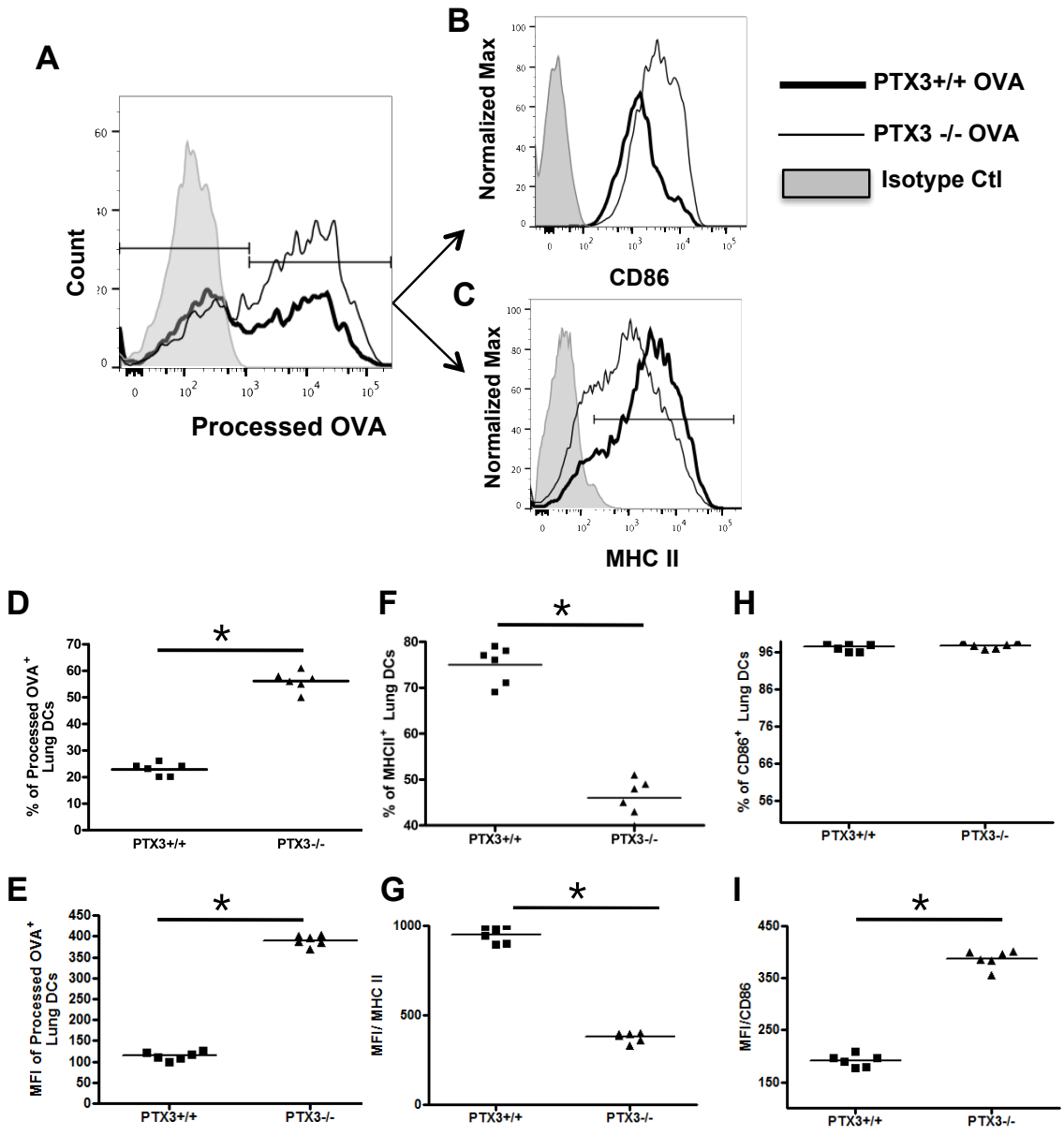

Figure 5.15: *PTX3-/- lung DCs showed enhanced OVA uptake and processing. DQ-OVA was administered intranasally to PTX3-/- and PTX3+/+ mice and lung DCs were assessed for processed OVA signal (FITC) 16 hrs post administration (A, D and E). Surface expression of CD86 (B, H and I) and MHCII (C, F and G) was determined on processed OVA+ lung DCs. Quantification and statistical analysis of Flow cytometry data is shown as graphs (D-I). n=6-8 / group, *p<0.01*

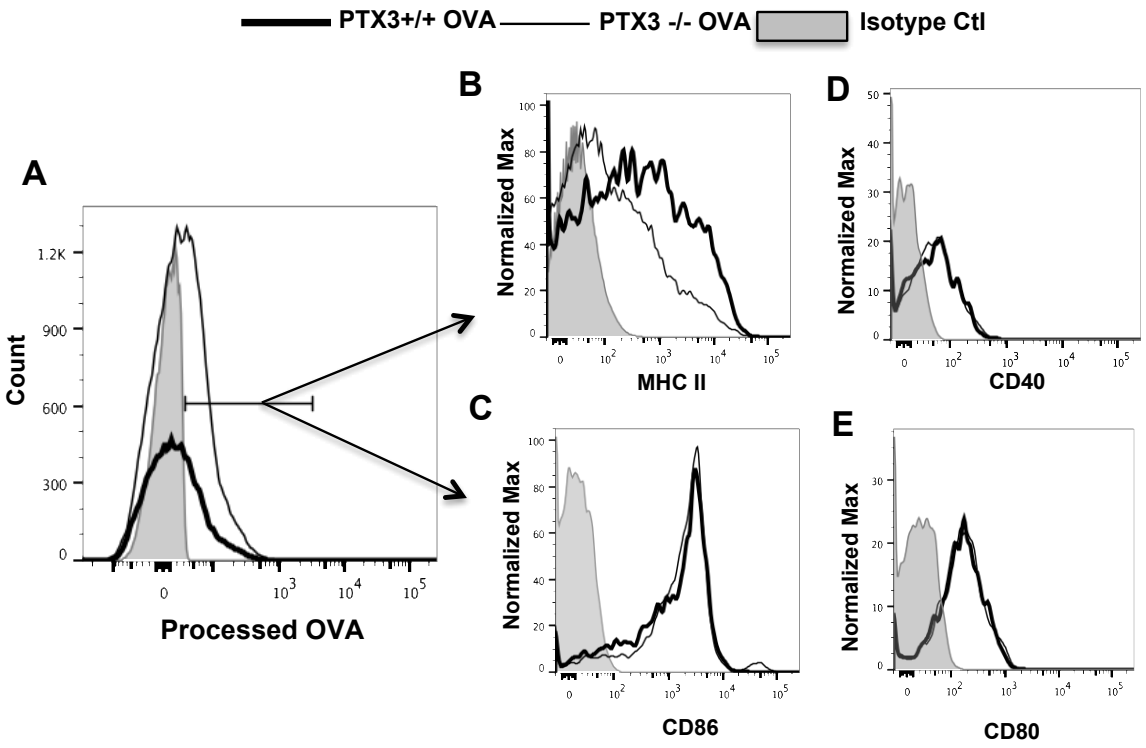

Figure 5.16: *PTX3-/- mice exhibited increased accumulation of OVA containing DCs in MLN. (A) PTX3-/- MLN DCs that contained processed OVA were compared with those from PTX3+/+ mice 24 hrs post i.n administration of DQ-OVA. Surface expression of MHCII, CD86, CD40 and CD86 was determined on processed OVA+ PTX3+/+ and PTX3-/- MLN DCs. n=4-5 / group.*

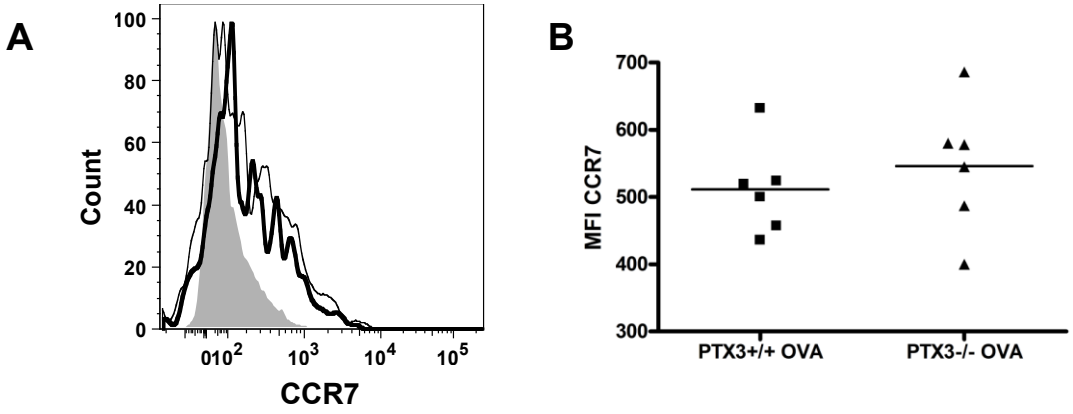

Figure 5.17: *PTX3 deletion does not affect expression of CCR7 chemokine receptor on lung DCs. OVA exposed lung DCs were assessed for the expression of CCR7, a chemokine receptor for CCL19 and CCL21. (B and C) Quantification and statistical analysis of Flow cytometry data is shown as graphs. n=6-8 / group.*

As soon as dendritic cells receive an inflammatory signal, these cells upregulate the surface expression of CCR7 that allow them to migrate to the T cell areas in the draining lymph nodes. Interestingly, lung PTX3-/- and PTX3+/+ $CD11c^{+}CD11b^{+}$ DCs showed comparable CCR7 expression, suggesting that enhanced accumulation of DCs in the MLN of PTX3-/- mice might be independent of CCR7 (Figure 5.17).

PTX3 deletion promotes IL-6 and IL-23 production by $CD11c^{+}CD11b^{+}$ DCs

Previously, I showed that $PTX3^{-/-}$ mice exhibit an IL-17A-dominant CD4 T cell phenotype upon OVA sensitization and challenge. Also, I found an enhanced production of IL-17A in gamma delta T cells and innate lymphoid cells. Therefore, I next assessed the role of lung CD11c+CD11b+ DCs in generating an IL-17A-dominant response in $PTX3^{-/-}$ condition. As production of IL-17A is mainly regulated by IL-6 and IL-23 (445), I compared production of IL-6 and IL-23 by lung $CD11c^{+}CD11b^{+}$ DCs from $PTX3^{+/+}$ and $PTX3^{-/-}$ mice. $PTX3^{-/-}$ mice exhibited increased accumulation of IL-6 (Figure 5.18A and B) and IL-23 (Figure 5.18C and D) producing lung $CD11c^{+}CD11b^{+}$ DCs post OVA challenge.

MLN CD11c+CD11b+ DCs also produces increased level of IL-6 (Figure 5.19A, B, E and F) and IL-23 (Figure 5.19C, D, G and H) in OVA exposed $PTX3^{-/-}$ mice. Not only the percentage of IL-6 (Figure 5.19E) and IL-23 (Figure 5.19G) producing DCs increased in MLNs but also there was an apparent increase in their production (Figure 5.19F and H). Collectively it suggests that deletion of PTX3 promotes the production of Th17 polarizing cytokines IL-6 and IL-23.

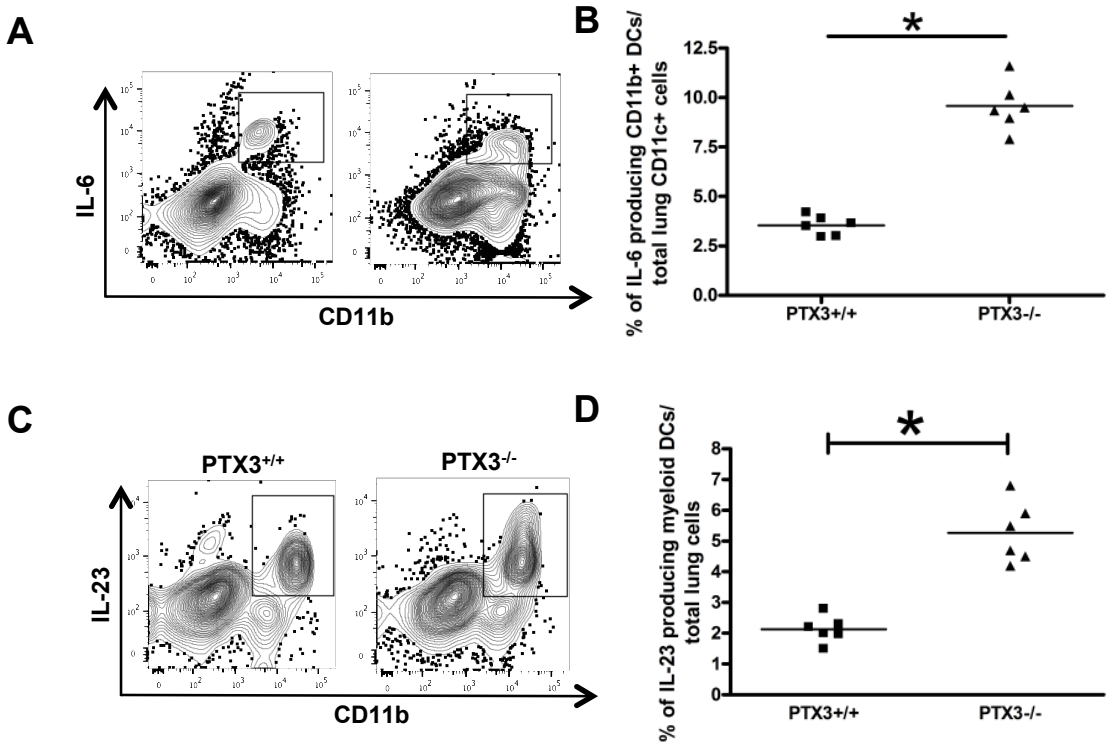

Figure 5.18: *OVA exposure results in increase in IL-6 and IL-23 producing DCs in PTX3$^{-/-}$ mice. (A) Lung CD11c^{+} CD11b^{+}DCs from OVA-exposed PTX3$^{-/-}$ and PTX3$^{+/+}$ mice were assessed for the production of IL-6 (A and B) and IL-23 (C and D) by flow cytometry. (B and D) Quantification and statistical analysis of Flow cytometry data is shown as graphs. n=6-7 / group, *p<0.01*

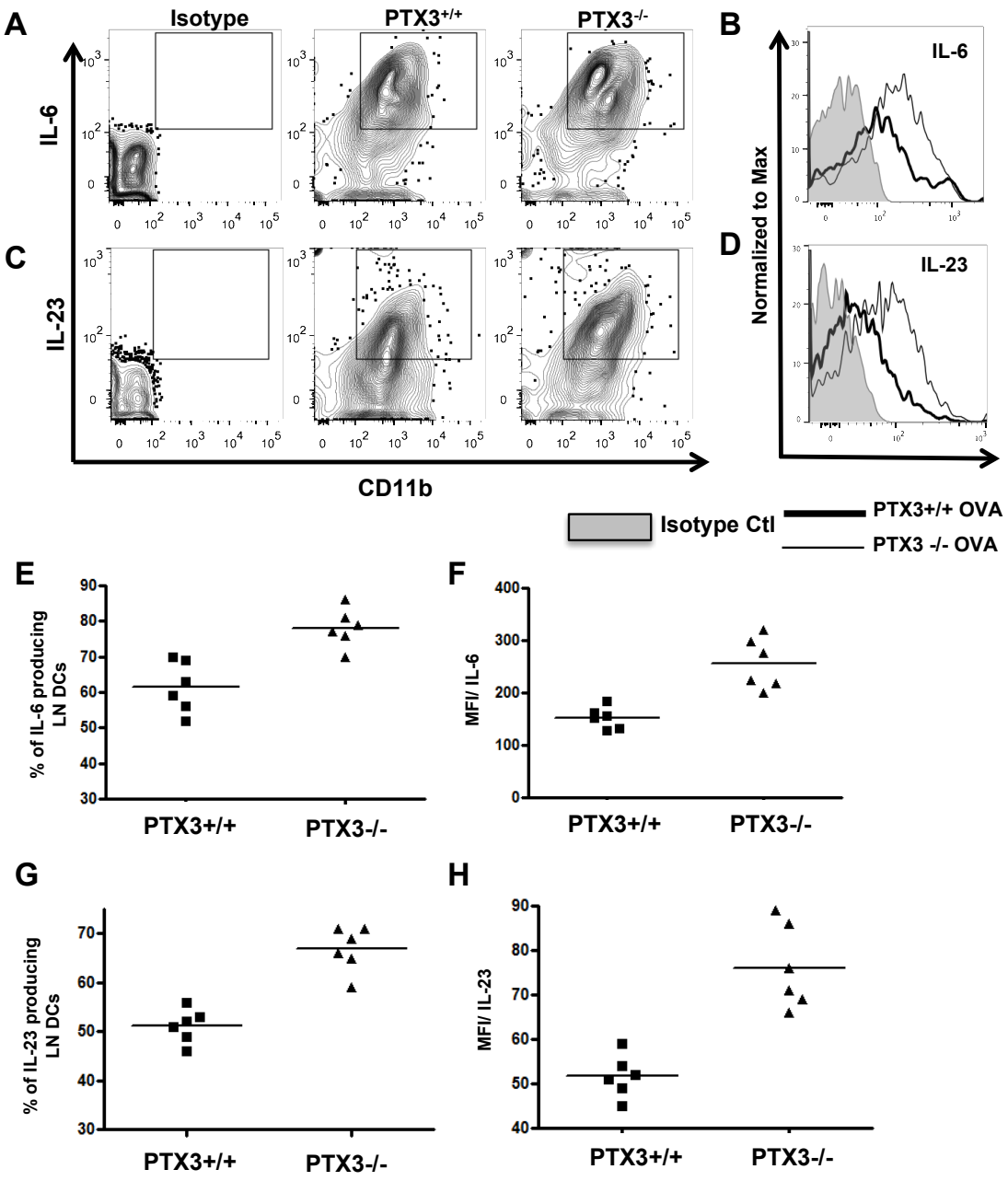

Figure 5.19: *PTX3-/- MLN DCs also showed increase in IL-6 and IL-23 producing upon OVA exposure. (A) MLN CD11c^{+} CD11b^{+}DCs from OVA-exposed PTX3$^{-/-}$ and PTX3$^{+/+}$ mice were assessed for the production of IL-6 (A, B, E and F) and IL-23 (C, D, G and H) by flow cytometry. (E-F) Quantification and statistical analysis of Flow cytometry data is shown as graphs. n=6-7 / group, *p<0.01*

PTX3-/- lung DCs promote Th17 phenotype in CD4 T cells

As $PTX3^{-/-}$ and $PTX3^{+/+}$ DCs showed differential production of IL-6 and IL-23, we next asked whether these DCs would show differential ability to induce Th17 phenotype. We purified lung DCs from OVA exposed $PTX3^{+/+}$ and $PTX3^{-/-}$ mice and co-cultured with CFSE labeled naïve $PTX3^{+/+}$ CD4 T cells. After 4 days of culture, naïve CD4 T cells co-cultured with $PTX3^{-/-}$ DCs showed significant increase in the number of IL-17A producing CD4 T cells (Figure 5.20A and B) and production of IL-17A (Figure 5.20C) as compared to naïve CD4 T cells co-cultured with $PTX3^{+/+}$ DCs.

Co-culture of OVA exposed $PTX3^{+/+}$ and $PTX3^{-/-}$ DCs with naïve CD4 T cells from OTII mice also showed a similar response (Figure 5.20 D and E). Collectively, it indicates towards an increased ability of OVA primed PTX3-/- DCs as compared to their PTX3+/+ DCs to promote IL-17A dominant phenotype in CD4 T cells.

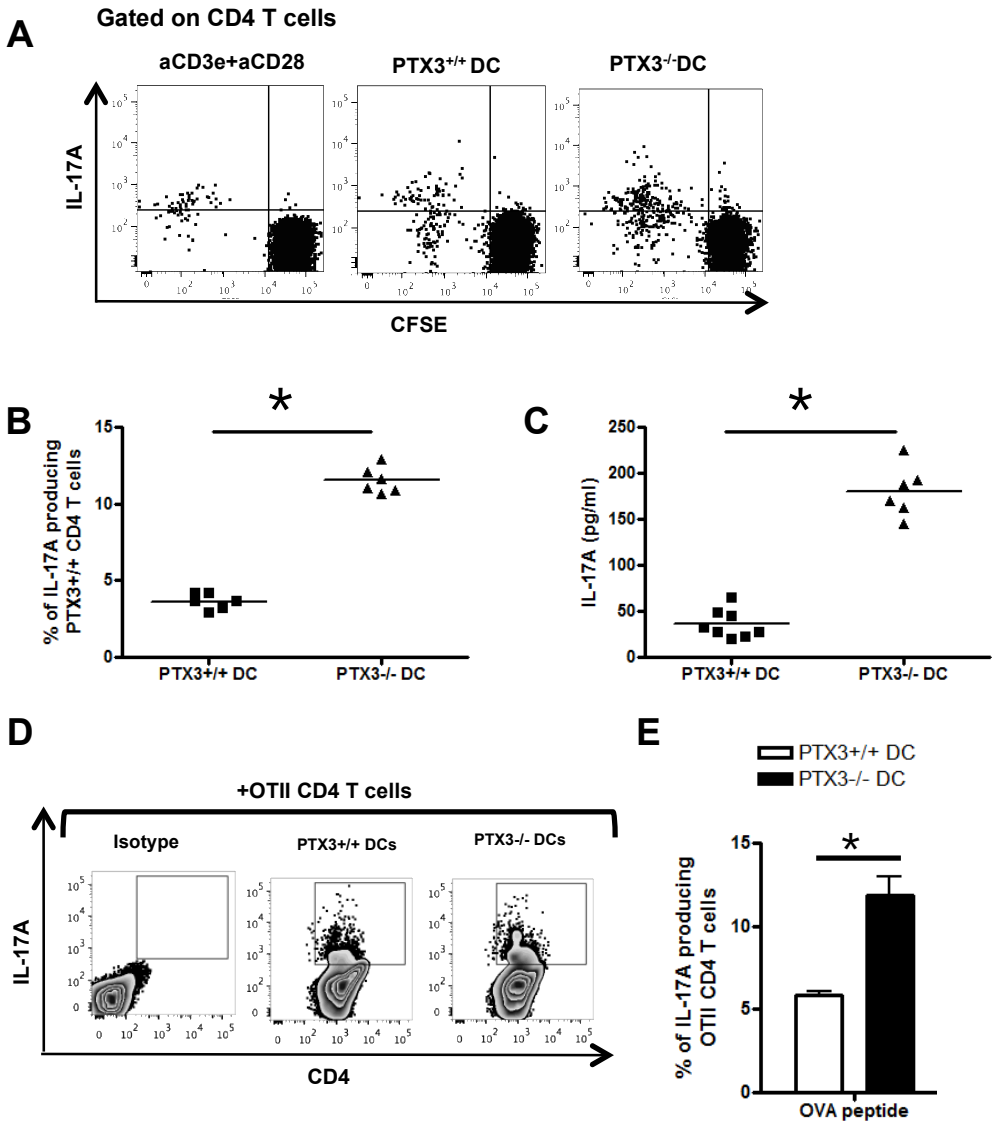

Figure 5.20*: PTX3-/- DCs promote increased IL-17A production by CD4 T cells. (A and B) Purified CFSE labeled PTX3$^{+/+}$ spleen naïve CD4 T cells were co-cultured with lung DCs from OVA exposed PTX3$^{-/-}$ and PTX3$^{+/+}$ mice for 4 days and number of IL-17A producing CD4 T cells were quantified by flow cytometry. (C) Level of IL-17A in these co-cultures was detected by ELISA. n=6-8/group, *p<0.01. (D and E) lung DCs from OVA exposed PTX3$^{-/-}$ and PTX3$^{+/+}$ mice were co-cultured with OTII mice naïve CD4 T cells and number of IL-17A producing CD4 T cells was determined after 4 days, n=3.*

5.2 Effect of PTX3 deletion on the phenotype and functions of BMDCs

In my study, I found increased accumulation of lung DCs in PTX3-/- mice upon OVA exposure as compared to PTX3+/+ mice. I characterized that deletion of PTX3 resulted in increased OVA uptake and processing, reduced MHCII surface expression, increased CD80 and CD86 on OVA exposed lung DCs as compared to their wild type counterparts. Given that isolation of lung CD11c+CD11b+ DCs is challenging as cells recovered are insufficient and isolation method that includes positive selection enrichment followed by FACS may affect the phenotype and function of these DCs, I explored whether I could use bone marrow derived DCs to expand my understanding of the effect of PTX3 deletion on dendritic cells. This strategy has been employed by several published reports. Therefore I sought to characterize the phenotype and function of PTX3+/+ and PTX3-/- BMDCs. I cultured bone marrow cells from PTX3+/+ and PTX3-/- mice with GM-CSF for 7 days and stimulated BMDC maturation with LPS, a widely used agent to study maturation in BMDCs. Previously LPS have been shown to induce robust production at least in human DCs. In my study, I showed that LPS can also induce production of PTX3 in murine DCs derived from bone marrow progenitors as determined by ELISA (Figure 5.21).

PTX3-/- BMDCs exhibited reduced expression of MHCII, CD80, CD86 and CD40

I then compared the phenotype of DCs from PTX3-/- and PTX3+/+ mice. Typically BMDCs grow as adherent monolayer that acquire stellate appearance with dendrites upon maturation post-LPS stimulation. However, PTX3-/- BMDCs remained circular in shape (Figure 5.22). CD11b expression, a signature of myeloid origin, was also reduced in PTX3-/- BMDCs as compared to PTX3+/+ BMDCs (Figure 5.23 A). In addition to dendrite growth, I also determined the expression of maturation markers on the surface of BMDCs from both genotypes. LPS treatment induced increase in the cell surface

expression of maturation markers including MHCII, CD40, CD80, and CD86 in BMDCs from both genotypes as compared to their medium controls. However, PTX3-/- BMDCs exhibited reduced expression of MHCII, CD80 and CD86 as compared to PTX3+/+ BMDCs upon LPS treatment (Figure 5.23 B, D and E). CD40 expression, however, remains unaffected by PTX3 deletion (Figure 5.23 C). I also determined whether treatment of BMDCs culture would compensate for PTX3 loss. BMDCs were treated with 50ng/ml PTX3 and expression of maturation cell surface markers were assessed. PTX3 treatment did not affect the expression of any of the cell surface markers on PTX3+/+ and PTX3-/- naïve or LPS stimulated BMDCs (Figure 5.24 and 25). Altogether it suggests that deletion of PTX3 affected LPS-induced BMDCs maturation and that addition of exogenous PTX3 could not compensate for PTX3 loss.

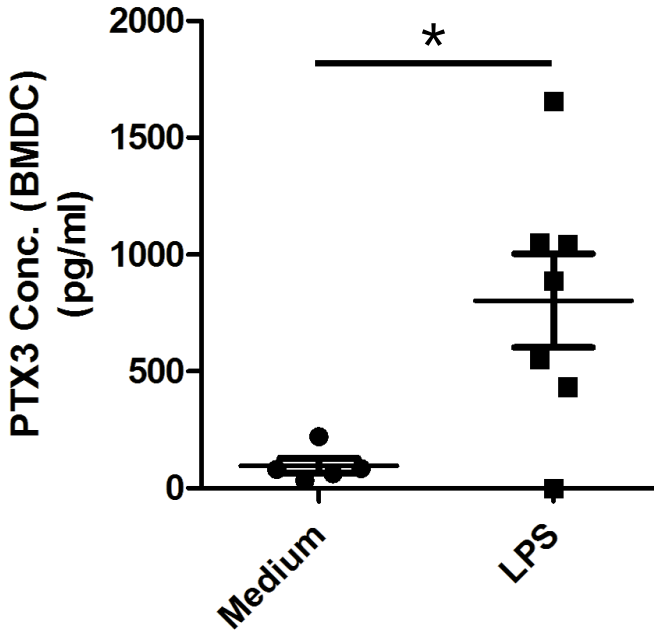

Figure 5.21*: LPS stimulation induces PTX3 production. PTX3+/+ BMDCs were stimulated with LPS and level of PTX3 was determined by ELISA. n=6/ group, *p<0.01*

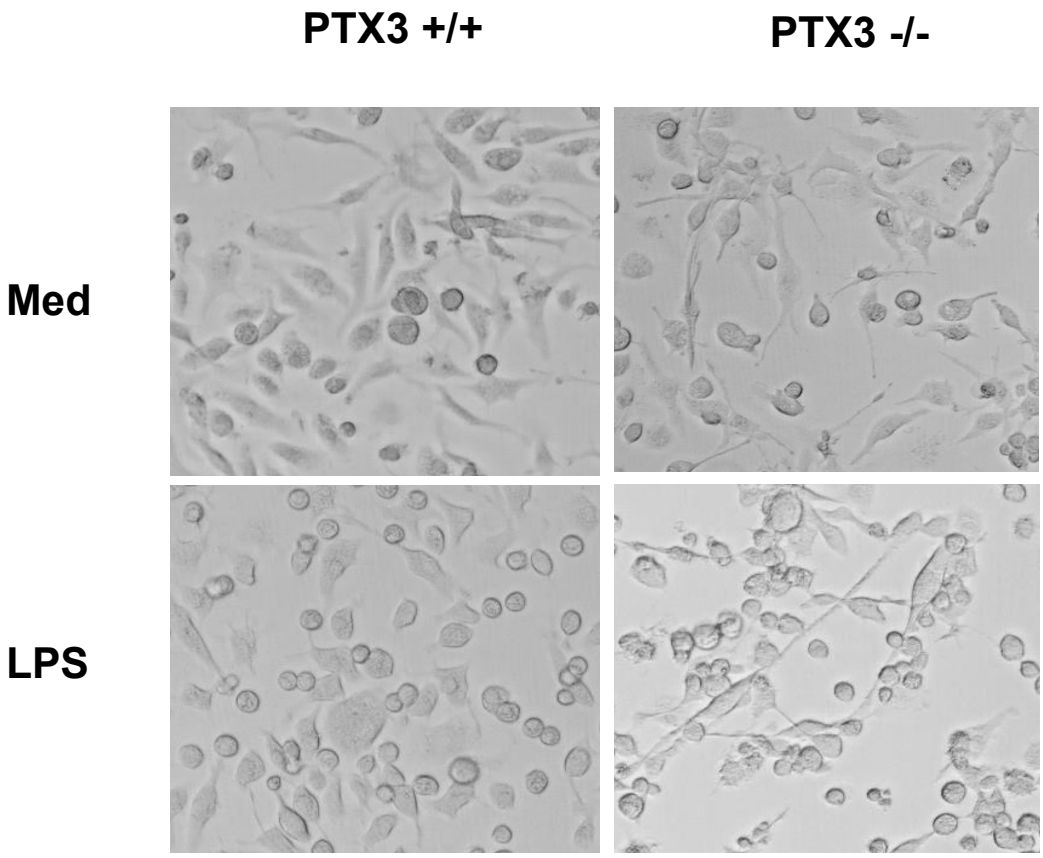

Figure 5.22*: Deletion of PTX3 results in aberrant morphology of BMDCs. Morphology of PTX3+/+ and PTX3-/- BMDCs was recorded using light microscopy, 200X*

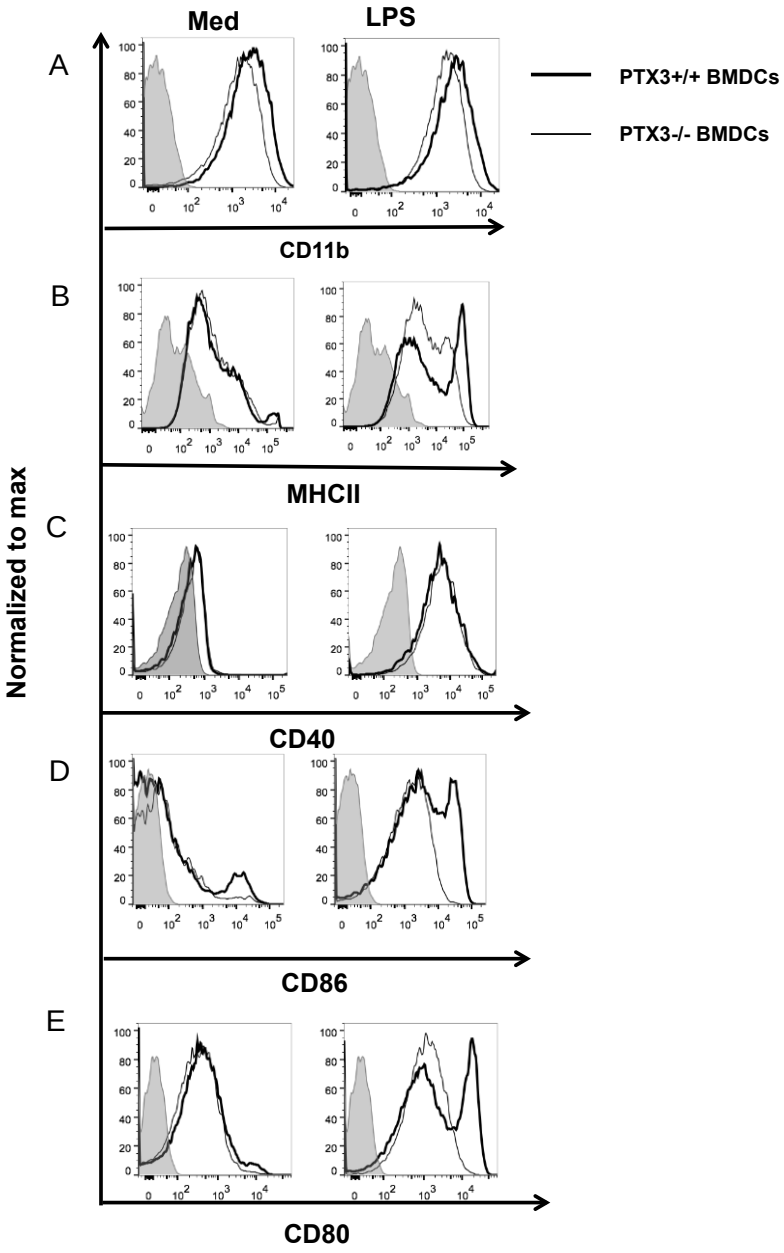

Figure 5.23: *PTX3 deletion results in reduced surface expression of CD80 and CD86 in response to LPS. Expression of CD11b (A), MHCII (B), CD80 (C), CD40 (D) and CD86 (E) was assessed by flow cytometry BMDCs upon LPS stimulation. Grey shaded area is respective isotype control. Quantification and statistical analysis of Flow cytometry data is shown as graphs in figure 5.2.5. n=6/ group*

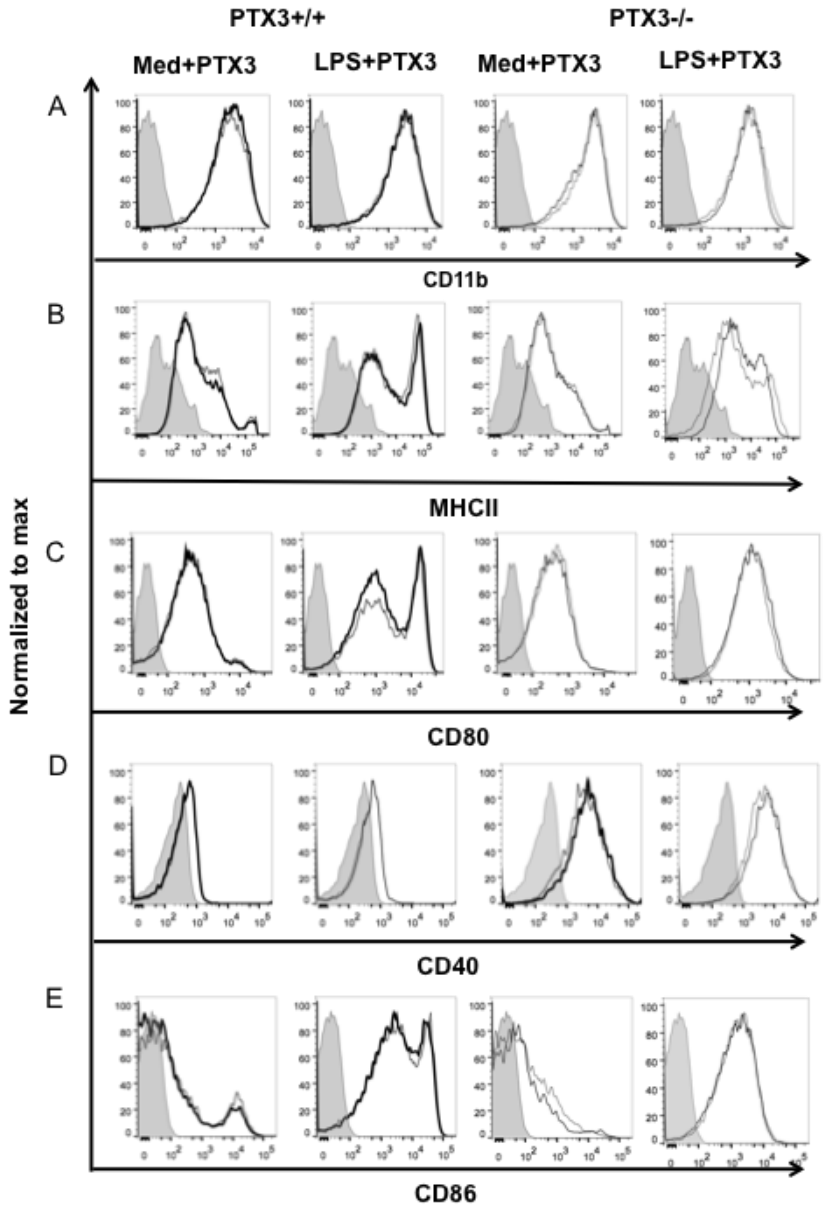

Figure 5.24*: PTX3 treatment did not compensate for PTX3 loss. Expression of CD11b (A), MHCII (B), CD80 (C), CD40 (D) and CD86 (E) was assessed by flow cytometry on PTX3+/+ and PTX3-/- BMDCs upon LPS stimulation in the presence and absence of 50ng/ml PTX3. Grey shaded area is respective isotype control, dotted histogram represents respective PTX3 treated group. Quantification and statistical analysis of Flow cytometry data is shown as graphs in figure 5.2.5. n=6/ group*

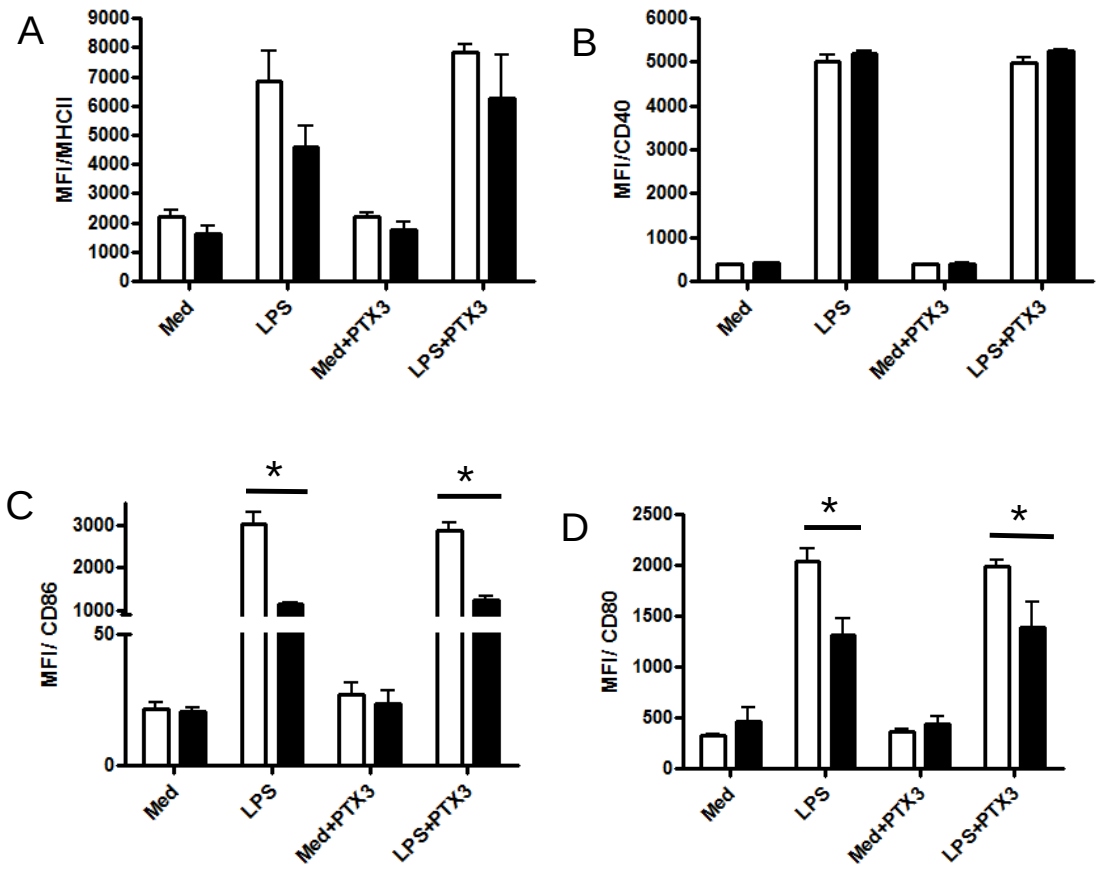

Figure 5.25*: Quantification and statistical analysis of Flow cytometry data shown in figure 5.23 and 5.24. White bars represent PTX3+/+ and black bars represent PTX3-/-. n=6/ group, *p<0.01.*

PTX3-/- BMDCs produced lower level of inflammatory cytokines upon LPS stimulation

In addition to cell surface maturation markers, LPS stimulation induces the production of proinflammatory cytokines through which DCs manifest their functions. Therefore I next detected the production of inflammatory cytokines by PTX3+/+ and PTX3-/- BMDCs upon LPS stimulation. LPS stimulation resulted in increased level of IL-6, IL-10 and IL-12p40 as detected by ELISA in PTX3+/+ BMDCs (Figure 5.26). However, PTX3 deletion significantly inhibited production of these cytokines. Since IL-12p40 subunit is shared by Th1 polarizing IL-12 and Th17 polarizing IL-23, I next assessed IL-12 and IL-23 production by flow cytometry. Concurrent to ELISA data, PTX3-/- BMDCs showed reduced production of IL-23 (Figure 5.27 A-C) and IL-12 (Figure 5.27 D-F) as compared to PTX3+/+ BMDCs upon LPS exposure. Collectively my data suggest that deletion of PTX3 impairs the ability of BMDCs to produce inflammatory cytokines in response to LPS.

PTX3-/- BMDCs exhibited impaired antigen uptake and processing

The primary function of DCs is to sample antigens/ allergens, process and present them to T cells through MHCII. Further, DCs supply secondary signals that altogether activate T cells. Given that PTX3-/- BMDCs showed reduced expression of maturation markers including MHCII, CD80 and CD86, I next questioned the ability of PTX3-/- BMDCs to uptake and process antigen. I used DQ-OVA as it allows us to track uptake and processing as compared to APC or FITC labeled OVA that can only be used to determine uptake. PTX3+/+ and PTX3-/- BMDCs were first stimulated with LPS for 30 minutes, followed by treatment with DQ-OVA. Florescence signal that represents the quantity of processed OVA was determined 3 hrs post treatment. As expected PTX3-/- BMDCs exhibited reduced OVA uptake and processing as compared to PTX3+/+ BMDCs (Figure 5.28).

Together this and other results suggest that deletion of PTX3 affected the overall ability of BMDCs to respond to the external insult.

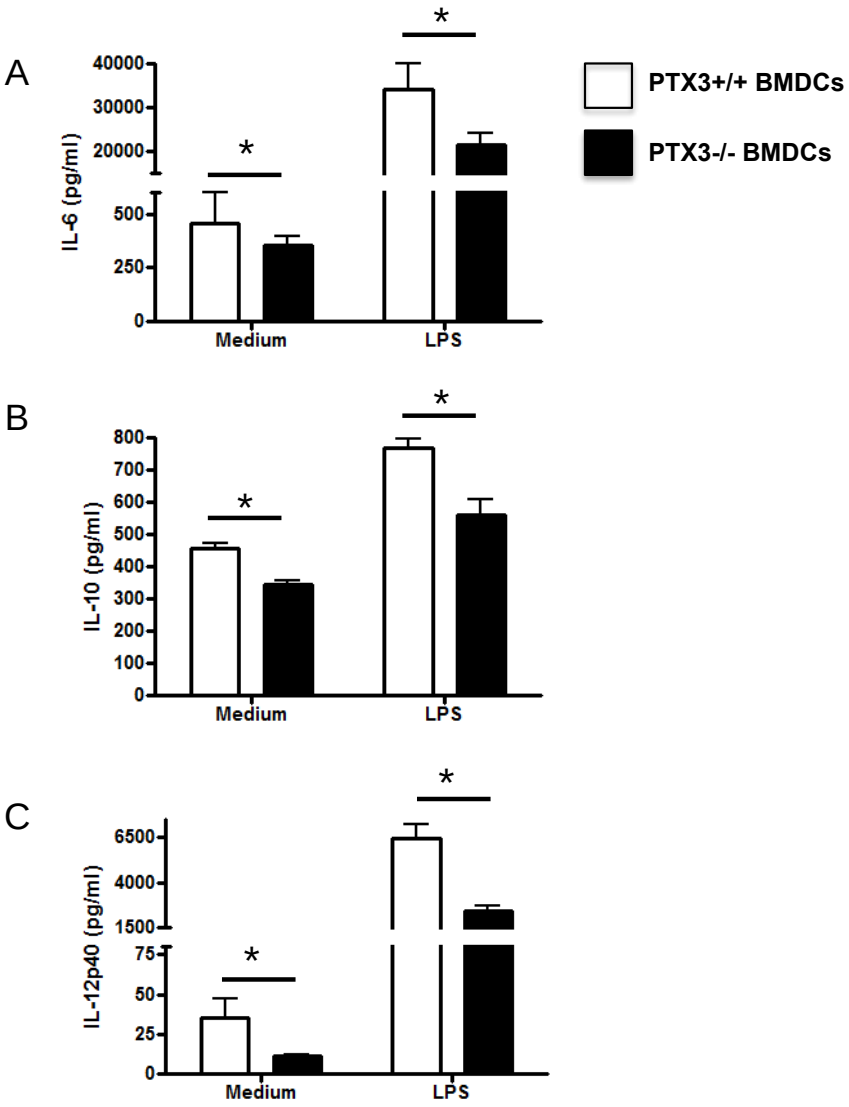

Figure 5.26: *PTX3-/- BMDCs exhibited reduced production of LPS induced inflammatory cytokines. Level of IL-6 (A), IL-10 (B) and IL-12p40 (C) in PTX3$^{+/+}$ and PTX3$^{-/-}$BMDCs cultured in the absence and presence of LPS as determined by ELISA, n=5-10/ group, *p<0.01.*

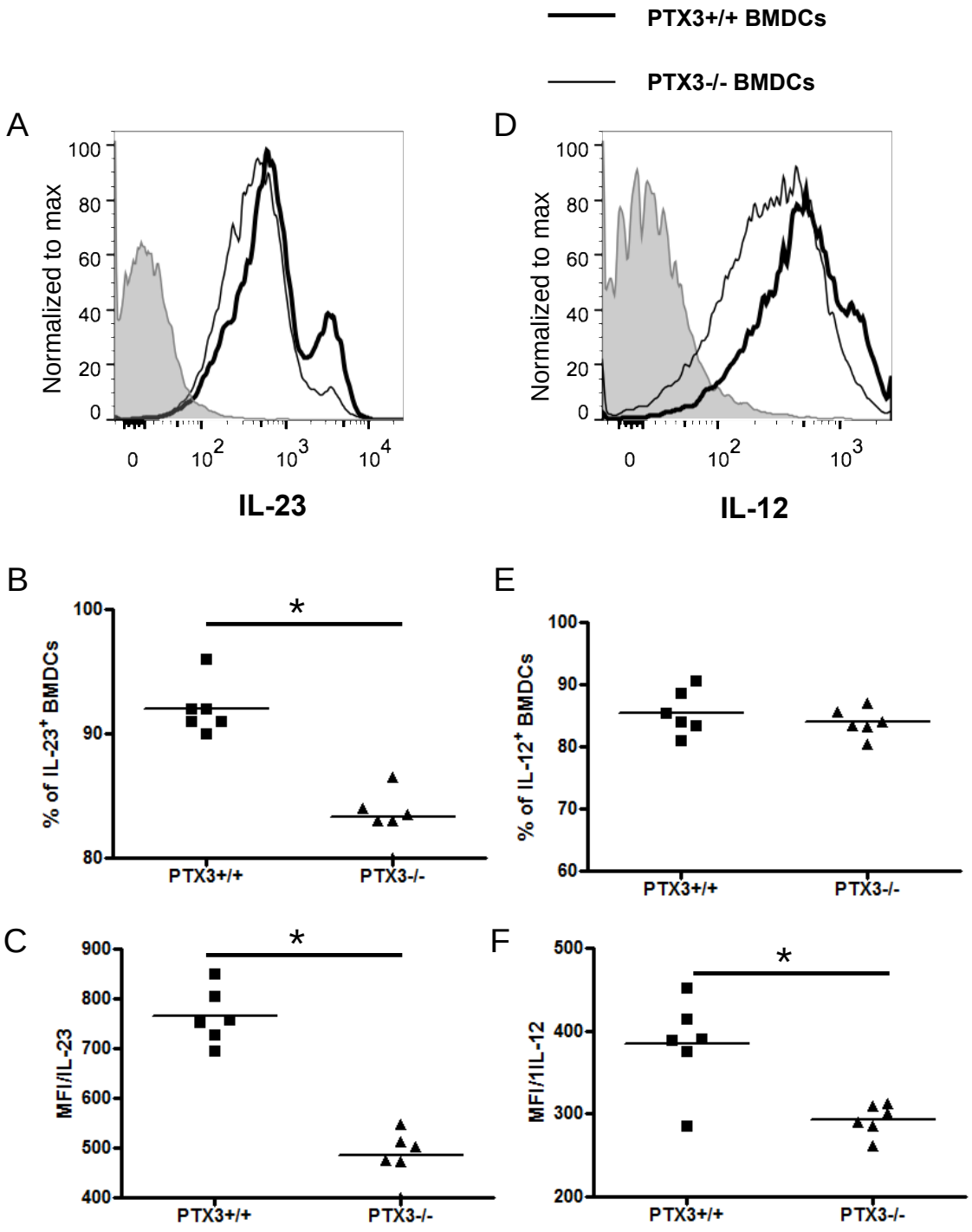

Figure 5.27: *PTX3-/- BMDCs exhibited reduced production of IL-23 and IL-12. Level of IL-23 (A-C) and IL-12 (D-F) in PTX3$^{+/+}$ and PTX3$^{-/-}$BMDCs cultured in the absence and presence of LPS as determined by flow cytometry, n=5-6/ group, *p<0.01.*

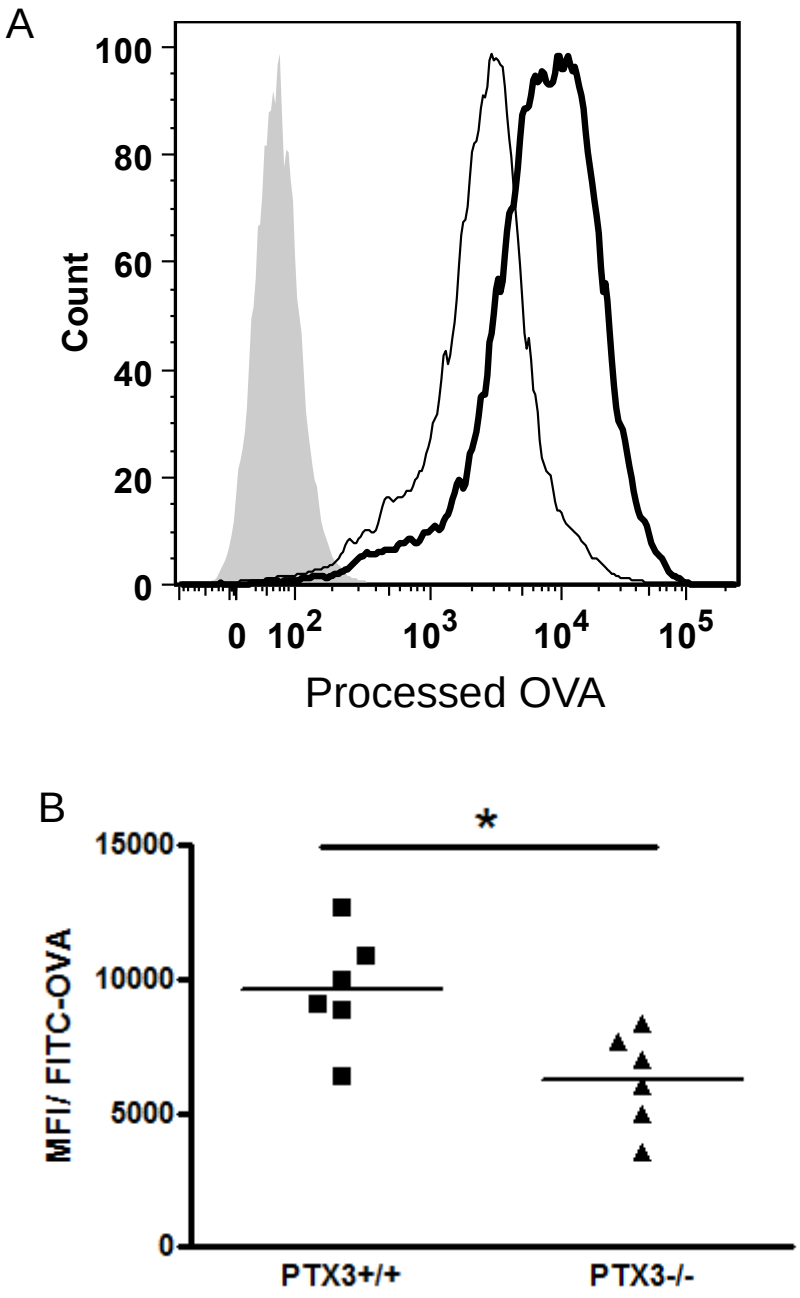

Figure 5.28: *PTX3-/- BMDCs showed reduced OVA uptake and processing. BMDCs were treated with LPS followed by treatment with DQ-OVA and BMDCs were assessed for processed OVA signal (FITC) 3hrs post treatment (A). Quantification and statistical analysis of Flow cytometry data is shown as graphs (B). n=6-8 / group, *p<0.01*

Chapter 6: Discussion

The role of PTX3 in the regulation of innate immune system has been extensively investigated in the last decade. A key conclusion that has emerged from these findings is that PTX3 provides protection against specific infections including SARS, *Pseudomonas aeruginosa*, CMV infection, pneumonia and aspergillosis (401). In mucosal adaptive immune response, particularly in asthma, the role of PTX3 has not been previously studied. In this report, I have revealed an association between PTX3 and allergic inflammation as observed in asthma. Consistent with my BALF data from severe allergic asthmatics, the lungs of allergen-exposed mice displayed a highly significant level of PTX3 compared to saline controls. These findings are in agreement with my previously reported data in bronchial biopsies obtained from severe asthmatics (400). However, serum concentration of PTX3 remains unaffected by allergen exposure that raised the possibility of local a consequence of PTX3 than imparting a systemic effect.

I demonstrate for the first time that features of allergic asthma such as AHR, airway remodeling, and inflammation were heightened in the absence of PTX3 in a murine model of OVA-induced experimental asthma. Moreover, the absence of PTX3 promotes Th17-dominant CD4 T cell response in the lungs combined with enhanced survival of CD4 T cells, suggesting a critical function of PTX3 in the preferential maintenance of specific CD4 T cell population at least in OVA-induced inflammation. Enhanced production of IL-6 by PTX3-/- DCs, a Th17-inducing proinflammatory cytokine, provides an additional explanation accounting for an IL-17A dominant allergic inflammation in mice. Collectively, my data suggest a hitherto unrecognized function of PTX3 in the regulation of events involved in the pathogenesis of asthma associated allergic inflammation.

Enhanced airway inflammation in PTX3-/- mice upon OVA exposure

Compared with wild-type littermates, OVA exposed *PTX3*$^{-/-}$ mice showed enhanced inflammation that was characterized by increased accumulation of neutrophils and eosinophils within the airways and the lung tissue. This phenotype was accompanied by an IL-17A-dominant cytokine production. IL-17A, a prototype of Th17 phenotype has been implicated in allergen-induced neutrophilia and eosinophilia at least via production of chemokines including keratinocyte chemoattractant (KC) (446, 447), which is a chemoattractant for neutrophils and eosinophils (448, 449). Along the same line, an enhanced KC production (data not shown) was detected in the lungs of OVA-exposed *PTX3*$^{-/-}$ mice that may account for an exaggerated neutrophilia and eosinophilia in *PTX3*$^{-/-}$ mice. Previously, IL-17A was shown to enhance airway hyperresponsiveness through airway smooth muscle-dependent mechanism (286, 450). Also, IL-17 family is involved in the upregulation of mucus genes, specifically *muc4ac* and *muc5b* (276). These reports are in accord with an enhanced IL-17A-dominant response, increased AHR and mucus production in OVA-exposed *PTX3*$^{-/-}$ mice in comparison with *PTX3*$^{+/+}$ mice.

IL-17A-dominant inflammation in OVA-exposed PTX3-/- mice

Lung CD4 T cells from OVA exposed *PTX3*$^{-/-}$ mice displayed enhanced activation compared to *PTX3*$^{+/+}$ mice as detected by CD69 and CD25 surface staining. This result is in line with a recent study showing that PTX3 treatment suppressed CD25 and CD69 in OVA-specific CD4 T cells (451). IL-17A production by CD4 T cells is mediated by a combination of IL-6/23-dependent mechanisms (452, 453) via at least STAT3 activation (454). Heightened IL-17A production with increased level

of phosphorylated STAT3 in the lungs of OVA exposed *PTX3*$^{-/-}$ mice is in accordance with previously published data.

IL-2 is a key negative regulator of Th17 phenotype (455) that is suppressed by STAT3 activation in T cells (232). Indeed, compared to *PTX3*$^{+/+}$ mice, reduced production of IL-2 in OVA-exposed *PTX3*$^{-/-}$ CD4 T cells was observed which may provide a reasonable mechanism accounting for the enhanced IL-17A production and STAT3 activation in my model.

In concert with my observation, *Moalli et al.* (429) and *Paroni et al.* (430) reported reduced levels of IL-17A upon treatment with exogenous PTX3 in murine models of chronic *P. aeruginosa* lung infection and cystic fibrosis, respectively. My findings combined with the above studies suggest an inverse correlation between PTX3 and IL-17A dependent pathway. Also, *D'Angelo et al* (431) and *Zhu et al* (432) showed that PTX3 suppressed IL-17A-mediated inflammation in aspergillus-induced chronic granulomatous disease and an ischemia-reperfusion injury model, respectively. Although both later studies mainly suggested that PTX3 suppressed IL-17A production by restricting γδT cells expansion, my study highlights an additional mechanism through which PTX3 may regulate IL-17A production by CD4 T cells. Altogether, the data points to a unique function of PTX3 in counter-regulating IL-17A/ Th17-dominant immune response. Interestingly, basal (saline) level of IL-17A and other detected proinflammatory cytokines are low in PTX3-/- mice as compared to their wild type counterparts. Whether this phenomenon indicates why and how PTX3 deleted mice are more susceptible to external insults including allergen exposure is worth studying.

Increased survival of OVA-exposed PTX3-/- CD4 T cells

Development of immune response in asthmatic airways is primarily driven by increased cell infiltration and/ or prolonged survival of proinflammatory cells (456). Cell death of proinflammatory cells is a regulatory mechanism, which limits inflammation-induced tissue injury and promotes resolution (456). Enhanced survival of T cells has been reported in the airways of asthmatics (457-459). Furthermore, BALF T cells from allergen-exposed asthmatic individuals were shown to be less susceptible to apoptosis inducing agents through Bcl-2 enhanced expression (460). In my study, I found that OVA exposed PTX3$^{-/-}$ CD4 T cells are more resistant to post activation-induced apoptosis and displayed increased Bcl-2 expression. Since naïve CD4 T cells also show a tendency to resist basal apoptosis, it is tempting to hypothesize that internal ability of CD4 T cells acquired upon PTX3 deletion contributes to enhanced survival of PTX3-/- CD4 T. This phenotype is however, more apparent in OVA-induced inflammation, plausibly through increased IL-17A production, STAT3 phosphorylation and reduced IL-2 production (232, 461, 462). *In vivo,* increased IL-6 production by DCs, which is known to promote cell survival either by directly activating STAT3 signaling pathway or by promoting IL-17A production (463, 464) **(463, 465, 466)** serves as an additional mechanism favoring T cell survival in PTX3 -/- mice.

Previously, *Lech et al* (467) reported splenomegaly and lymph node hyperplasia of PTX3/*lpr*$^{-/-}$ mice in systemic lupus erythematosus (SLE) condition that was also associated with an exaggerated inflammation in the lungs. Whether it was due to an enhanced survival of lymphocytes in the absence of PTX3 will be interesting to study. The analysis conducted herein suggests greater longevity of PTX3$^{-/-}$ CD4 T cells that may possibly drive enhanced allergen-induced airway inflammation in PTX3$^{-/-}$ mice.

Other sources of enhanced IL-17A production in OVA-exposed PTX3-/- mice

Furthermore, PTX3 deletion resulted in an apparent expansion of γδ T cell population under OVA sensitization/ challenge regime. Recently, *Zhu et al.* [51] provided evidence that exogenous PTX3 treatment reduced IL-17 levels by inhibiting γδ T cells. Thus far, my and others data collectively suggests that PTX3 regulates γδ T cell population and that absence of which renders their expansion and enhanced cytokine production. Although γδ T cells represent a minor population of total lung T lymphocytes, their critical role in constituting a major IL-17 producing innate cells has previously been studied [52]. γδ T cells through the production of IL-17 and related cytokines have also been involved in the initiation of psoriasis [54]. In Mycobacterium chronic infection, γδ T cells are shown to be crucial for neutrophil influx into the tissue [53]. Similarly, in my study, I found a parallel increase in IL-17A producing cells including γδ T cells and neutrophilia at the inflammation site.

Pantelyushin et al. [54] also stressed on the involvement of ILC in the development of IL-17-dependent inflammation in an Aldara psoriasis model. ILC3 are a newly characterized population of pulmonary innate lymphoid cells, capable of secreting IL-17 unlike other ILCs found in the lungs [14]. I observed notably more ILC3 in the lungs of PTX3-/- mice. Regardless of their minute population, IL-17 producing ILC3 by themselves are capable of inducing AHR [14]. Together, PTX3 might seem to regulate IL-17A production by innate inflammatory cells, including γδ T cells and ILC3 in allergen-induced allergic inflammation in mice. Given that PTX3 is a PRR and its production starts during the early phases of the diseases or infections (reviewed in [4]), PTX3 may be acknowledged to regulate the inflammatory mechanisms that are orchestrated long before the activation of the adaptive immune system.

Increased accumulation of lung DCs in OVA-exposed PTX3-/- mice

DCs are strategically located in the epithelium so as to capture antigens/ allergens and orchestrate immune response according to the type of antigen. They are equipped to process allergens and present them to naïve CD4 T cells (111, 145, 152-157, 164). Deletion of PTX3 resulted in an enhanced IL-17A-dominant inflammation in OVA-sensitized/challenged mice. Although isolated PTX3-/- CD4 T cells were capable of increased polarization to Th17 cells even without the presence of DCs, cytokines that were present in polarization cocktails are typically produced by inflammatory cells such as DCs *in vivo*. Given that DCs are located in close proximity with T cells and are involved in directing their functions as discussed in the introduction, I was interested to understand the role of DCs in shaping such a Th17 dominant phenotype in *in vivo* OVA-induced allergic inflammation in mice. In this study, I showed increased infiltration of CD11b+CD11c+ DCs into the lungs of PTX3-/- mice as compared to PTX3+/+ mice upon OVA exposure. Unlike PTX3-/- CD4 T cells, PTX3-/- DCs did not show any apparent difference in survival as compared to PTX3+/+ DCs. A plausible explanation of such an increase in inflammatory CD11c+CD11b+ DCs could be enhanced generation of CMP in the bone marrow, which was observed in PTX3 depleted mice in contrast to their wild type counterparts. *Manz et al (436)* reported that CMP in bone marrow give rise to GMP which then give rise to functional DCs *in vitro and in vivo.* Although in my study I did not find any difference in GMP population while CMP count was significantly increased in PTX3-/- mice as compared to PTX3+/+ mice. Monocytes constitute an intermediate stage between GMP and tissue DCs. During inflammation, DCs in peripheral tissue originate from monocytes, which infiltrate into the tissue upon inflammation (98, 146, 166, 167). In order to understand whether increased accumulation of lungs DCs was dependent on differentiation of monocytes into DCs, I monitored monocytes in the

bone marrow, blood and the lungs. Reduction of monocytes in bone marrow and blood but increase in their count in the lungs of PTX3-/- mice upon OVA exposure explains increased accumulation of their differentiated product, that were the lung CD11c+CD11b+ DCs. Such monocytes, which are able to differentiate to functional DCs upon inflammation, are known as inflammatory monocytes (98, 146, 166, 167). These are characterized by high expression of CCR2 in contrast to patrolling monocytes, which are $Ly6C^{low/\ int}$ $CCR2^{low}$. CCR2 plays a critical role in facilitating egress of inflammatory monocytes from the bone marrow to blood and from blood to the lungs in response to allergen challenge (440). In my study, I observed that inflammatory monocytes were $CCR2^{high}$ in both genotypes. As CCR2 expression on monocytes at basal level was comparable in PTX3-/- and PTX3+/+ mice, it rules out the plausibility of an effect of PTX3 deletion on CCR2 expression in naïve state. Although I did not find a significant difference in the CCR2 expression on BM, blood and lung monocytes, lung DCs showed upregulation of CCR2 expression upon OVA exposure. Such observation could stem from two possibilities: PTX3-/- lung DCs with increased CCR2 represent monocytes that had increased CCR2 which now due to foreign stimulus have differentiated into DCs and / or increase in CCR2 in PTX3-/- DCs regulate their accumulation in the lungs independent of their such a role on monocytes. Previously, diesel particles induced inflammation was shown to upregulate the expression of CCR2 in the lungs (468). Recruitment of inflammatory monocytes, monocytes derived DCs and induction of subsequent pulmonary inflammation was also shown to be CCR2 dependent in this study. Similarly, CCR2 dependent recruitment of monocytes-derived DCs has also been reported upon influenza infection, exposure to LPS and particulate antigens (437, 468, 469). Loss of CCR2 could not be compensated by other chemokine- chemokine receptor systems (440).

Other chemokine receptors including CCR5 and CCR6 have also been involved in directing monocytes to the tissue sites. Both of these chemokine receptors are expressed on immature DCs and regulate their immigration to the tissue sites (470, 471). Previously *Norata et al (8*) also showed enhanced CCR5 and CX3CR1 expression in the vascular wall of PTX3-/- apoE-/- mice as compared to their PTX3+/+ counterpart. In my study, I observed increased expression of CCR5 and CCR6 on naïve supposedly immature lung DCs in PTX3-/- mice that were parallel to increased DC accumulation in the lungs upon OVA exposure. Surface expression of CCR5 and CCR6 downregulate as DCs undergo maturation. Similarly, I also found reduction in their expression 12 hrs post OVA challenge. This reduction was however, more prominent in PTX3-/- mice. Interestingly, their expression increased 24 hrs post challenge, became similar to their expression on lung DCs at naïve state, suggesting possibility of acquiring naïve like phenotype by lung DCs 24 hrs after the challenge. Altogether it seems likely that increased CCR5 and CCR6 on naïve PTX3-/- lung DCs renders them more responsive to chemokines, which are released immediately after exposure to foreign antigens. CCR5 expression is associated with Th1 cells (472). CCR6, on the other hand, regulates migration of Tregs and Th17 cells into the inflammatory region (473). Whether altered expression of these chemokine receptors on lung DCs is also involved in the regulation of Th17 promoting cytokines would be interesting to study.

According to traditional paradigm, CCR2 ^{high} inflammatory monocytes express low levels of CX3CR1 as compared to CCR2 ^{low} patrolling monocytes (474, 475). CX3CR1 facilitate long-range crawling of monocytes on resting epithelium (442). Furthermore, CX3CR1 reduces monocytes motility in the bone marrow, a mechanism contradictory to that of CCR2, through which it retains monocytes in the bone marrow (476). However, some recent reports showed an active role of CX3CR1 in directing inflammatory monocytes to inflammation site (477-480). *Tacke et al* showed

the requirement of CX3CR1 on blood inflammatory monocytes along with CCR2 and CCR5 so as to render their accumulation within atherosclerotic plaques in mouse. In my study, I found increased CX3CR1 expression on OVA challenged PTX3-/- blood monocytes, plausibly suggesting a supporting role of CX3CR1 in recruitment of monocytes in the lungs in response to OVA exposure.

PTX3 interacts with P-selectin and inhibits migration of neutrophils to the peripheral tissues. This phenomenon was reversed upon disruption of PTX3 and P-selectin interaction either through PTX3 deletion or antibody-mediated inhibition. Furthermore, increased expression of P-selectin has been observed in PTX3-/- mice (481), further supporting likelihood of P-selectin-dependent migration of inflammatory cells in the absence of PTX3. As monocytes are also dependent on P-selectin for migration, it is tempting to hypothesize the involvement of this pathway in resulting enhanced monocytes and CD11c+CD11b+ DCs accumulation in the lungs.

Role of lung DCs in IL-17A-dominant inflammation in OVA-exposed PTX3-/- mice

In the lungs, DCs exist in an immature state, which upon allergen exposure initiate their maturation process. Mature DCs process allergen, present allergen epitopes through MHCII along with the expression of an array of costimulatory molecules including CD40, CD80, and CD86, which are well known as maturation markers of DCs. PTX3-/- CD11c+CD11b+ DCs showed greater ability to uptake and process OVA as compared to PTX3+/+ DCs. Surprisingly, PTX3-/- DCs expressed reduced level of MHCII. Whether lower expression of MHCII by PTX3 depleted DCs was due to increased recycling of MHCII or due to reduced biosynthesis or transport to the membrane, is not known (482). Engagement of CD80/86 by CD28 induces the production of IL-6 by DCs (483). In my study I observed enhanced expression of CD80 and CD86 by PTX3-/- CD11c+CD11b+ DCs in

contrast to PTX3+/+ CD11c+CD11b+ DCs, which is also concurrent with increased production of IL-6 by PTX3-/- DCs.

IL-6 acts on naïve CD4 T Cells and induce their differentiation to Th17 CD4 T cells (484). IL-23 acts sequentially and promotes the maintenance and survival of Th17 CD4 T cells (485). In addition to IL-6, depletion of PTX3 also resulted in increased production of IL-23, clearly elucidating a plausible DC-dependent mechanism, which leads to induction of Th17 dominant inflammation in PTX3-/- mice upon allergen exposure. Augmented Th17 CD4 T cell differentiation of naïve CD4 T cells from PTX+/+ and OTII mice upon co-culture with PTX3-/- DCs further validated the connection between increased production of Th17 polarizing cytokines by PTX3-/- DCs and IL-17A dominant inflammation in these mice.

Consistent with increased maturation, antigen uptake, and processing, I also observed greater migration of OVA peptide containing CD11c+CD11b+ DCs from lungs to the draining lymph nodes in PTX3-/- mice as compared to their wild type controls. CCR7 is a chemokine receptor, which regulates migration of lung DCs to the draining lymph nodes (486-488). Although PTX3-/- and PTX3+/+ DCs did not show any apparent difference in CCR7 expression, it seems that increased OVA peptide containing DC population in LN was the result of their increased number in the lungs and was independent of any differential expression of CCR7. Similar to lung DCs, OVA peptide containing PTX3 MLN CD11c+CD11b+ DCs also showed increased production of IL-6 and IL-23. Altogether my data explains how deletion of PTX3 rendered pulmonary DCs hypermatured upon OVA exposure and affected Th17 polarizing capacity of DCs.

Differential effects of PTX3 deletion on BMDCs and lung DCs

In contrast to my observation in the lungs, PTX3-/- BMDCs showed reduced expression of maturation markers and aberrant production of proinflammatory cytokines in response to an inflammatory stimulus, LPS. Furthermore, PTX3-/- BMDCs exhibited reduced ability to uptake and process OVA, an allergen that was also used in *in vivo* model of allergic asthma. Since structural cells and other inflammatory cells in the tissue sites regulate the phenotype and function of dendritic cells, it is tempting to hypothesize that absence of these factors may have affected the ability of PTX3-/- BMDCs to respond to LPS and OVA.

In summary, my study assigns a critical function of PTX3 in the etiology of allergic inflammation. Without dismissing the role of PTX3 in regulating innate inflammatory mechanisms, my study proposes that the absence of PTX3 results in dysregulated programming of inflammatory mechanisms involved in IL-17A production, leading to an exaggerated allergen-induced inflammation. IL-17A dominant immune response has previously been associated with steroid resistant severe asthma in humans (490). Therefore, my study may provide some novel insight in understanding mechanisms involved in the regulation of IL-17A-dominant immune response in severe asthma. Based on our current data, it is difficult to infer whether PTX3 treatment would provide protection against allergen-induced activation of an unwarranted immune system. However, our data clearly show that deletion of PTX3 plays a critical role in regulating functional capacity of immune system, thereby affecting immunological metrics in response to an allergen in a murine model of experimental asthma.

Chapter 7: General Discussion

Pentraxin 3 (PTX3) is a non-redundant molecule of the immune system that was previously known to regulate innate inflammatory mechanisms. Although previous literature suggested how PTX3 acts as a link between the innate immune system and adaptive immune system, most of the evidence focused on its role in innate arm particularly in infection models.

Through my thesis, I have studied PTX3 in context with a chronic disease, asthma that occurs in response to otherwise normal foreign agents such as ovalbumin (OVA). Allergic inflammation as observed in asthma is an example of coordination between innate and adaptive inflammatory mechanisms. The overarching goal of my study was to understand the role of PTX3 in regulating inflammation that is induced in response to allergens and to explore the possibility of using this information for effective management of asthma in humans. To achieve my goal, OVA was used as an allergen as it induces many features of asthmatic lungs including a robust allergic inflammation, airway hyper responsiveness and tissue remodeling in mice. Use of PTX3-/- mice furthered our understanding of the effects of its deletion on hallmark characteristics of allergic asthma, particularly on CD4 T cell-DCs-mediated inflammation. Thus my thesis can be divided into two major sections. In the first section (Chapter 4), I identified that deletion of PTX3 resulted in enhanced infiltration of inflammatory cells in the airways and the lungs, increased production of IgE and enhanced airway hyper reactivity in response to OVA sensitization/ challenge regime. Also, I identified that PTX3 deletion favored IL-17A dominant inflammation in the lungs and the draining lymph nodes that is at least in part due to Th17 CD4 T cells, gamma delta T cells and ILC3s

In the second section (chapter 5), I showed increased infiltration of CD11c+CD11b+ DCs in the lungs of OVA exposed PTX3 deleted mice. PTX3-/- DCs exhibited enhanced expression of CD80 and CD86 and increased production of Th17 polarizing cytokines IL-6 and IL-23. PTX3 deleted DCs showed increased uptake and processing of OVA. Co-culture of these DCs with PTX3+/+ CD4 T cells resulted in increased production of IL-17A. Interestingly, deletion of PTX3 seemed to affect peripheral tissue DCs and bone marrow-derived dendritic cells differently based on my observation of their response to inflammatory signals. Phenotypic analysis of LPS stimulated BMDCs revealed reduced expression of MHCII, CD80, and CD86 in the absence of PTX3. Deletion of PTX3 also resulted in defective OVA uptake/ processing and reduced production of inflammatory cytokines by PTX3-/- BMDCs as compared to their PTX3+/+ controls. Not only the finding of chapter 5 suggested that lung and LN DCs exhibited hyper responsiveness to allergen OVA and that this response favored IL-17-dominant inflammation, it also indicates differences in peripheral tissue DCs and BMDCs. It is due to these differences that BMDCs could not be used to study tissue DCs in my study. Whether these differences were due to varied niche in peripheral tissue or and bone marrow or differential function of PTX3 in different tissues are not clear and worth examining. Findings of my thesis not only provide insights of the role of PTX3 in regulating inflammation in murine model of allergic asthma, it also adds to the information on the regulatory role of PTX3 on CD4 T cell-DC axis (figure 7.1).

Clinical significance and Knowledge translation

My study assigns a critical function of PTX3 in the etiology of allergic inflammation. Without dismissing the role of PTX3 in regulating innate inflammatory mechanisms, my study proposes that the absence of PTX3 results in dysregulated programming of inflammatory mechanisms involved in IL-17A production, leading to an exaggerated allergen-induced inflammation. IL-17A dominant

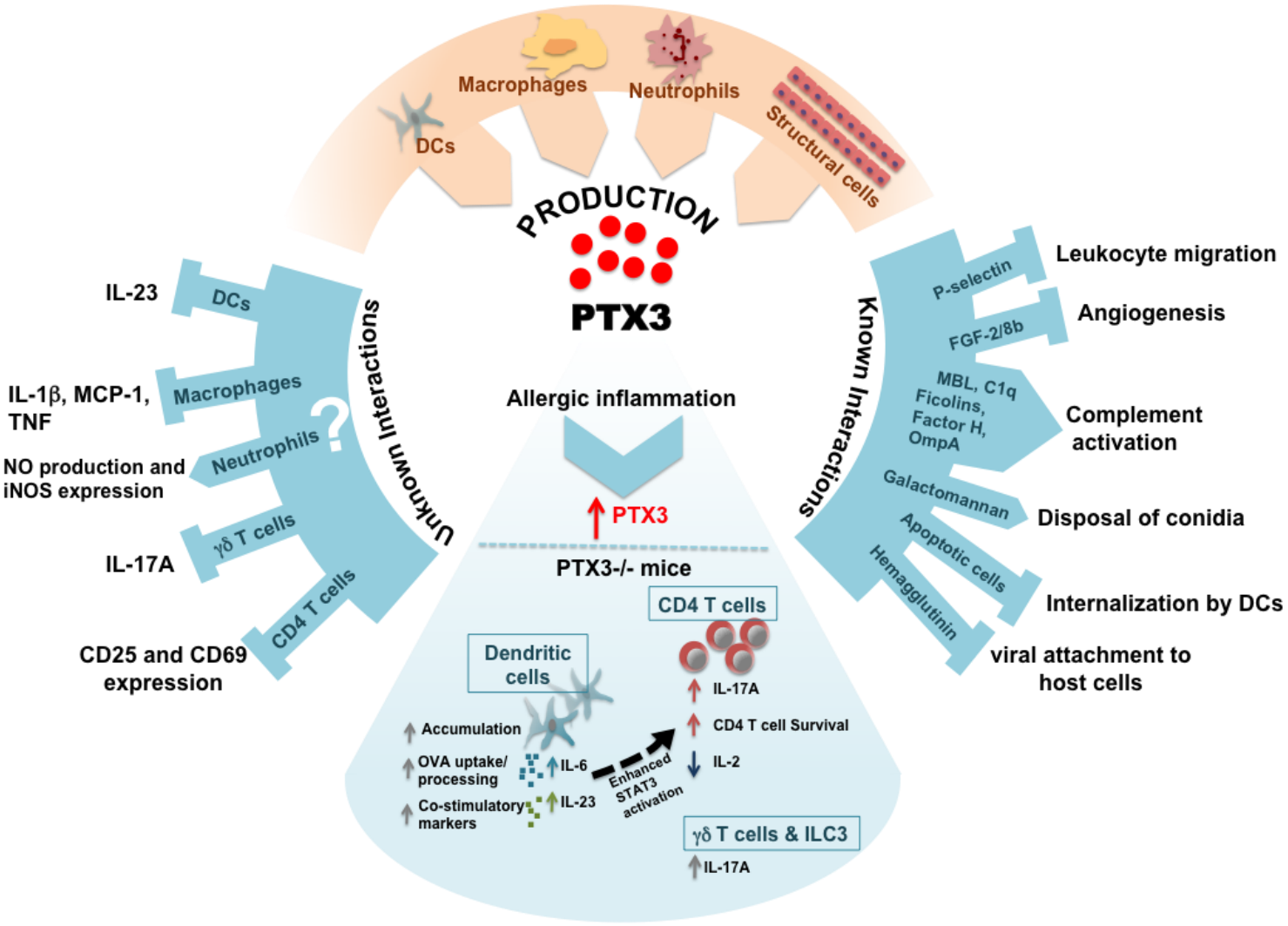

Figure 7.1*: Immunobiology of PTX3: Model-showing findings of this study and other previously published reports in context with production, interactions and functions of PTX3. OmpA: Outer membrane protein A of K. pneumoniae*

immune response has previously been associated with steroid resistant severe asthma in humans (58). Therefore, my study may provide some novel insight in understanding mechanisms involved in the regulation of IL-17A-dominant immune response in severe asthma.

Given that PTX3 deletion renders mice susceptible to allergic inflammation, I expected that overt expression of PTX3 would be helpful in resolving asthma inflammation. On the contrary, subjects that showed increased PTX3 levels in their BALF and in the lung tissue (as shown by others in my lab) had severe asthma. So far the role of increased PTX3 in asthmatics or whether it was a consequence of the inflammation or steroid treatment of these patients, is unclear. Therefore based on our current data, it is difficult to infer whether PTX3 treatment would provide protection against allergen-induced activation of an unwarranted immune system. If, however, further research shows promising information on the possibility of using exogenous administration of PTX3 for therapeutic purpose, it would serve as an interesting and in fact more suitable therapeutic approach to target severe forms of asthma particularly IL-17 dominant phenotype. In that case, suitable administration route and dose needs to be studied in detail. PTX3 is produced by a variety of cells in almost all tissues. Since PTX3 is shown to play anti-inflammatory and pro-inflammatory roles, inappropriate route and dose may disturb the balance of its functions.

Very recently *Cinha et al* (489) reported the existence of G-A/G-A (h2/h2) SNP in hematopoietic stem cell transplant donors, which renders recipients more susceptible to invasive pulmonary aspergillosis. h2/h2 SNP results in PTX3 deficiency that affects anti-fungal function of neutrophils in transplant recipients. Accordingly if there were asthma patients with similar deficiency, my study would provide key insights in understanding the development of asthmatic inflammation. That said,

determination of PTX3 levels in asthma subjects may serve as a suitable too to study etiology of the disease.

In summary, data shown in this thesis clearly shows that deletion of PTX3 plays a critical role in regulating functional capacity of immune system, thereby affecting immunological metrics in response to an allergen in a murine model of experimental asthma.

Future Directions

In my thesis, I have studied the response of PTX3+/+ and PTX3-/- mice to allergen OVA. Although OVA sensitization/ challenge regime reproduces asthma-like symptoms in mice, the clinical relevance of OVA as an allergen is debatable. Therefore, in order to simulate real sensitization to aeroallergens, researchers use alternate allergens including house dust mite, ragweed. and cockroach extract. Therefore, the response of mice to HDM as an allergen may be studied upon PTX3 deletion will be studied.

In other studies, it has been reported that PTX3 binds specifically to certain species but not all fungus. Similarly, my attempt to delineate allergic inflammation in the absence and presence of PTX3 in response to other allergens would help us understand whether regulation of inflammation in response to a wide variety of allergen is specific or not.

While the effect of PTX3 deletion was investigated in the context of CD4 T cells in chapter 4, I showed an increase in IL-17A producing γδT cells and ILC3s in the lungs of OVA exposed PTX3-/- mice. Since γδT cells and ILC3s play critical role in early stages of inflammation, it remains an interesting question whether deletion of PTX3 would affect these cells in a similar manner at an early stage and if the effect of PTX3 deletion is dependent on IL-17A favoring CD4 T cell-DC axis.

In my thesis, experiments were performed on female mice PTX3-/- mice were bred in house. Since PTX3 deletion renders female infertile, I could not breed knock-out male to knock-out females. Therefore I bred PTX3-/- male with PTX3+/- females that oftentimes resulted in insufficient number of females for experiments. As PTX3 deletion affects female reproduction, it could be a possible reason that PTX3-/- females were heavier in weight as compared to their PTX3+/+ counterparts. Recently, the relation between obesity, airway hyper reactivity and ILC3 mediated IL-17 prominent inflammation. This observation raised a question whether increased AHR and IL-17 inflammation in PTX3-/- females was due to obesity or vice versa? Therefore, I may design studies in future to delineate the involvement of obesity-pathway in PTX3-/- females and whether this pathway is IL-17A dependent or *vice versa*. Interaction of these pathways in asthma in context with PTX3 will be studied.

In the thesis, PTX3-/- and PTX3+/+ mice were of 129SvEvC57B6 or C57B6 background. In response to OVA, PTX3-/- mice showed an IL-17A dominant inflammatory response. For future studies, I may cross-breed these mice to A/G mice that are widely used to study IL-17 dominant inflammation. Given that Balb c mice develop a robust Th2 biased immunological response, I may validate my observation by cross breeding these mice to Balb c mice.

BMDCs are widely used to understand the phenotype and functions of DCs that are found at the site of inflammation. However, I observed phenotypic differences between lung DCs and BMDC DCs in PTX3-/- mice due to which I could not use BMDCs to study lung DCs. In future, I may attempt to investigate the cause of such differences between DCs from BM and the lungs in PTX3-/- mice. Furthermore, since there are no negative selection kits available for selecting different subsets of DCs, it is difficult to obtain unperturbed lung or LN DCs that hinders studying molecular mechanisms regulating their phenotype.

Altogether, my experimental data as reported in this thesis have laid the foundation of the project that aims to understand the role of PTX3 in allergic asthma. In future, our lab may administer exogenous PTX3 to evaluate whether PTX3 can be used as a therapeutic molecule to manage asthma.

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