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The prolactin inducible protein/gross cystic disease fluid protein-15 deficient mice develop anomalies in lymphoid organs

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ABSTRACT

The Prolactin Inducible Protein (PIP) is a 15 kDa protein secreted by normal apocrine glands, including salivary, lacrimal and sweat glands. PIP levels are normally low in the mammary glands of healthy individuals, but high levels have been observed in pathological conditions of the breast such as benign breast cystic disease and breast cancer. While the function of PIP is not well elucidated, accumulating evidence strongly point to a role in both innate and adaptive immunity. Using PIP deficient mice (*Pip* $^{-/-}$ mice) our laboratory demonstrated that loss of PIP function led to impaired T helper type 1 response and cell mediated immunity. In the present study we provide additional supporting evidence showing abnormal lymphocytic distribution in primary and secondary lymphoid organs of *Pip*$^{-/-}$ mice. Significant morphological changes in the Eustachian tube, an immune-protected site where PIP is normally found, were also associated with the absence of PIP. Collectively, these results further support an immuno-regulatory role for PIP and have implications for a spectrum of immune-related illnesses including otitis media and hearing loss as well as breast cancer.

1. Introduction

The human prolactin inducible protein (PIP), also known as gross cystic disease fluid protein 15 (GCDFP-15) is a 15 kDa glycoprotein which is highly abundant in the secretions of the lacrimal, salivary and sweat glands, as well as in human seminal plasma (Autiero et al., 1995, 1991). The expression of the human *PIP* gene has been linked with some diseases of the breast as PIP is not expressed in normal breast but is highly expressed in several benign and malignant diseases of the mammary gland (Haagensen and Mazoujian, 1986). PIP is regulated by several hormones in human breast cancer cell lines, most potently by androgens, glucocorticoids and interleukins (Blais et al., 1996, 1995, 1994; Murphy et al., 1987; Simard et al., 1989). The function of PIP is unknown, however accumulating evidence suggests that it plays a role in innate and adaptive immunity (Hassan et al., 2008; Umadat et al., 2013).

PIP expression has been observed in tissues found at strategic ports of pathogen entry, such as the mouth, skin, ears and eyes (Haagensen and Mazoujian, 1986; Mazoujian et al., 1983) where it is thought to be involved in innate and passive mucosal immunity. PIP is also detected in the saliva, sweat, ear wax, tears, amniotic fluid and milk (Murphy et al., 1987). It is speculated that PIP may contribute to some form of protection from infection for the developing embryos and newborns, lending support for a role in innate immunity. Indications that PIP also plays a role in adaptive immunity come from studies which showed that PIP acts as a ligand for the CD4 molecule of T cells, which serves an important function in the immune system (Autiero et al., 1995; Gaubin et al., 1999). PIP has been implicated in inhibiting HIV infection because it was shown to interfere with the interaction between CD4 and HIV envelope glycoprotein (gp) 120 (Autiero et al., 1997). Additionally, PIP is known to bind potently to the Fc fragment of IgG, further supporting a role as an immunomodulatory protein (Autiero et al., 1997, 1995). As well, the crystalline structure of PIP reveals binding sites for immunoglobulin molecules and PIP is a binding partner for zinc alpha

Abbreviations: PIP, prolactin inducible protein; GCDF-15, gross cystic disease fluid protein-15; ZAG, zinc alpha 2-glycoprotein

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2-glycoprotein (ZAG) (Hassan et al., 2008). Interestingly, PIP was shown to reside in a cleft within the ZAG molecule and that both molecules have common immunoglobulin-like folds. Studies have also shown that PIP and ZAG are induced by androgens in human breast cancer thereby suggesting a role in tumor progression as well (Hassan et al., 2008). Notably, the tissue specific expression of PIP in lacrimal and salivary glands is preserved in mice and rats, while its sequence is also conserved across several species thereby suggesting its functional importance (Mirels et al., 1998; Myal et al., 1994).

The mouse homologue (Pip), also referred to as mouse submaxillary/submandibular gland protein (SMGP), is highly similar to its human counterpart. Both mouse (Pip) and human (PIP) prolactin inducible protein show similar pattern of tissue expression (Nistor, 2008). Pip is also strategically located at various ports of entry for invading organisms such as skin, oral cavity, eyes, ears and bronchus (Hassan et al., 2009). Antibacterial activities of Pip related to host defense have also been reported by us (Blanchard et al., 2009; Lee et al., 2002; Nistor, 2008; Umadat et al., 2013), and others (Schenkels et al., 1997). We have shown that Pip binds to both mouse and human bacterial strains that inhabit the oral cavity, particularly those within the genus *Streptococcus*, thereby promoting their aggregation and inhibiting their proliferation and migration (Lee et al., 2002).

These important functional and tissue specific similarities between the human PIP and the mouse Pip therefore made the mouse an ideal model and an excellent tool to investigate PIP function. In light of such similarities, our laboratory generated the first *Pip*$^{-/-}$ mouse to address Pip function *in vivo* (Blanchard et al., 2009). The initial characterization of the *Pip*$^{-/-}$ mouse has been reported earlier (Blanchard et al., 2009). Our recent studies now support a role for Pip in adaptive immunity as well. Although no overt difference in phenotype was observed, differences in immune rigor were observed as *Pip*$^{-/-}$ mice were found to be more susceptible to invading organisms (Li et al., 2015). As well, differences in the bacterial flora within the oral cavity of *Pip* $^{-/-}$ mice compared to control mice were also observed. Most notable, was that bacteria of the genus *Streptococci* represented a higher percentage of the flora that resided in the oral cavity of the *Pip* $^{-/-}$ mice, and those of the genus *Neisseria,* were absent, when compared to wild type (WT) mice. Gram negative rods were also notably absent in the *Pip* $^{-/-}$ mice (Nistor, 2008). Further, in support of our hypothesis that PIP is involved in host defense we recently demonstrated that *Pip* $^{-/-}$ mice are unable to control *Leishmania major* infection (Li et al., 2015). We determined that on stimulation with toll-like receptor (TLR) agonists, bone marrow derived dendritic cells (BMDCs) from *Pip* $^{-/-}$ mice displayed significantly lower expression levels of co-stimulatory molecules and pro-inflammatory cytokines. Moreover, we observed a significantly impaired differentiation of naïve CD4^{+} T cells from *Pip* $^{-/-}$ mice into IFN-γ producing type 1 T helper (Th1) cells both *in vitro* and *in vivo* (Li et al., 2015).

The present study was designed to investigate whether Pip loss of function also affects the development of primary and secondary lymphoid organs. Here, we show that loss of Pip function resulted in distinct morphological changes and alterations in immune cells composition in both primary and secondary lymphoid organs.

2. Methods

2.1. Animals

Eight male mice (8–9 months old; 4 wild type (WT) – CD1, and 4 *Pip* $^{-/-}$ mice) were used for histological analysis of the middle ear. An additional group of 4 *Pip* $^{-/-}$ female mice (9 months old) and matching WT controls were euthanized and evaluated for pathological changes in the thymus, spleen and cervical lymph nodes. Same aged WT mice were purchased from Charles River Breeding Laboratory (Wilmington, MA. USA) to be used as controls. All mice were housed in the Central Animal Care Services Facility at the University of Manitoba. The study was approved by the University of Manitoba Animal Care and Use Committee.

2.2. Histological analysis of immune tissues

Animals were anesthetized in a chamber infused with 4% isoflurane, followed by euthanasia by cervical dislocation. Spleens, thymuses and cervical lymph nodes were dissected, fixed in 10% buffered formalin solution (Fisher scientific, Fair Lawn, NJ) for 18–24 h and paraffin embedded. Hematoxylin and eosin (H&E) staining was performed on 5 μm sections of the mouse tissues.

2.3. Histological analysis of the Eustachian tube

2.3.1. Animal perfusion

Following anesthesia induction by intraperitoneal (i.p.) injection of avertin, mice were euthanized by intra-cardiac perfusion with 3% paraformaldehyde (Fisher scientific, Fair Lawn, NJ) for 10 min, followed by a perfusion with 2.5% buffered glutaraldehyde solution for an additional 10 min. This procedure involves the dissection with open exposure of the thoraco-abdominal cavity, insertion of a catheter into the left ventricle and the creation of an opening in the right atrium. Under these conditions, the solutions perfused rapidly through the tissues inducing an almost instantaneous death of the animals.

2.3.2. Tissue fixation and decalcification

Following dissection, the cervical nodes and the middle ear were fixed in 2.5% glutaraldehyde solution overnight. After 24 h, the nodes were paraffin embedded and sent for sectioning and staining with Hematoxylin and Eosin (Sigma, St. Louis, MO). The ears were suspended in 4% isotonic ethylene diamine tetra-acetic acid (EDTA, Fisher scientific, Fair Lawn, NJ) solution under constant stirring for 3 weeks at 4 °C for demineralization. This procedure allowed for easy dissection of the middle ear structure with the bulla attached to preserve middle ear contents. The tissues were then washed with cold phosphate buffer and their post-fixation was done using 1% osmic acid solution for 2 h at room temperature under the fume hood. The sections were dehydrated in increasing concentrations of acetone, and after dehydration the sections were placed in different proportions of acetone and Epon (Sigma, St. Louis MO) mixture at room temperature to achieve the proper infiltration.

2.3.3. Tissue embedding and sectioning

The ear specimens were sectioned and embedded in pure Epon in plastic beam capsules and left in the oven at 60 °C for 48 h for the Epon to polymerize. Then, the embedded structures were taken out of the plastic capsules, trimmed and sent out to the Department of Human Anatomy and Cell Science at the University of Manitoba for sectioning and staining with 1% blue toluidine. The most representative sections of middle ear from WT and PIP$^{-/-}$ mice were then selected for light microscopy.

3. Results

3.1. Deficiency of PIP leads to morphological changes in lymphoid organs

Our recent studies (Li et al., 2015) demonstrated for the first time that loss of Pip function leads to impaired Th1 immunity. Although *Pip* $^{-/-}$ mice exhibit no overt phenotypic changes and appear to grow and develop normally (Blanchard et al., 2009), they however displayed many anomalies that point to defects in immune responses and immune cells. Further, enlarged lymphoid organs were often observed in several *Pip* $^{-/-}$ mice. In this study histological analysis was carried out to determine whether there were morphological and structural changes in immune organs associated with Pip deficiencies in *Pip* $^{-/-}$ mice.

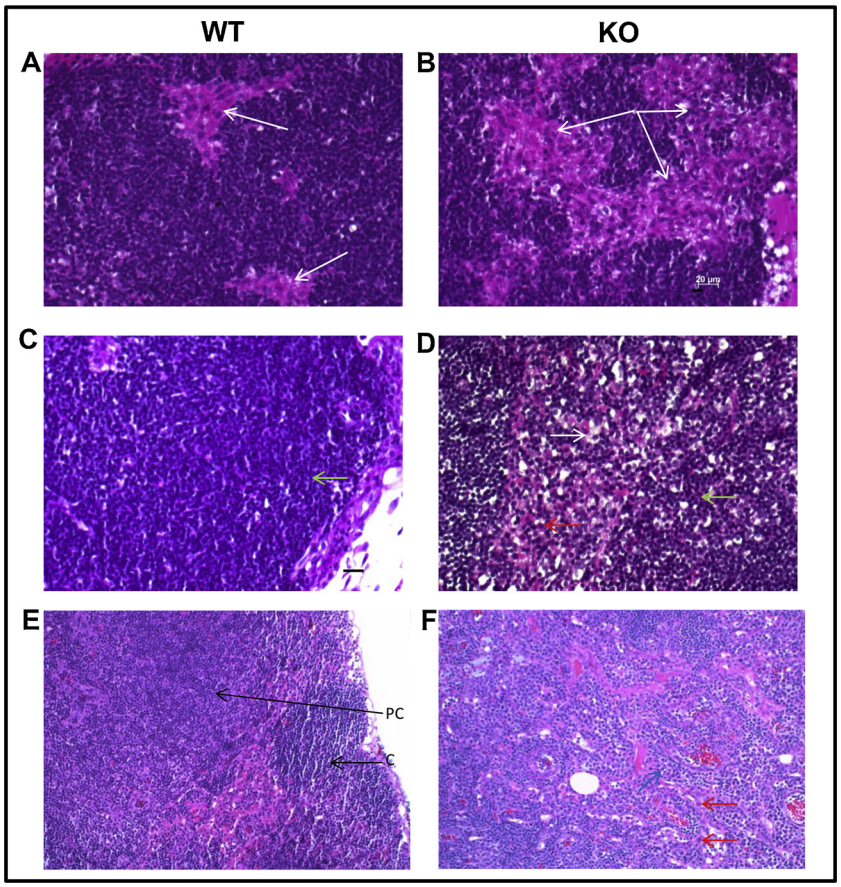

Fig. 1. Morphological features of WT and *Pip*$^{-/-}$ cervical lymph nodes. A. Para-cortical area in a normal cervical lymph node of a wild type (WT) mouse. B. The para-cortical area of a lymph node of a *Pip*$^{-/-}$ mouse shows increased number of sinuses filled with histiocytes. C. The cortical area of a lymph node of a WT mouse showing small lymphocytes. D. The cortical area of a *Pip* $^{-/-}$ lymph node showing small lymphocytes, plasma cells, scattered large lymphoid cells, and occasional histiocytes. E. Lymph node of a WT mouse showing cortical (C) and para-cortical (PC) areas. F. Prominent medullary cords filled with plasma cells in the *Pip* $^{-/-}$ mouse. White arrows: histiocytes; green arrows: small lymphocytes; red arrows: plasma cells; blue arrow: medullary cords. Scale bar: 20µm.

3.1.1. Morphological changes in cervical lymph nodes

Cervical lymph nodes as well as lymph nodes in the submandibular glands were dissected from *Pip* $^{-/-}$ mice and histological analysis carried out. Distinct morphological changes were observed compared to those of the WT control mice (Fig. 1). Lymph nodes from *Pip* $^{-/-}$ mice were often slightly enlarged with dilated sinuses. In the normal lymph nodes, the cellular compartments are distributed among three discrete regions: the cortex, paracortex, and medullary cords whereas in the *Pip* $^{-/-}$ mice, the distribution between these regions were disproportionate. Significant expansion of white pulp with prominent germinal centres were also obvious in the medulla and corticomedullary junction with increased number of plasma cells. In the normal lymph nodes, the medullary cords in the inner area of the lymph node contain T cells, plasma cells, B cells, as well as macrophages and dendritic cells.

3.1.2. Morphological changes in the spleen

Histological analysis was also carried out on spleens of *Pip* $^{-/-}$ mice and compared to their WT controls. As with the lymph nodes, we observed altered distribution of the compartments within the spleen. Normal spleen has two major compartments: red pulp and white pulp, and the organization is similar to that of the lymphoid tissue of lymph nodes. The most pronounced difference in the *Pip* $^{-/-}$ mice was that they displayed large expansion of the white pulp region of the spleen (Fig. 2).

3.1.3. Morphological changes in thymus

The medulla of the thymus contains dendritic cells that are similar to cutaneous Langerhans cells and lymph node interdigitating dendritic cells. Histological analysis of thymi of *Pip* $^{-/-}$ mice reveal frequent expansion of the medulla compared to morphology observed in control mice (Fig. 3) as well as an increase in plasma cells.

3.2. Loss of Pip function affects middle ear development in Pip $^{-/-}$ mice

The impairment in Th1 responses has been associated with otitis media, a middle ear infection caused by *Streptococcus pneumonia,* which can frequently lead to hearing loss. We have now demonstrated for the first time that loss of function of Pip leads to impaired Th1 immunity. Additionally, in previous reports, we showed that Pip binds to, and aggregates bacterial strains, most frequently, those of the genus *Streptococci*. Therefore, we examined whether a loss of Pip would also have an effect on middle ear development.

3.2.1. Morphological changes in Eustachian tube

Examination of the developing Eustachian tube revealed morphological differences between the *Pip* $^{-/-}$ mice and control WT mice. The epithelium of the Eustachian tube undergoes a transition from a ciliated columnar epithelium with goblet cells (at its origin in the middle ear) to a stratified squamous non-keratinized epithelium (at its opening into the naso-pharynx). Fig. 4A shows the epithelium of the Eustachian tube in the vicinity of the opening into the middle ear of the WT mouse. At this site the epithelium is ciliated with goblet cells. In Fig. 4B, at approximately the same site in the *Pip* knockout mouse, the ciliated columnar epithelium shows a tremendous increase in goblet cells when compared to the WT. This increased presence of goblet cells in the *Pip* knockout mice was the only observable significant change throughout the epithelium. These results suggest that Pip is important for the normal development of the Eustachian tube.

4. Discussion

The suggestion that Pip participates in innate immunity and protection of host against microbial infections is based on the observation that significant amounts of Pip is found in strategic locations that serve

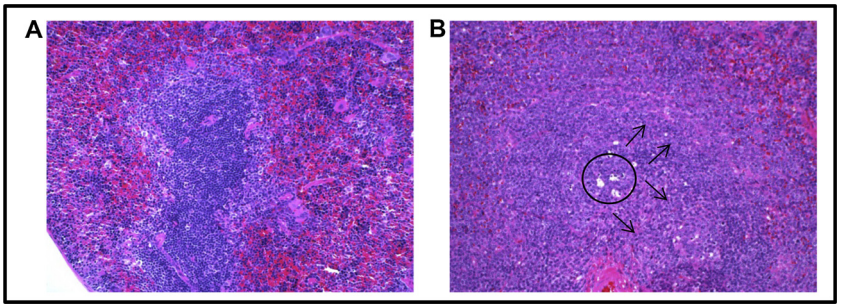

Fig. 2. Histological anomalies in spleen tissues of *Pip*$^{-/-}$ mice. A. Spleen tissue from a WT mouse. B. Expansion of **white**pulp (arrows) with germinal centres (circle) in the *Pip* $^{-/-}$ spleen.
Scale bar: 20μm.

as the first line of defense against invading organisms. These locations include mucosal-type tissues, bronchial submucosal glands, apocrine glands of the skin, saliva, and lacrimal fluid as well as immune-protective sites such as the middle ear (Umadat et al., 2013; Urbaniak et al., 2018). This hypothesis is further supported by studies from our laboratory which demonstrated that Pip can bind to various species of bacteria, particularly those of the genus *Streptococci*, thereby aggregating these organisms and inhibiting bacterial colonization of the oral cavity (Lee et al., 2002). Loss of Pip in mice have also been shown to significantly alter the oral microbiota. *Streptococci*, a Gram-positive bacterium, was found to represent a higher proportion of the bacteria inhabiting the oral cavity of *Pip* $^{-/-}$ mice (Nistor, 2008). Conversely, we showed that Gram negative bacteria such as *Neisseria* and Gram-negative rods were notably absent in the oral cavity of *Pip* $^{-/-}$ mice (Nistor, 2008).

Pip has also been reported to interact with various immune related molecules like the CD4 molecule, Immunoglobulin G (IgG), actin, and the zinc α2- glycoprotein (Hassan et al., 2009; Umadat et al., 2013). Furthermore, we have also shown that Pip influences the adaptive immune response and cell mediated immunity (Li et al., 2015). *Pip* $^{-/-}$ mice displayed defective Th1 responses and we showed impaired polarization of naïve CD4^{+} T cells into interferon gamma producing Th1 cells. Impaired signalling was also observed in dendritic cells and macrophages derived from *Pip* $^{-/-}$ mice (Ihedioha et al., 2018; Li et al., 2015).

Based on such observations, we now investigated whether Pip deficient mice exhibited any defects in the lymphoid organs. We characterised and compared the morphology of the lymphoid organs, lymph node, spleen and thymus of *Pip* $^{-/-}$ mice and the WT controls. We observed that the lymph nodes from *Pip* $^{-/-}$ mice were often slightly enlarged with dilated sinuses (Fig. 1B). The distribution of cellular compartments among the cortex, paracortex and medullary cord regions were also disproportionate in *Pip* $^{-/-}$ mice compared to their WT counterparts. Also obvious was a significant expansion of the white pulp with prominent germinal centres observed in the medulla and corticomedullary junction, and increased number of plasma cells (Fig. 1C–F) suggesting that lack of Pip affected normal lymph node development and further suggesting an important role for Pip in immune tissue development. Lymph nodes are organized to facilitate effective communication and interaction between the various immune cells in a bid to filter lymph from the organ it drains, recognize foreign antigens and clear invading pathogens. It is therefore logical that any defect in the normal development of the lymph nodes would affect proper function including Th1 responses. Thus, it is not unexpected that we observed lymph node abnormalities in *Pip* $^{-/-}$ mice.

Anomalies were also observed in other immune tissues such as the spleen of *Pip* $^{-/-}$ mice. The spleen is the largest secondary lymphoid organ in the body and is well vascularized. It is involved in systemic immune responses as it filters the blood from various parts of the body. Our previous studies (Li et al., 2015) showed that the proportion of CD4^{+} T cells in the spleens of *Pip* $^{-/-}$ mice was decreased compared to WT control mice.

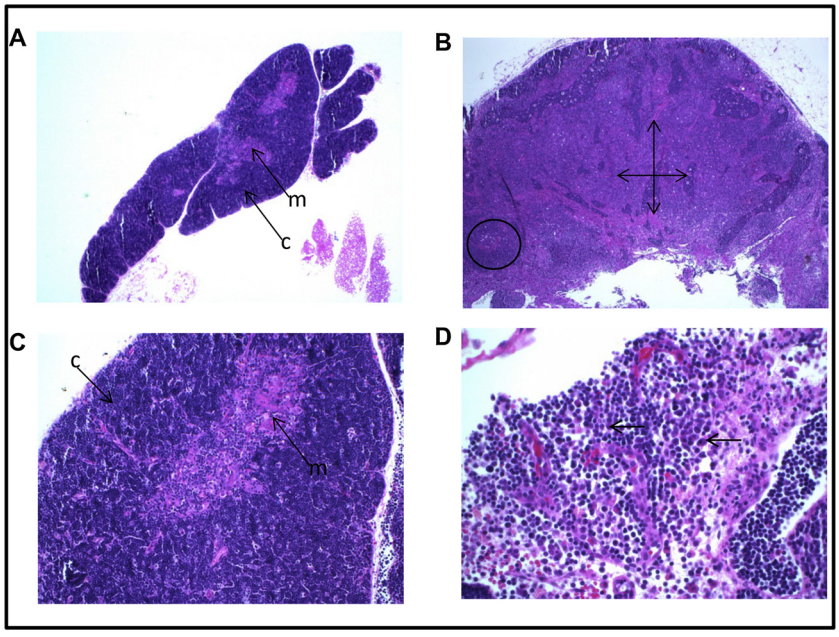

Fig. 3. Histological anomalies in thymus tissues of *Pip*$^{-/-}$ mice. A. Thymus from a wild type (WT) mouse shows primarily cortical region (c) with small medullar area (m). B. Expansion of medulla (arrows), and germinal centers (circle) in the thymus from a knockout (*Pip* $^{-/-}$) mouse. C. WT thymus showing no significant expansion of medulla; D. *Pip* $^{-/-}$ thymus with increased plasma cells (arrows).
Scale bar: 20μm.

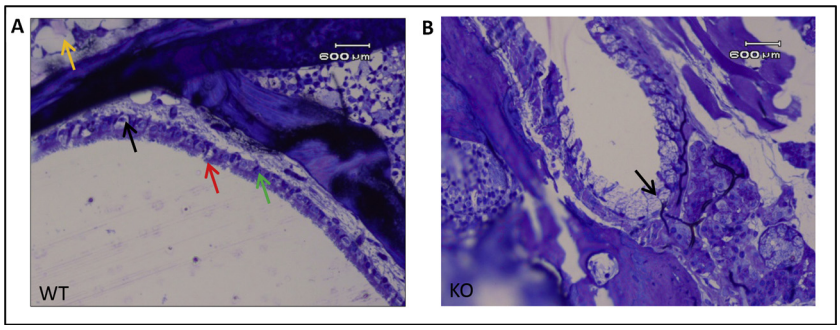

Fig. 4. Histological anomalies in the Eustachian tube of *Pip*$^{-/-}$ mice. A. A cross section of the Eustachian tube in the middle ear of wild type mice showing a thin, ciliated epithelium with clear staining goblet cells **(black**arrow, goblet cells; red arrow, cilia; green arrow, epithelium; yellow arrow, fat cells). B. Morphology of the middle ear in *Pip* $^{-/-}$ mice. Shows the structure of Eustachian epithelium with hyperplasia of goblet cells and disappearance of ciliated epithelia within the tube.
Scale bar: 600µm.

Similarly, abnormalities were also observed in the thymus. The thymus is an important immune organ in host defense. T cells undergo development and maturation in the thymus and are important in the adaptive immune system where they recognize the wide array of antigens encountered in the body. The medulla compartment of the thymus contains dendritic cells that are like cutaneous Langerhans cells and lymph node interdigitating dendritic cells. We observed an expansion of the medulla and an increase in plasma cells in the thymus of *Pip* $^{-/-}$ mice when compared to their WT counterparts. However, when we examined the relative proportions of thymocytes in *Pip* $^{-/-}$ mice and WT, they were comparable (Li et al., 2015), suggesting that the expansion of the medulla did not affect the numbers per se, but may have affected their function.

PIP is also found in high levels in the secretions of the ear canal (Haagensen and Mazoujian, 1986). We investigated whether Pip deficiency would impact ear canal development. *Pip* $^{-/-}$ mice showed increased goblet cells in the Eustachian tube epithelium in the middle ear (Fig. 4). Interestingly, Pip has been associated with otitis media, a middle ear infection, which affects almost 80% of all children before the age of 3 and can lead to hearing loss (Bergenfelz and Hakansson, 2017). Strikingly, otitis media is also known to be caused by *Streptococcus pneumoniae,* a genus of bacteria whose expansion has been shown to be inhibited by Pip (Lee et al., 2002). Moreover, *S. pneumoniae* ear infection has been associated with impairment in Th1 immune response and we have demonstrated that Pip deficiency results in Th1 impairment. Furthermore, an increase in goblet cells in the Eustachian tube in response to *S. pneumoniae* infection has been reported (Lin et al., 2012). In the *Pip* $^{-/-}$ mice, we observed an increase in the number of goblet cells in the middle ear epithelium, strongly suggesting that Pip may play an important role in Eustachian tube development and function. *S. pneumoniae* is an important human pathogen that colonizes the upper respiratory tract. In addition to otitis media, it causes potentially life-threatening diseases such as pneumonia, septicemia and meningitis. Our previous demonstration of the binding of Pip to *Streptococcus* (Lee et al., 2002) suggests that it provides protection against infections caused by *S. pneumonia*.

In conclusion, we observed that the deficiency of Pip led to anomalies in the histology of lymphoid organs including the lymph nodes, spleen and thymus. We also observed anomalies in the morphology of the Eustachian tube and tympanic membrane in Pip deficient mice. Collectively, these data strongly suggest that Pip is an important regulator of immune responses and may have implications in a wide range of diseases including *Leishmania* and otitis media. The high level of PIP in some breast cancers suggest that it may have an immune regulatory role in this disease as well.

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Author contribution

Chidalu Edechi contributed to data interpretation, compilation and manuscript writing, AK conducted ear experiments, MRN and HQ conducted pathological assessment of tissues, AAB undertook mouse experiments, JEU and YM for project supervision and intellectual contributions. Cynthia Ellison for intellectual contribution and technical input.

Declaration of Competing Interest

None.

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