The mast cell – B cell axis in lung vascular remodeling and pulmonary hypertension

Inaugural-Dissertation
to obtain the academic degree
Doctor rerum naturalium (Dr. rer. nat.)

submitted to the Department of Biology, Chemistry and Pharmacy
Freie Universität Berlin

submitted by

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August 2016

Research for the here presented doctoral thesis was conducted between September 2011 and August 2015 at the Keenan Research Centre for Biomedical Science of St. Michael's Hospital affiliated with the University of Toronto, in Toronto, Canada under the supervision of Prof. Dr. Wolfgang M. Kuebler.

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Date of defense: 21. February 2017

ACKNOWLEDGMENTS

Many people contributed to the success of my PhD and I want to thank all of them. Most of all I want to thank Dr. Wolfgang Kuebler, the best mentor a student could wish for. He supported me through both good and difficult times of my PhD project and always believed in me and my success. His door was always open for good advice and stimulating conversations. I feel privileged that I have been his student.

My gratitude also goes to Dr. Petra Knaus, who agreed to be my co-supervisor at the Freie Universität Berlin. Without her commitment, I could not have done my PhD in Toronto.

Furthermore, I want to say thank you to Dr. Martin Witzenrath, from the Charite Universitätsmedizin Berlin. He was an excellent mentor during my diploma thesis in his lab and he was the one who connected me with Dr. Kuebler, thus making this extraordinaire time in Toronto possible.

Thank you to Dr. Roland Buelow, for providing me with the B-cell deficient *JH*-KO rats, which were an important part of my experiments.

I also want to thank all the people from the 6th floor, the core facilities, the vivarium and the rest of the building of the Keenan Research Centre in Toronto for their amazing support. I especially would like to thank Asela Gamage, Julie Khang, Dr. Warren Lee, Dr. Katalin Szaszi, Dr. Andras Kapus, Xiaofeng Lu, Christopher Spring, Dr. Caterina Di Ciano-Oliveira, Lauren McKeeman and Danielle Gifford.

Not to forget the amazing people of the Kuebler lab in Toronto, I will miss you all, a big thank you to all of you: Jun Yin, Liming Wang, Simon Rozowsky, Hanpo Yu, Arata Tabuchi, Nicole House, Richa Parihar, Cecile Chupin, Adrienn Krauszman, Krishnan Ravindran, Ran Mo, Neil Goldenberg, Nataliya Potapova, Diana Zabini, Mark McVey, Sarah Weidenfeld, Mylash Mylvaganam, Maz Maishan, Jeremy Huang, Kevin Tran, Mariana Carreira Geralde and Yijie Hu as well as Julia Hoffmann from the Kuebler lab in Berlin.

Last but not least, I want to thank my family, who has been supporting me throughout my live, my mom and dad; Barbara and Werner Breitling, my grandparents; Gieselheid and Werner Süss and Paul and Frida Breitling, as well as my aunt in Vancouver, Ingrid Ehmann.

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1. INTRODUCTION

1.1 Pulmonary Hypertension

1.1.1 Definition and Classification

The German physician Ernst von Romberg was likely the first person to describe pulmonary hypertension (PH) in 1891. It was described by him, in an autopsy report, as “pulmonary vascular sclerosis” (109). However, several decades after Romberg’s discovery, PH was still regarded as an obscure disease and knowledge about the underlying pathological mechanisms were scarce (153). Interest gained traction in the late 1960s after a sudden and more than 20-fold increase in PH incidences, associated with the appetite suppressant aminorex fumarate, occurred (7, 45, 80). Based on these events, in 1973 the world health organization (WHO) in Geneva, Switzerland, hosted the first world symposium on PH. At this meeting, PH was defined by an increase in mean pulmonary arterial pressure (mPAP) to ≥25 mm Hg at rest (58), assessed by right heart catheterization, compared to a mPAP at rest of approximately 14 mm Hg in the healthy population (75). Furthermore, a simple classification of PH in primary (no known cause) and secondary (with known cause) PH was proposed (58). In the following decades the understanding of the pathophysiology of PH increased substantially. At the second world symposium on PH, hosted 25 years later in 1998, in Evian, France, PH was categorized into 5 different groups, reflecting the variety of underlying etiologies (108, 121). In brief this 5 groups consisted of (1) pulmonary arterial hypertension (PAH), (2) PH owing to left heart disease (PH-LHD), (3) PH owing to lung diseases and/or hypoxia, (4) chronic thromboembolic PH, and (5) PH with unclear multifactorial mechanisms. This categorization has since been maintained in its general form and has been modified only marginally at the following world symposia on PH in Venice, Italy in 2003, in Dana Point, California in 2008 and at the most recent one being held in Nice, France in 2013 (46).

1.1.2 Pathology

On a symptomatic level PH commonly presents with dyspnea on exertion, edema, fatigue, and chest pain and is often diagnosed only at a late, progressive disease stage (13). At the pathophysiological level (also see fig. 1), remodeling of the small to medium sized distal pulmonary arteries and endothelial dysfunction are hallmarks of the disease in all forms of PH, resulting in elevated pulmonary vascular resistance (PVR) vascular tone and arterial pressure (>25 mm Hg). As a consequence the right ventricle has to cope with an increased afterload, leading to right ventricular hypertrophy and ultimately death by right ventricular failure (11). The disease process can be subdivided into two parts. A first initial/ rapid response, which is characterized by injury and disruption of the endothelial cell layer, causing irreversible damage and cell death, as a result of predisposing genetic factors, such as mutations in the bone morphogenetic protein receptor type 2 (BMPR2) and/or molecular substrates or exposure to certain environmental or biological mediators or mechanical injury (77, 83). This can be for example the use of certain anorexigenic agents in case of PAH or vascular congestion in case of pulmonary hypertension due to left heart disease. The early stage is accompanied by a significant increase in oxidative stress and the infiltration of inflammatory cells, causing DNA damage and cell death (145). In a second stage apoptosis resistant pulmonary artery endothelial and pulmonary smooth muscle cells are excessively proliferating, contributing to vascular remodeling, which is characterized by intimal and adventitial thickening and increased perivascular fibrosis, which finally results in narrowing and occlusion of the pulmonary vasculature (83, 145). A central histological feature of severe IPAH are the presence of concentric neointimal and complex plexiform lesions, albeit their exact contribution in PH remains controversial (102). The vascular damage and remodeling is further aggravated by endothelial dysfunction, characterized by an imbalance of vasoconstrictive and proliferative over vasodilatory and anti-proliferative signaling pathways, affecting smooth muscle cells, prothrombotic mediators and inflammatory cytokines. One of the most prominent vasodilatory and anti-proliferative pathways, in the lung, is nitric oxide (NO) signaling, it is crucially involved in vascular homeostasis. NO levels are markedly reduced in PH patients, thus contributing to the disease process (74). The endothelin pathway,

on the other hand, is one of the most prominent vasoconstrictive and proliferative pathways, as described in more detail in section 1.1.5 (83). Since this work has its focus on class 1 and 2 of PH, namely pulmonary arterial hypertension and pulmonary hypertension due to left heart disease, both will now be discussed in more detail, in the following two sections, 1.1.3 and 1.1.4.

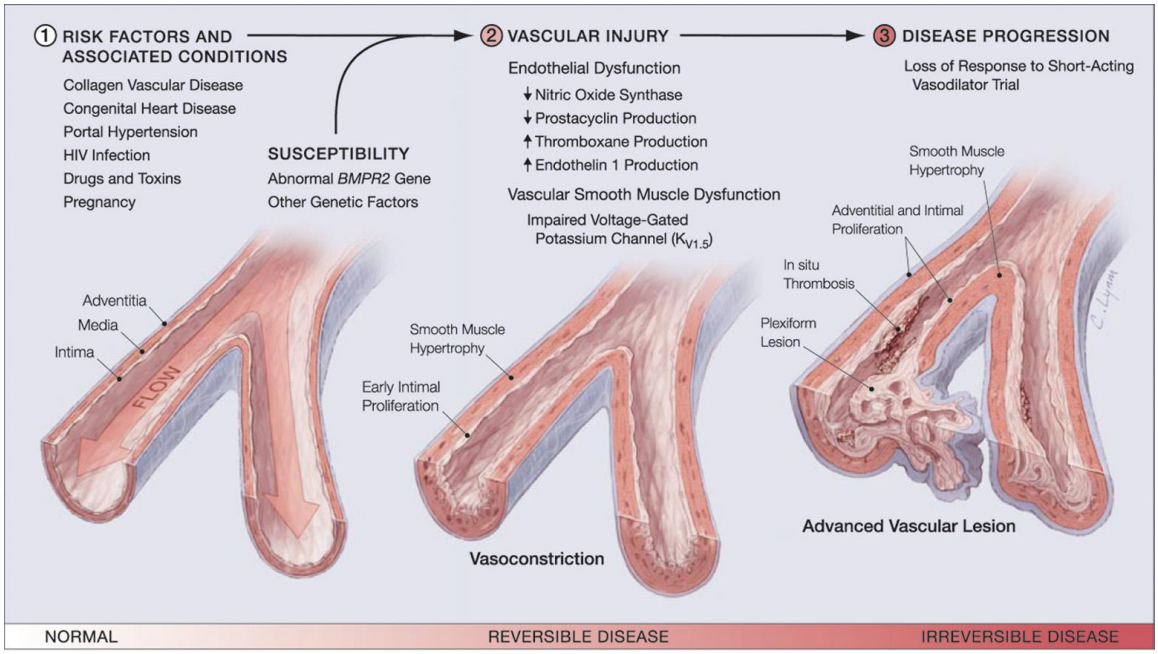


Fig. 1: Pathophysiology of PH. Reprinted from Gaine et al, *JAMA, 2000;* 284**:**3160–3168. Copyright © American Medical Association. Used with permission.

1.1.3 Pulmonary arterial hypertension

Prevalence in PH varies considerably based on the underlying etiology. While PAH is by far the most studied form of PH, it is regarded a rare disease, with a prevalence of about 15 cases/1 million adults (69). PAH may be heritable, idiopathic, or associated with drug or toxin exposures, or other medical conditions including connective tissue disease or human immunodeficiency virus (HIV) infection (122)**.** Several mutations have been associated with the development of PAH. Mutations in the gene encoding for the BMPR2, a member of the transforming growth factor beta (TGF-β) receptor family, have been detected in approximately 70% of patients with PAH of heritable origin and in 11-40% of patients with sporadic, idiopathic PAH, with no identified risk factors and no family

history (131). Notably patients with a BMPR2 mutation are diagnosed at an earlier age, are more likely to be unresponsive to vasodilator reactivity and appear to have more severe PAH at diagnosis, compared to patients without a BMPR2 mutation (39, 112, 131). In recent years, additional mutations associated with the formation of PAH have been discovered, including mutations in the activin-like kinase-type 1 (*ALK1*), endoglin (*ENG*), caveolin-1 (*CAV1*) and *KCNE3* also called *Task-1,* gene. However, these mutations appear to be less prevalent compared to mutations in the BMPR2 gene (81, 127). Despite its orphan status, several drugs have been developed and clinically approved for the treatment of PAH, as described in more detail in section 1.1.5.

1.1.4 Pulmonary hypertension due to left heart disease

Knowledge about the pathophysiology of PH-LHD is still scarce and currently no therapies directly targeting PH in LHD are available. Yet PH-LHD is presumably the PH group with the most incidences. Between 60%-80% of patients with chronic heart failure of systolic or diastolic origin develop PH-LHD (49, 78). PH-LHD is commonly defined by an increase in pulmonary capillary wedge pressure (PCWP) >15 mm Hg, concomitant with an increase in PAP. PH-LHD is often described as postcapillary PH, in contrast to all other groups of PH with a normal PCWP <15 mm Hg which are therefore described as precapillary PH (111). The formation of PH is caused by a backwards transmission in left arterial filling pressures, due to a chronic elevation in left atrial pressure as a consequence of left heart disease. Notably, an increase of 1 mm Hg in left arterial pressure corresponds with an increase of 1 mm Hg in diastolic PAP (17), illustrating the connection between LHD and the development of PH. However, it is important to point out that PH-LHD is not the sole result of an increase in passive, congestive vascular pressure. Instead it is often exacerbated by a reactive rise in PVR, which increases right ventricular afterload and thus, promotes right ventricular failure (78). Notably, an elevation in PAP is directly associated with increased mortality in heart failure patients. Based on a Cox proportional hazards model in a cohort of 400 patients with PH and LHD, a 9% increase in mortality per 5 mmHg increase in right ventricular systolic pressure was calculated, making increased PAP a strong independent prognostic

marker in heart failure patients (73) and underlining the need for therapies specifically targeting the lung in PH-LHD.

1.1.5 Current therapies

Untreated PAH is associated with high mortality. Data from the National Institute of Health (NIH) in the USA, following up 194 PAH patients between 1981 and 1985, at a time when there were no specific drugs against PAH available, show 1-year, 3-year, and 5-year survival rates of 68%, 48%, and 34%, respectively (26). In contrast, the recently conducted REVEAL study, in which 2635 PAH patients were enrolled between 2006 and 2009, shows markedly improved 1-year, 3-year, and 5-year survival rates of 85%, 68%, and 57%, respectively. Nevertheless, the mortality rate in PAH is still alarmingly high and still no ultimate cure is available. However, within about 20 years median survival rates more than doubled, from 2.8 years to more than 7 years, therefore demonstrating the advances made in PAH therapy (9), which have contributed to an increase in live expectancy, exercise capacity and overall quality of life, for PAH patients. However, it also needs to be pointed out that most progress has been made in WHO group 1 of PAH, while knowledge and therapeutic options for all other groups are still lacking. Over the past decades, three classes of drugs have been introduced and approved for the treatment of PAH, targeting the prostacyclin pathway, the nitric oxide pathway, and the endothelin pathway, thus primarily aiming at vasoactive and anti-mitogenic pathways (6) (fig. 2). In 1995 Epoprostenol, a synthetic prostacyclin (prostaglandin I_2) analogue, was the first drug approved by the FDA for PAH treatment (43). Prostacyclin is derived from arachidonic acid and is predominantly released from the endothelium. Prostacyclin is characterized by a range of beneficial properties, it is a strong vasodilator, anti-inflammatory, and inhibits platelet aggregation and smooth muscle cell growth. Its effects are mediated by activating G-protein-coupled receptors, leading to the generation of cAMP (18, 43). A second class of PAH drugs are phosphodiesterase 5 inhibitors, targeting the NO pathway. In PAH patients NO levels are generally low, while PDE5 levels tend to be elevated. Endothelial NO release activates soluble guanylate cyclase which subsequently promotes cGMP generation and as a consequence vasodilation. PDE5 promotes the degradation of cGMP resulting in an increase in intracellular

calcium levels and thus in vasoconstriction. PDE5 is the main phosphodiesterase in the lung and present in high quantities. Therefore PDE5 inhibitors such as Sildenafil result in increased cGMP levels thus supporting the vasodilative and anti-proliferative effects of NO (3).

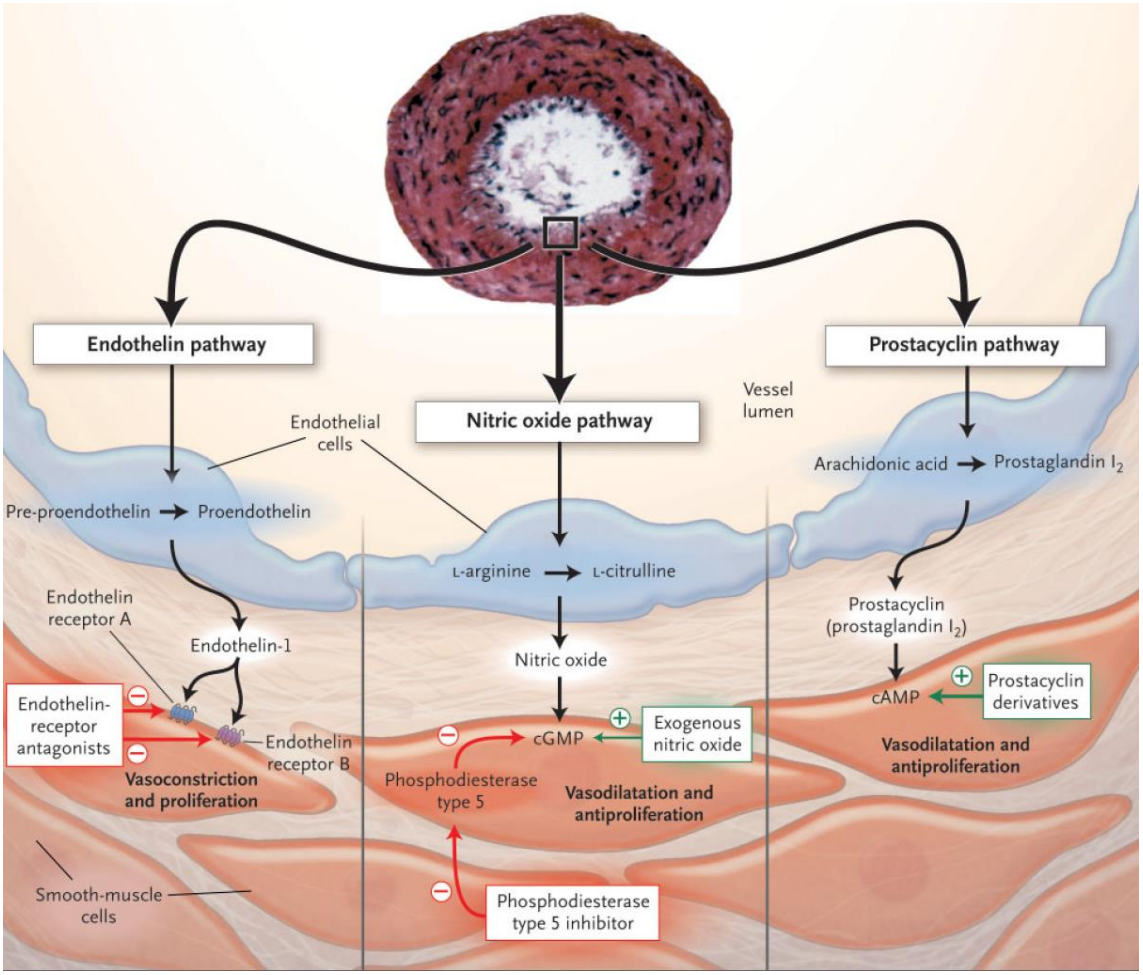


Fig. 2: Current therapies in PH. Reproduced with permission from Humbert et al., N Engl J Med 2004; 351:1425-143, Copyright © Massachusetts Medical Society

A second drug, which has been developed recently and which is also targeting the NO pathway, is Riociguat. It is a soluble guanylate cyclase stimulator and for the first time offers a treatment option for patients with chronic thromboembolic pulmonary hypertension (CTEPH) (120). Moreover, Riociguat has been demonstrated to significantly improve exercise capacity and pulmonary hemodynamics in PAH patients (50).

While NO levels are commonly low in PH, endothelin-1 levels are elevated. Endothelin-1 exerts its effects mediated by ET_A and ET_B receptor signaling. The ET_A receptor is supposed to elicit strong vasoconstrictive and cell proliferative effects, while ET_B receptors are supposed to contribute to ET-1 clearance and the production of the vasodilators NO and prostacyclin. Specific antagonists have been developed, which either block both ET_A and ET_B receptors, so called dual receptor antagonists, such as Bosentan or in order to maintain the beneficial properties of the ET_B receptor, single receptor antagonists, which only block the ET_A receptor, such as Ambrisentan (43). If pharmacological therapies fail, lung transplantation is the only definite cure for PH. However, mortality is high, the post–lung transplant survival rate for IPAH patients resides at 69.4% and 53.9% after 1-year and 3-year respectively; thus only patients with a live expectancy of less than 3 years should be considered for lung transplantation (152).

The general aim of this work is to expand the knowledge about the role of inflammation, in the development of PH. Specifically the focus lies on the interplay of innate and adaptive immunity and here most of all on mast cells and B-cells. Therefore, the following section (1.2) titled "*The immune system*", wants to give an overview on this topic, in particular on the cells (mast cells and B-cells), mediators (Cytokines) and mechanisms (autoimmunity), which are important to understand this work.

1.2 The immune system

1.2.1 The pulmonary immune defense

The lung comprises the largest epithelial surface area of the body and is in a steady contact with all kinds of particulate pollutants, allergens and infectious microorganisms. Different mechanical barriers, such as the hair in the nostrils, the cough and sneeze reflex or the mucociliary clearance, but also structural cells such as epithelial cells or fibroblasts act as the first line of defense and help to keep pathogenic microorganisms away from their target cells (31, 130). In case the first line of defense fails, the body has an elaborate system in place, consisting of the innate and the adaptive (acquired) immune system. The innate immunity provides an immediate, though non-specific response, which can act within minutes and which can generally be defined by its lack of memory (32). It consists of phagocytic cells (neutrophils, monocytes, macrophages), cells that release inflammatory mediators (basophils, mast cells, and eosinophils), and natural killer cells (fig. 3). Moreover, the innate immunity consists of molecular components including antimicrobial peptides (e.g. defensins, cathelicidins and lysozymes), complement, acute-phase proteins and cytokines. In the healthy lung, alveolar macrophages represent the most abundant cell type, with approximately 85% of all alveolar inflammatory cells (31). The adaptive immune response is consisting of B- and T-lymphocytes. It is defined by its high specifity and in contrast to the innate immunity, needs days to weeks to reach its full capacity, but it builds up a memory which in turn allows for a more rapid and effective response upon reinfection (130). B-cells possess immunoglobulins on their surface, when activated they can be secreted as soluble antibodies, from then called plasma cells, to provide protection against pathogens in the extracellular space. T-cells recognize pathogens, through MHC receptors. T-cells can be subdivided into different subsets, among them cytotoxic CD8 T-cells, which kill infected cells and regulatory CD4 T-cells which can activate other immune cells or offer help to B-cells (fig. 3) (94).

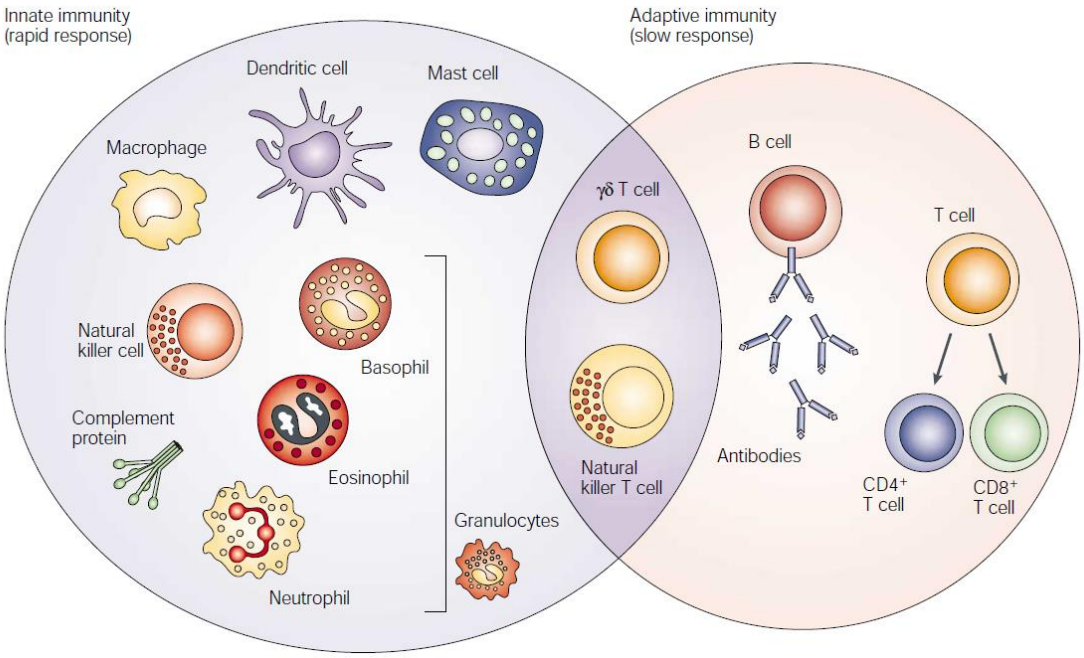


Fig. 3: Cells of the innate and adaptive immune system. Reprinted by permission from Macmillan Publishers Ltd: Dranoff et al., Nat Rev Cancer. 2004; 1:11-22, Copyright ©2004

1.2.2 Mast cells

Mast cells are long-lived mononuclear cells, with a size of 5 to 20µm. Based on the presence of granules in their cytoplasm they are, together with neutrophils, basophiles and eosinophils, categorized as granulocytes (88). Mature mast cells are tissue resident and do generally not circulate in the blood stream. They are found in most tissues, but are especially abundant in the perivascular space, around nerves and in tissues with direct contact to the external environment, such as the skin, the gastrointestinal tract, or the airways (23, 88). It is estimated that all mast cells taken together in their mass would equal the size of the spleen (146). Mast cells are derived from hematopoietic progenitor cells in the bone marrow from where they are released as undifferentiated CD34+ cells into the blood stream. Once they have arrived in their target tissues, mast cells develop and proliferate based on the surrounding microenvironment (79). One of the predominant drivers of mast cell differentiation is the cytokine stem cell factor, which interacts with the c-KIT receptor (CD117), which is present on the surface of all mast cells. However, many growth factors, cytokines and chemokines can influence the number and phenotype of mast cells (47). Mature mast cells possess a great heterogeneity in

their phenotype between different species as well as between different tissues in regard to their morphological, biochemical and functional properties. In rodents, mast cells are categorized based on their location, as mucosal (MMC) and connective tissue (CTMC) mast cells, respectively. MMCs are primarily located in the mucosa of the gastrointestinal tract and in the lamina propria of the respiratory tract, while CTMCs are primarily located in the submucosa of the gastrointestinal tract, in the skin, and in the peritoneum. In humans a clear classification based on their localization is, however, not feasible. Human mast cells are generally categorized based on their protease content, in those that contain only tryptase (MC_T) or only chymase (MC_C) and those that contain both tryptase and chymase (MC_{TC}) (30, 146). Concerning the lung, the MC_T is the most abundant subtype in the lung parenchyma, bronchial lamina propria and bronchial epithelium, while the MC_{TC} subtype surrounds pulmonary blood vessels with close proximity to vascular endothelial cells (23). Mast cell activation can be subdivided in FcεRI dependent and FcεRI independent activation. Mast cells express the high-affinity immunoglobulin E (IgE), F_C-epsilon receptor I (FcεRI) on their surface. The crosslinking of the FC receptor bound IgE, initiated by binding of multivalent antigens, leads to their activation. This triggers a signaling cascade via tyrosin kinases, phospholipase Cγ, inositol trisphosphate, diacylglycerin and protein kinase C which leads to the release of Ca^{2+} from intracellular stores, influx of extracellular Ca^{2+} and reorganization of the cytoskeleton and finally the release of mast cell mediators (23, 88). The FcεRI independent activation is triggered through many different mediators, such as specific microorganisms and their products, by components of the complement system, cytokines, neuropeptides, venoms, or physical stimuli (85, 115). Upon their activation, mast cells can then release a wide variety of different preformed and newly synthesized mediators such as histamine, heparin, tryptase, chymase and a whole array of cytokines including IL-4-, IL-6, IL-13 and TGF-β. There is evidence that mast cells critically contribute to inflammatory and fibrotic lung diseases, of which asthma is probably the best studied, however, there is also evidence for a mast cell involvement in idiopathic pulmonary fibrosis, chronic obstructive pulmonary disease or in pulmonary hypertension, as established by our own group and others (23, 66). Although, the view of mast cells as cells negatively contributing to allergic and inflammatory diseases may dominate, mast cells also play a critical role in a successful immune

response. Mast cells can neutralize venoms, they participate in direct killing of pathogens by phagocytosis and generation of reactive oxygen species and they recruit adaptive and innate immune cells to sites of infection, and likely have additional immune modulatory properties (30).

1.2.3 B-cells

B-cells and T-cells, are both lymphocytes and together constitute the adaptive (acquired) immunity. B-cells are best known for their role as antibody expressing and secreting cells. However, in addition they play a role as antigen presenting cells, are important for lymphoid organogenesis and they can have immune regulatory properties (36). B-cells are continually produced, initially in the fetal liver, later from haematopoietic stem cells in the bone marrow. The development to mature B-cells is primarily defined by the sequential steps of assembly and expression of antigen receptor genes. B-cell development starts at the pre-pro-B-cell stage, with the formation of the immunoglobulin heave chain (Igμ), which involves the RAG (recombination-activation gene) mediated rearrangement of variable (V), diversity (D) and joining (J) gene segments. The successful assembly of the Igμ chain leads to the association with the surrogate light chain to form a pre B-cell receptor (BCR). Subsequently, Pre-BCR signaling initiates the IL-7 mediated proliferation of pre-B-cells. The newly formed pre-B-cells then start with the rearrangement of the light chain, which this time only involves the V and J segments, culminating in the assembly and expression of the IgM BCR. This so called V(D)J recombination, is crucial for the enormous variability of B-cell receptors. It is estimated that B-cells are able to produce 10^{15} different antibody variable regions that originate from less than 400 genes. A high percentage of BCRs show auto reactivity; however, subsequent antigen dependent negative selection by receptor editing, clonal deletion or anergy ensures that under physiological conditions only BCRs with no auto reactivity are allowed to further develop. The naive immature B-cells are then leaving the bone marrow and are homing to the secondary lymphoid tissues, where they mature into either marginal zone or follicular B-cells (19, 32, 63, 118). If a B-cell with its unique BCR recognizes an antigen, it starts to proliferate rapidly. B-cell proliferation is

accompanied by a process called somatic hypermutation, small changes in the amino acid composition of the BCR, which ensures strong antigen binding. The proliferation of B-cells ultimately results in two distinct subsets, antibody producing plasma cells, and long lasting memory cells. For most antigens, B-cell activation requires the direct contact with T-helper cells, which also need to specifically recognize the same antigen, with their T-cell receptor. The activation and proliferation of B-cells is located in lymphoid tissues, since this is the only place where the density of lymphocytes is high enough that matching B- and T- cells can come together. In addition to B-cell activation, T-cells regulate the switching to different antibody isotypes, since naive B-cells only express IgM and IgD receptors on their surface. Based on the C terminal amino acid sequence of the constant region of the heavy chain, five classes of immunoglobulins can be specified; IgG, IgA, IgM, IgD, and IgE, plus four subclasses of IgG, and two subclasses of IgA (32, 94). They all have distinct functional characteristics and are specifically distributed in the body. Altogether IgG is the most frequent isotype (70, 148). All antibody isotypes have a common Y formed structure, consisting of two identical heavy and two identical light chains. At their N terminus they have variable regions, which contribute to the antigen binding. The variable N terminal site is commonly called Fab, while the C terminal site is called the FC region (32, 94). The so far discussed B-cells are categorized as so called B2 – B-cells. Apart from B2-cells there is a second subset of B-cells so called B1-cells. Depending on age, they make up between 1 and 9% of all B-cells (53). It is thought that B1-cells originate early in ontogeny (i.e. B1) in the fetal liver and later are self-renewing. B1-cells constitutively secrete antibodies, independent of activation or the presence of pathogens. These antibodies are termed natural antibodies. B1-cells primarily secrete IgM and IgA; an estimated 80-90% of all IgM and an estimated 50% of all IgA is derived from B1-cells. Moreover, B1-cells present antigens, stimulate T-cells and play a role in disposing cellular debris and molecular toxins. The question whether B1-cells may form memory cells is still under investigation; however, based on their properties, B1-cells are rather seen as cells of the innate immunity (114, 135). Finally, there is a third class of B-cells, so called regulatory B-cells (Breg). Bregs are associated with the suppression of excessive inflammatory responses, by secretion of anti-inflammatory cytokines, such as IL-10 or IL-35. Currently it is thought that any B-cell can differentiate into a Breg cell, at any

developmental stage (i.e. immature B cells, mature B cells, and plasma cells). The differentiation into Bregs seems to be triggered by its specific microenvironment (113).

1.2.4 Cytokines

The word “cytokine” is derived from the Greek word *cyto* meaning “cell” and *kinin* meaning “movement” (14). Cytokines are a diverse group of small signaling proteins with a size of around 25kDa (94). They include monokines, lymphokines, interleukins, interferons, colony stimulating factors and chemokines (14). Cytokines are secreted and are recognized by a multitude of both immune and non-immune cells and play a critical role in health and disease. Their effects can be autocrine, effecting the cytokine secreting cell, paracrine, effecting the cells in their close vicinity or endocrine, effecting distant cells (94), though, most cytokines have a short half-life and exert their biological effects in their immediate microenvironment (93). Cytokines modulate inflammation, immunity and hematopoiesis and can be pro-inflammatory such as TNFα, IL-1 or IL-6 as well as anti-inflammatory such as IL-4, IL-10, TGFβ or IL-13 (14, 54). In addition to their classical role in inflammation and immunity, cytokines play a critical role in regulating cell metabolism and organ function (129). Since cytokines are such powerful effector proteins, their signaling pathways are tightly regulated. Usually cytokines are only secreted temporarily, as reaction to a certain stimulus and subsequently are rapidly degraded again. A whole range of inhibitory mechanisms contribute to the termination of cytokine signaling. One such mechanism is the cytokine inhibition through tyrosine kinases, which act by dephosphorylating cytokine receptors. Another mechanism terminating cytokine signaling is through negative feedback loops, here the presence of cytokines activate specific inhibitors (94). Considering the far reaching role of cytokines in human physiology, it is not surprising that cytokines are linked to a multitude of different diseases, ranging from acute and chronic inflammatory and autoimmune diseases to neurodegenerative disease, the joint epidemics of diabetes and obesity and even depression (48, 129).

1.2.5 Autoimmunity

Autoimmunity is defined as a process by which the adaptive immune system reacts against self-antigens. It is characterized by the rise of autoreactive effector cells and self-directed antibodies, so called autoantibodies. If autoimmunity is not properly contained it can result in damage and dysfunction of tissues and in the development of a wide variety of autoimmune diseases. About 5% of the population is affected by an autoimmune disease, whereas the prevalence is generally greater in females than in males. Autoimmune diseases can be categorized as systemic for example rheumatoid arthritis or systemic lupus erythematosus or as organ specific, for example type 1 diabetes or Crohn´s disease (29, 94). During the V(D)J recombination in lymphocyte development, antigen receptors are assembled in a random manner, therefore the chance for the formation of autoreactive B- and T-cells is rather high. However, several protective mechanisms such as clonal deletion or anergy, are usually preventing the activation of autoreactive lymphocytes. It is assumed that in autoimmune diseases, these protective mechanisms fail, due to genetic susceptibility followed by the influence of certain environmental factors, including the exposure to infectious agents, to dietary components, to chemicals, xenobiotics, toxins or stress. The progression towards a chronic immune disease is then further driven by dysregulation of immune regulatory and inflammatory processes, such as an imbalance of pro- and anti-inflammatory cytokines or an abnormal autoantigen scavenging machinery (95, 96).

1.3 Pulmonary hypertension and inflammation

The general view of a range of diseases has been redefined over the past 50 years by linking them to inflammatory disorders, including heart disease, Alzheimer's disease, type 1 and type 2 diabetes and obesity (143). In a similar fashion inflammation is currently reshaping the general view on PH. There is increasing evidence for a key role for inflammation in the development of PH in humans as well as in experimental models (fig. 4). PH is often connected to inflammatory and autoimmune diseases, such as HIV or scleroderma. According to a French registry, 15.3% of PAH patients have a connective tissue disease (140). Moreover, 20% of schistosomiasis patients, a parasitic infection that causes a strong inflammatory response, develop PAH (116). However, an increased accumulation of B- and T-cells, macrophages, dendritic cells and mast cells have also been observed in plexiform lesions and around other remodeled vessels, in experimental models as well as in IPAH patients, independent of any comorbidities (60, 101, 142). Evidence for a functional role of mast cells was provided by our own group, in that mast cell deficiency, in the aortic banding model of PH-LHD, in *Ws/Ws* rats, significantly improved vascular remodeling and PH (66). In a later clinical pilot study, by Farah and colleagues, treatment with the mast cell inhibitor cromolyn proved beneficial, in a group of PAH patients (41). The notion of a functional role of the innate immune system in PH is increasingly expanded to the adaptive immune system. B-cells might promote vascular remodeling by cytokine and autoantibody production. Evidence for this provoking hypothesis has been provided recently, by Perros and colleagues, who for the first time described the presence of tertiary lymphoid tissue (tLT) in the lungs of iPAH patients, which to a large extend consists of B-cells (100). While Colvin and colleagues gave prof for the presence of lung tLT derived pathologic autoantibodies in PH (20). Moreover, the presence of autoantibodies against various targets such as anti-endothelial cell antibodies or anti–endothelin receptor type A antibodies have been described before (4, 8), as will be disused in more detail in the discussion (section 5.3). T-cells also play a role in PH, they seem to have detrimental as well as beneficial effects. Athymic rats, which lack T-cells, were reported to be more prone to the development of PH (137). On the other hand, depletion of CD4+ T cells, antigen-specific T helper type 2 (Th2) response, or the pathogenic Th2 cytokine interleukin

13 significantly improved pulmonary arterial muscularization (28). Notably, the infiltration of innate and adaptive immune cells is associated with – and likely orchestrated by – a concomitant release of inflammatory cytokines. Patients with idiopathic or associated PAH exhibit higher circulating levels and/or pulmonary expression of tumor necrosis factor, IL-1b, IL-4, IL-6, IL-8 and of a series of chemokines including CCL2 (formerly called monocyte chemoattractant protein (MCP)-1), CCL5 (RANTES) and CX3CL1 (fractalkine) (107, 140). Some of these cytokines and chemokines have been linked with a worse clinical outcome in PAH patients (104). Intriguingly, cytokines including IL-4 and IL-6, as well as other factors are capable of stimulating B-cell proliferation and the secretion of auto-antibodies (103). Especially IL-6 seems to stand out in the pathophysiology of PH. In various studies it has been described as an important effector molecule in PH (44). There are studies indicating increased IL-6 expression in experimental models as well as in PAH patients (10, 68). Furthermore, overexpression of IL-6 has been described to be sufficient to induce PH, in a transgenic mouse model (128). Notably, in an experimental model it has been demonstrated that inflammation in general is sufficient to cause vascular remodeling and muscularization of lung arteries. Mice that were exposed to either Aspergillus fumigatus antigen or to OVA, developed severe pulmonary arterial remodeling. This response required the presence of $CD4^+$ T-cells. Furthermore, the severity was correlated with Ig serum levels (28). Together with the observation that in experimental models, inflammation appears before the onset of vascular remodeling, it strongly suggests that altered immunity is a cause rather than a consequence of vascular disease (104). Interestingly there is increasing evidence, suggesting a link between mutations in the BMPR2 gene, which is associated with an increased risk for PAH, and inflammation. Recently it has been reported that BMPR2 deficiency increases the LPS stimulated lung and circulatory IL-6 and IL-8 production, in patients with BMPR2 deficiency as compared to control subjects (125). In an earlier study it was shown that there is a negative feedback loop between IL-6 and BMPR2 signaling, both *in vitro* and *in vivo*. Therefore a BMPR2 mutation might result in a dysregulation of the cytokine microenvironment and thus promote inflammation (55).

Overall, the current literature points to an active involvement of both innate and adaptive immunity, in all forms of PH. However, many gaps still await to be filled. Ultimately, the immune system could emerge as a rewarding new target, in the treatment of PH.

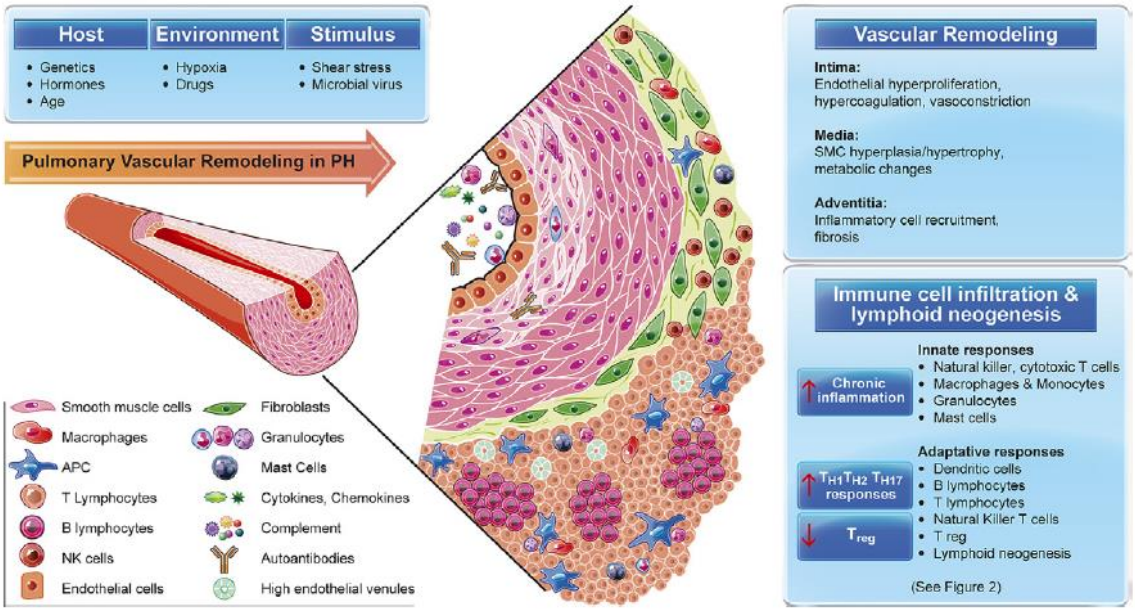


Fig. 4: Inflammation in pulmonary arterial hypertension. Rabinovitch et al. Circ Res. 2014;115:165-175, Copyright © American Heart Association, Inc. All rights reserved, reprinted with permission.

2. AIMS

Despite the progress made in recent years, effective treatment options, which could actually cure PH are still missing. More and more the role of the immune system is emerging as an interesting field in the research for novel therapies. Infiltration of cells of both the innate and the adaptive immune system in PH has been described in various studies. However, the mechanisms underlying the role of inflammation in PH are still poorly understood. In our own group we could demonstrate an important functional role for mast cells in PH. In order to gain mechanistic insights into the involvement of mast cells in PH, we performed a gene array analysis. Here we detected constituents of immunoglobulins as the most prominently mast cell dependent regulated genes in PH, thus hinting towards a mast cell – B-cell axis. We here propose a critical role of both, the innate and the adaptive immune system in the development of vascular remodeling and PH. Moreover, based on the well described role of IL-6 as a key effector molecule in PH, we propose IL-6 as the driver of this axis.

In particular we aim to test the hypothesis that:

1. mast cells exert their previously demonstrated pathogenic effect in PH via the recruitment of B-cells, the formation of tLTs and the development of autoimmunity which then promotes vascular remodeling and PH

   and

2. that mast cell mediate B-cell recruitment is regulated via release of IL-6.

3. MATERIAL AND METHODS

3.1 Materials

3.1.1 Chemicals, solutions antibodies

If not stated otherwise in the method section, salts and buffers were purchased from Sigma-Aldrich (St. Louis, MO), Thermo Scientific (Waltham, MA) or BioShop Canada Inc (Burlingtion, ON). Antibodies were purchased from Biolegend (San Diego, CA), Abcam (Cambridge, MA) or from R&D Systems (Minneapolis, MN).

Antibodies:

Antibody Name	Vendor	Product#	Assay
CD20	Genentech	n/a	neutralizing
IL-6	R&D	AF506	neutralizing
IL-6	Abcam	ab6672	IF
Tryptase	Abcam	ab2378	IF
CD45RA	Biolegend	202301	IF
CD45RA	Biolegend	202307	FC
Anti-rat IgG	Biolegend	405420	autoab detection
Alexa Fluor 555	Life Technologies	A21422	sec. antibody
Alexa Fluor 488	Life Technologies	A11034	sec. antibody

3.1.2 Commercially purchased kits

- Lung dissociation Kit mouse (Miltenyi Biotec, Auburn, CA)
- IgG rat ELISA kit (Abcam)
- IL-6 rat ELISA Kit (Thermo Scientific)
- BCA Protein Assay Kit (Thermo Scientific)

3.1.3 Animals

Male Sprague-Dawley rats were purchased from Charles River Laboratories, St. Constant, QC. Male JH-KO rats were generously provided, as part of a material transfer agreement, from OMT Inc. Palo Alto. All animal procedures were approved by the Animal Care Committee of St. Michael´s Hospital and complied with the principles and guidelines of the Canadian Council of Animal Care.

3.2 Methods

3.2.1 Aortic banding model

The aortic banding model is commonly used in our group to mimic PH secondary to left heart disease (62, 72, 151). For this procedure Sprague-Dawley rats with a body weight of 100±10g were anesthetized by intraperitoneal injection of ketamin (100 mg/kg bw) and xylazine (10 mg/kg bw). Subsequently rats were placed in the supine position, the chest wall was shaved and sterilized, and a left thoracotomy was performed in the third intercostal space while rats were ventilated with 100% O_2. In the aortic banding group, the ascending aorta was isolated from the connective tissue and partially occluded by a titanium clip (Hemoclip^{TM}, Teleflex Medical, Markham, ON) with a pre-defined internal diameter of 0.8 mm (fig. 5).

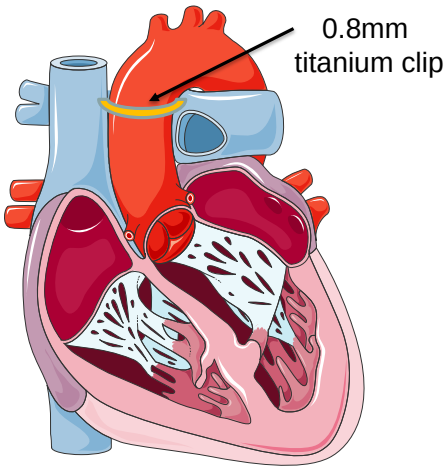


Fig. 5: Aortic banding. Schematic of the heart, illustrating the supracoronary placement of the titanium clip around the ascending aorta. The figure was produced using Servier Medical Art, available from www.servier.com/Powerpoint-image-bank.

Sham operated rats underwent the same surgical procedure including the isolation of the ascending aorta, but the banding clip was positioned in the surrounding mediastinal tissue instead of around the aorta. The thoracic cavity and subsequently the skin were carefully resealed by surgical sutures, and rats were allowed to recover from anesthesia. Buprenorphine (0.05mg/kg) subcutaneous and Anafen (5mg/kg) subcutaneous were administered once intra-operatively. For postoperative analgesia, rats were given 0.1 mg/kg Buprenorphine (Buprenex) subcutaneously at the first two postoperative days. After 9 weeks rats developed moderate PH, concomitant with congestive heart failure, evident by the appearance of RV hypertrophy, elevated PVR, vascular remodeling, endothelial dysfunction, and increased vessel tone, with aortic flow, systemic arterial pressure, and left ventricular ejection fraction (LVEF) being normal (150).

3.2.2 Monocrotaline (MCT) model

MCT is a plant alkaloid, derived from *Crotalaria spectabilis* seeds (90) and has been used since the 1960s to study PH in animal models (16). For our experiments, male Sprague-Dawley rats of 210±10g body weight received a single injection of MCT (Sigma Aldrich) intraperitoneally at a dose of 60mg/kg at the inception of the study. The MCT was initially dissolved in a 0.5N solution of HCl and subsequently neutralized with a solution of 0.5N NaOH to a physiological pH of 7.4. Control rats received a single intraperitoneal injection of saline. Final measurements were taken 3 weeks after MCT injection, at which point, rats had established severe vascular remodeling and PH. The MCT model is commonly seen as a model mimicking PAH (98).

3.2.3 Sugen-Hypoxia (SuHx) model

Male Sprague-Dawley rats of 210±10g body weight received a single subcutaneous injection of 20 mg/kg SU5416 (Tocris Bioscience, Ellisville, MO) suspended in CMC (0.5% carboxymethyl cellulose, 0.9% sodium chloride, 0.4% polysorbate 80, 0.9% benzyl alcohol in deionized water), at the inception of the study or diluent alone as vehicle control. For the first three weeks, rats were housed under hypoxic conditions with 10% O_2 in a ventilated chamber (Biospherix,

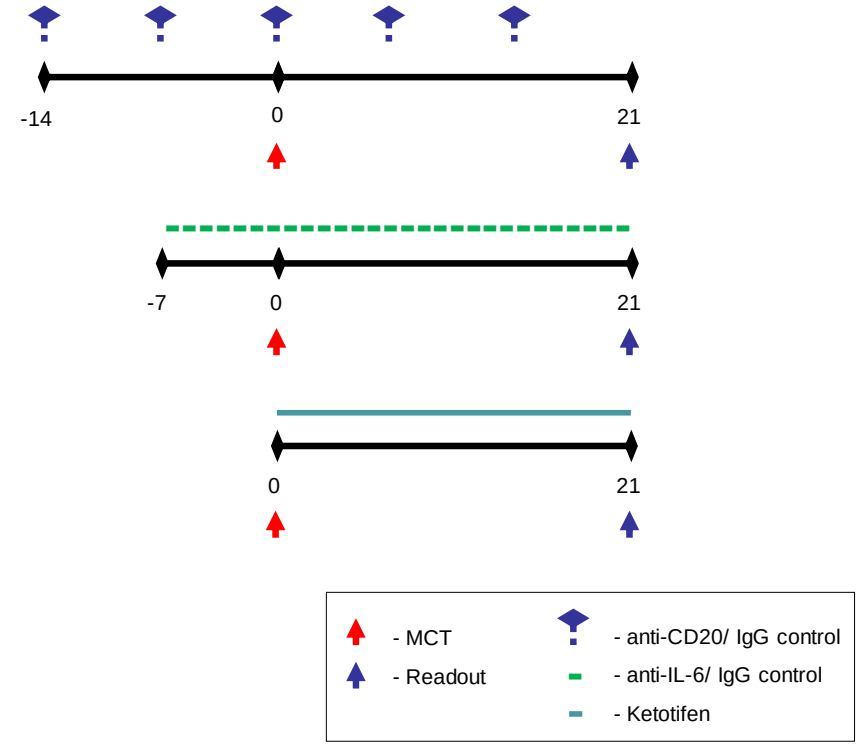
Lacona, NY), followed by two weeks of housing under normoxic conditions in normal room air. The model has been described for the first time by Taraseviciene-Stewart and colleagues in 2001 and has since been established as a widely accepted model for PAH (136).The advantages of this model are especially seen in the occurrence of so called plexiform lesions, which are a hallmark of severe human PAH and are absent in most of the other experimental PAH models (1).

3.2.4 Treatment regimes with ketotifen, anti-CD20 and anti-IL-6 neutralizing antibodies

B-cells were depleted in SuHx or MCT treated rats by weekly intraperitoneal administration of 5mg/kg of a murine monoclonal anti-CD20 antibody. SuHx and MCT controls received normal mouse IgG (generously provided by Genentech, San Francisco, CA) to exclude non-specific effects of immunotherapy. Injections started two weeks prior to the induction of PH by MCT treatment or SuHx respectively.

For *in vivo* neutralization of IL-6, affinity purified polyclonal anti-IL-6 neutralizing antibodies raised in goats immunized with purified *E.coli*-derived recombinant rat IL-6 (R&D Systems) was administered intraperitoneally at 1 µg/rat/day. The amount of the IL-6-specific antibodies administered was chosen according to the neutralization dosage indicated by the manufacturer, and to previous studies (5, 124). IL-6 depletion was started one week prior to the induction of PH by either SuHx or MCT treatment. A control group was injected with normal goat IgG.

Ketotifen can inhibit the release of mast cell mediators and is therefore termed a mast cell stabilizer (67). For our experiments, ketotifen fumarate (Sigma Aldrich) was administered orally with the drinking water at a dose of 1mg/kg bw/day, starting at day 1 of MCT treatment, a dose which has been shown to be effective in blocking mast cells, in previous studies (66). An overview of the treatment regimes is given in figure 6.



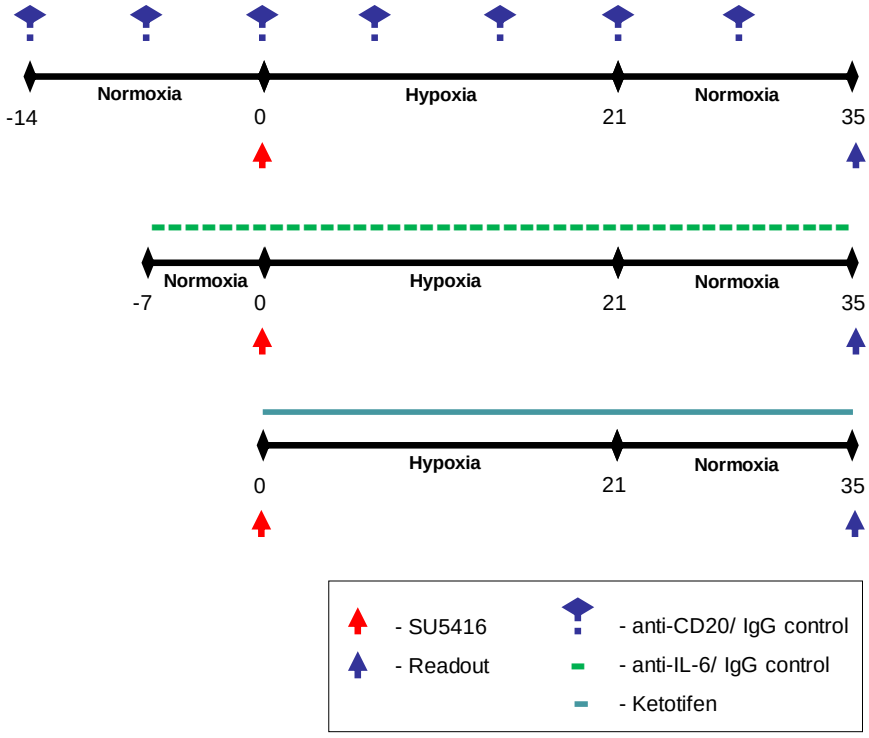


Fig. 6: Flow chart depicting the different treatment regimes for anti-CD20, anti-IL-6 and ketotifen, in the MCT (above) and SuHx (below) model respectively.

3.2.5 Right ventricular systolic pressure measurements

Final hemodynamic characterization (terminal experiment) was performed nine weeks after induction of PH by supracoronary aortic banding, 5 weeks after inception of SuHx or 3 weeks after injection of MCT, respectively. To this end, rats were anesthetized by an intraperitoneal injection of a triple combination of medetomidine (0.5 mg/kg bw, Domitor®), fentanyl (0.05 mg/kg bw), and midazolam (5 mg/kg bw, Dormicum®) and depth of anesthesia was verified by checking the toe pinch reflex. Rats were tracheotomized, a tracheal canula was introduced and animals were ventilated with a tidal volume of 6 mL/kg. Next, a 1.4 mm pressure catheter (Millar Instruments, Houston, TX) was introduced via the right jugular vein and advanced to the right ventricle, for continuous monitoring of right ventricular systolic pressure, using LabChart software (AD Instruments, Colorado Springs, CO). The correct position of the catheter tip, was determined by the pressure tracings, which adopted a characteristic patern, once the catheter was placed in the right ventricle, as seen in figure 7. RVSP values were recorded for at least 30s and the average taken, for further analysis. At the end of the procedure, rats were euthanized by exsanguination, through cardiac puncture.

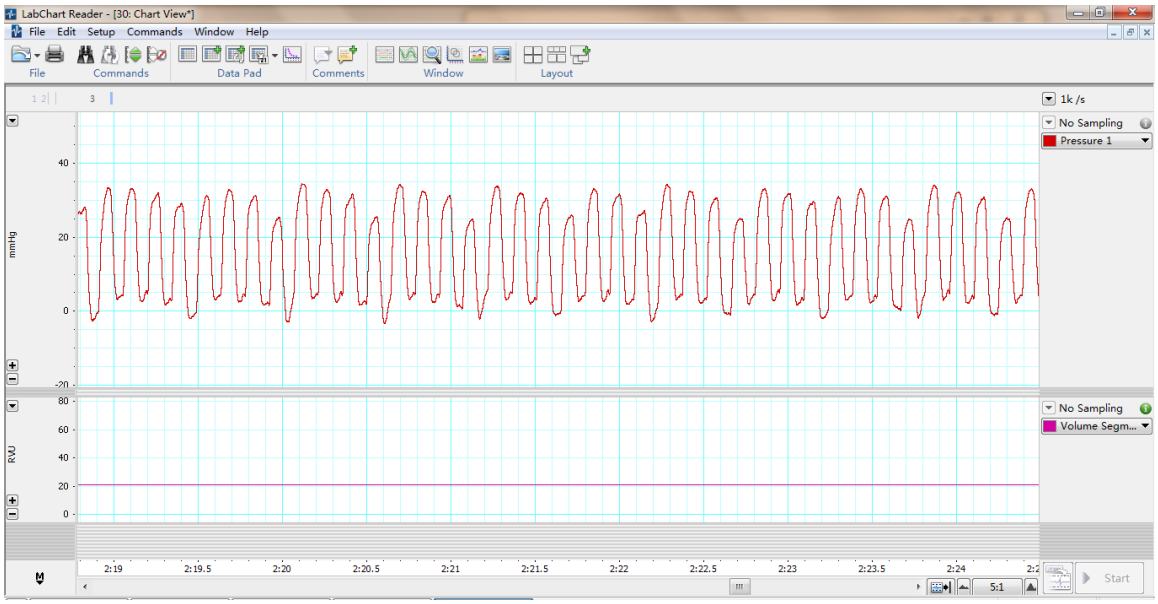


Fig. 7: Representative RVSP tracing, recorded using LabChart

3.2.6 Histology sample preparation and vascular remodeling

For histology, lungs were excised, intratracheally instilled with 4% paraformaldehyde (PFA) and fixed for 24h hours. After dehydration, lungs were paraffin-embedded and cut into 5µm slices. For CD45RA staining of slides from MCT-treated mast cell deficient *Ws/Ws* rats (141), histological samples from a prior study were used (66). For assessment of lung vascular remodeling lung sections were stained with haematoxylin and eosin. Pulmonary vessels were categorized based on their external diameter as small (20-50 µm) and medium (50-100 µm) sized. Medial wall thickness was determined as percentage of external vessel diameter. At least one slide with 20 vessels per rat was analyzed, with at least 4 rats per group.

3.2.7 Toluidine blue staining

For mast cell staining paraffin-embedded lung tissue sections were dewaxed, rehydrated and incubated with 0.05% w/v toluidine blue for three min. Mast cell numbers were counted in 20 fields per slide and expressed as mast cells per mm^2.

3.2.8 Immune fluorescence

For immune fluorescence staining, slides were deparaffinized and rehydrated. Next, slides were put in 100°C tris-EDTA buffer (10mM Tris Base, 1mM EDTA solution, 0.05% Tween 20, pH 9.0) for 20min for antigen retrieval. Slides were allowed to cool down and were put in a 0.3M glycine solution for 1h to block auto fluorescence. Slides were blocked with 10% BSA in PBS for 1h and subsequently were incubated with the primary antibody overnight, at 4°C. At the next day, slides were incubated with a fluorophore associated secondary antibody for 1h and finally covered with a DAPI containing mounting medium and a cover slide. As control, slides were incubated with secondary antibody only. For autoantibody measurements, rat plasma was used as the primary antibody, as previously described (20). The average fluorescence intensity was measured for at least 20 fields per slide. Images were acquired using an OLYMPUS BX50, for the BALT analysis and a Quorum WaveFX-X1 Borealis Spinning Disc Confocal System on

a Leica DM4000 Upright microscope for autoantibody detection and images were analyzed using ImageJ (National Institute of Health, Bethesda, MD).

3.2.9 ELISA

IL-6 and IgG ELISA measurements were performed using either lung homogenate or plasma. Blood was obtained by cardiac puncture after rats were sacrificed and blood was centrifuged at 2000 x g for 10min. Lung homogenate was prepared by putting a 30mg piece of lung into lysis buffer (Roche, Mini Complete) and homogenizing it with an electric homogenizer on ice. Samples were stored immediately at -20°C. Protein concentration in lung homogenate was measured using a BSA assay (Thermo Scientific) and 60µg of sample were loaded per well into a 96 well ELISA plate. ELISAs were then performed according to the manufacturer´s instructions.

3.2.10 Flow Cytometry

Flow Cytometry was performed to determine CD45RA positive cells (B-cells) in lung, spleen, lymph nodes, blood and bone marrow. Lung cells were separated using a mouse lung dissociation kit (Miltenyi Biotec) according to the manufacturers protocol. Spleen and lymph node cells were obtained by homogenizing the respective tissues through a 40µm cell strainer. Bone marrow was obtained by removing the tibia and femur and flushing out the respective cells with PBS. Red blood cells were depleted by ACK lysis buffer (1,5M Ammoniumchlorid (NH4Cl), 100mM Kaliumhydrogencarbonat (KHCO3) 10mM Triplex111 (EDTA-2Na)) treatment, in blood, spleen and in all other tissues as necessary. All cells were suspended in cell staining buffer (Biolegend, San Diego, CA) and stained for 20min with a PE labeled CD45RA antibody (Biolegend) (1µl of antibody per 10^6 cells in 100µl volume). Samples were analyzed using a MACS Quant flow cytometer (Miltenyi Biotec). Results were analyzed using MACS Quantify data acquisition and analysis software (Miltenyi Biotec).

3.2.11 Microarray analysis

Lungs of three control, three aortic banding alone, and three aortic banding plus ketotifen treated rats were harvested and the complete RNA extracted (Stratagene Absolute RNA Miniprep Kit). Three µg of RNA of each sample were processed according to the One-Cycle Target Labeling protocol (GeneChip Expression Analysis, Affymetrix, Santa Clara, CA). Before and after the amplification process, the concentration of RNA was measured by Nanodrop ND1000 (Thermo Scientific, Waltham, MA) analysis. The samples were hybridized to the GeneChip Rat Genome 230 2.0 Array. Gene Array data has been deposited at the NCBI's Gene Expression Omnibus under accession number GSE84704.

3.2.12 Data analysis

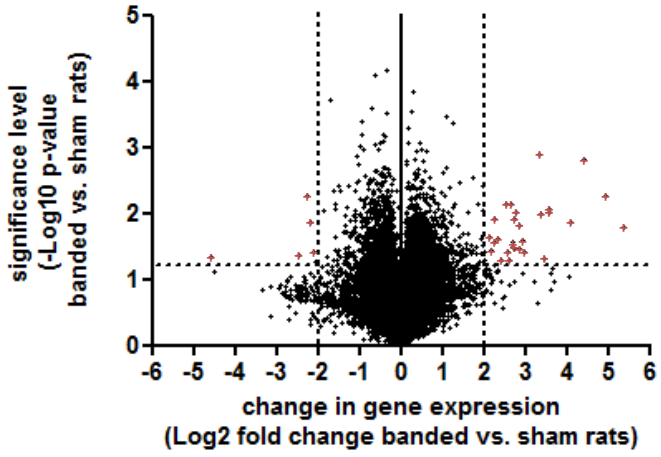
Data are given as means ± SEMs. Different groups were either compared by t-test or ANOVA followed by Dunnett's multiple comparison test (Prism, version 5.0, GraphPad Software Inc., La Jolla, CA). Statistical significance was assumed at $p < 0.05$.

4. RESULTS

4.1 Differentially regulated mast cell dependent genes

Mast cell infiltration has been described as a common feature in experimental PH as well as in PH patients of different ethiologis. An important functional role for mast cells in experimental PH was established before in our own group (27, 66). In order to gain further insights into the underlying mechanisms by which mast cells contribute to the development of PH, a gene array analysis was performed, using lungs of three healthy control rats, three aortic banding rats (mimicking PH-LHD) with no further treatment and three aortic banding rats treated with the mast cell stabilizer ketotifen.

A



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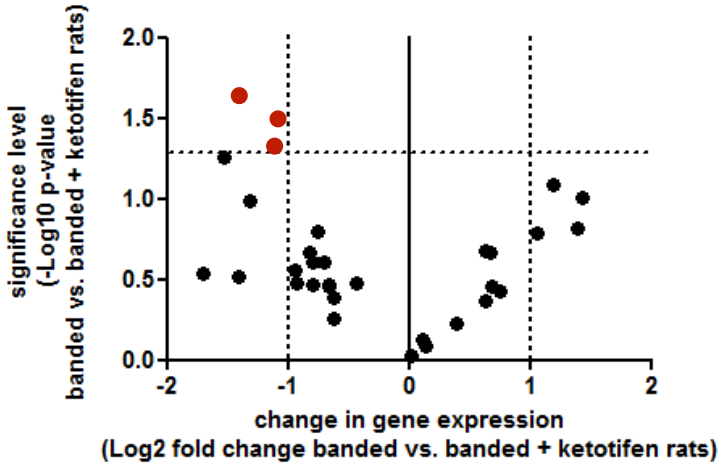


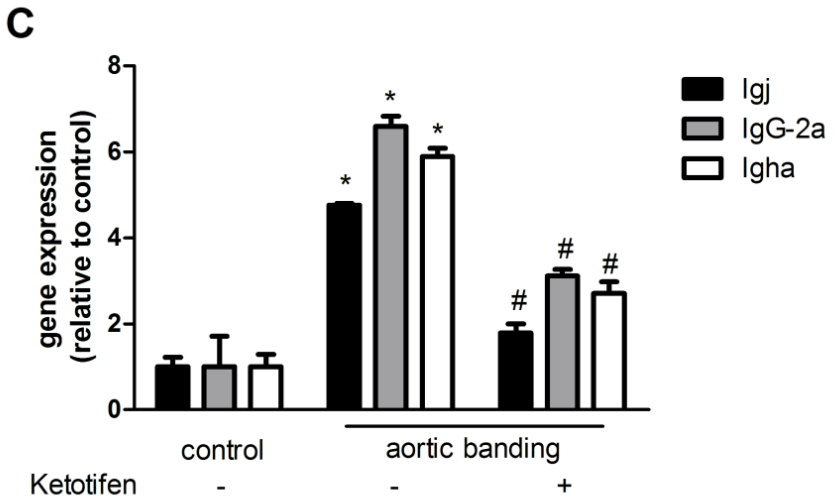


Fig. 8: Gene array analysis. A) Volcano blot from whole rat genome microarray analysis (31000 genes in total), 32 genes are significantly regulated, marked in red (≥4-fold change with $p<0.05$) in aortic banding compared to the control group. B) Volcano blot of these 32 genes, in banded alone compared to banded plus ketotifen treated. Marked in red are genes more than 2-fold and with $p<0.05$ regulated. C) Bar graph of the three genes (Igj, IgG-2a and Igha) differentially regulated in control vs. banding and again in banding vs. banding plus ketotifen (n=3).

Out of 31000 analyzed genes, 32 were differentially regulated (marked in red) with more than ≥4-fold change with $p<0.05$, in lungs of aortic banding compared to control rats, as depicted in a volcano blot in fig. 8A. Out of these 32 genes only three were counter regulated again by mast cell stabilization with more than 2-fold change and with $p<0.05$, marked in red, as depicted in another volcano blot in fig. 8B. All three genes were encoding for constituents of immunoglobulins, namely Igj, IgG-2a and Igha (fig. 8C) which were all upregulated in aortic banding rats as compared to controls, and again downregulated by treatment with the mast cell stabilizer ketotifen. These findings point to an important role of mast cells in the regulation of the adaptive immune response in PH.

4.2 Aortic banding time course analysis

To consolidate that mast cell accumulation may precede Ig production and hence, potentially regulate the adaptive immune response in PH, we next performed a time course analysis. The temporal sequence of immune cell infiltration and disease progression where recorded throughout the 9-week time course of the aortic banding model. For this purpose, right ventricular systolic pressure (RVSP), vascular remodeling, pulmonary mast cell infiltration and plasma IgG levels were analyzed at 10 different time points. RVSP went up quickly and plateaued at around day 20 (fig. 9A), while vascular remodeling went up more slowly and progressively (fig 9B), both parameters confirming the development of PH. Mast cell infiltration went up instantly and peaked already at around day 3 after which the further increase proceeded more moderately (fig 9C). IgG levels on the other side, stayed on a relatively low level for the first 10 days and then went up sharply, at the end of the time course at day 64, IgG levels had more than tripled (fig. 9D). These findings demonstrate that mast cell infiltration indeed precedes Ig production and therefore further solidifies the notion of a potential connection between innate and adaptive immunity in the development of PH.

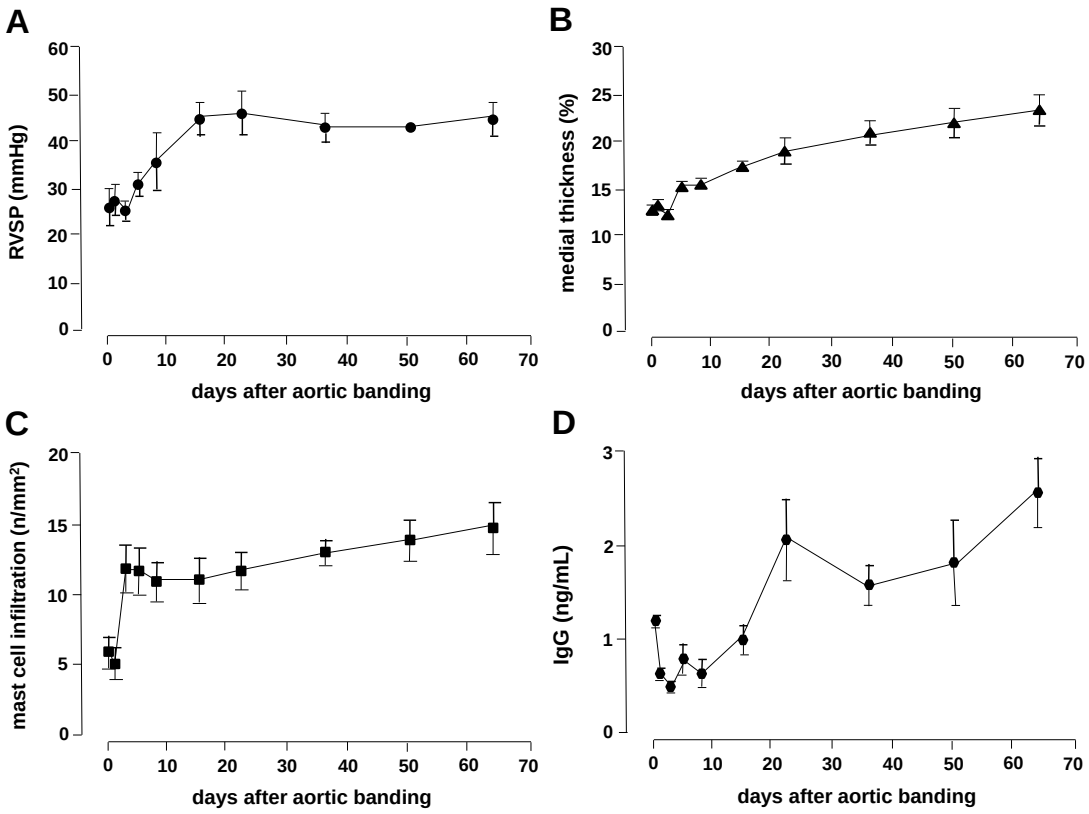
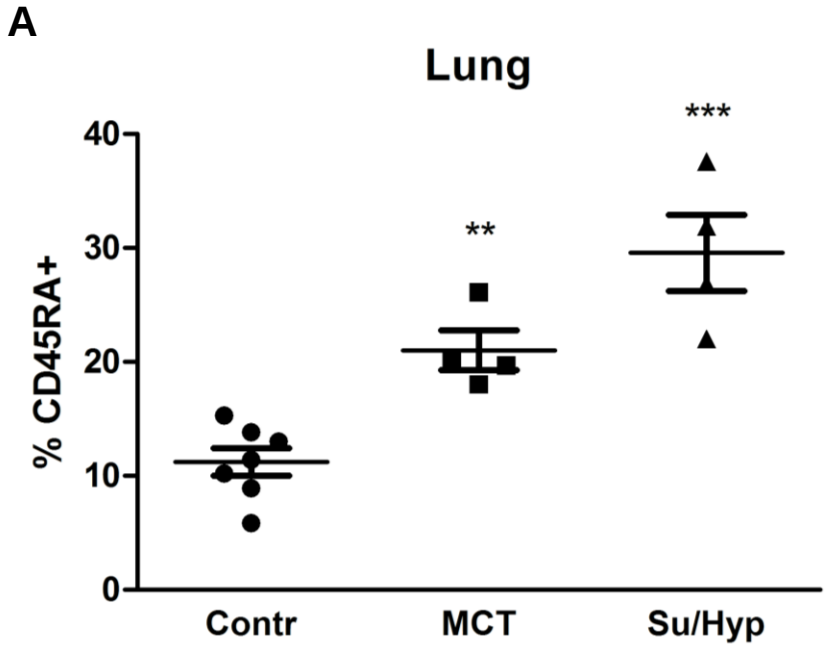


Fig. 9: Time course analysis. The development of right ventricular systolic pressure (RVSP), vascular remodeling (medial wall thickness), pulmonary mast cell infiltration and plasma IgG levels were recorded at 10 different time points during the 9-week aortic banding model. Each dot represents mean ± SEM of n = 6-8 experiments

4.3 B-cells accumulate locally and systemically

After determining the sequence of mast cell and Ig infiltration in PH, we next wanted to analyze, if an increase in B-cell numbers, as the source of the Igs, can be detected in lung tissue. For this purpose, Organ-specific B-cell accumulation was determined in the MCT and the SuHx model of PH, by analyzing the relative abundance of CD45RA positive cells through flow cytometry. CD45RA positive cells were significantly increased in lungs of both the MCT and the SuHx model compared to lungs from healthy control rats. Overall the increase in CD45RA positive cells showed a tendency for a more prominent increase in the SuHx model compared to the MCT model, however, without reaching statistical significance between the two groups (p=0.065) (fig. 10).



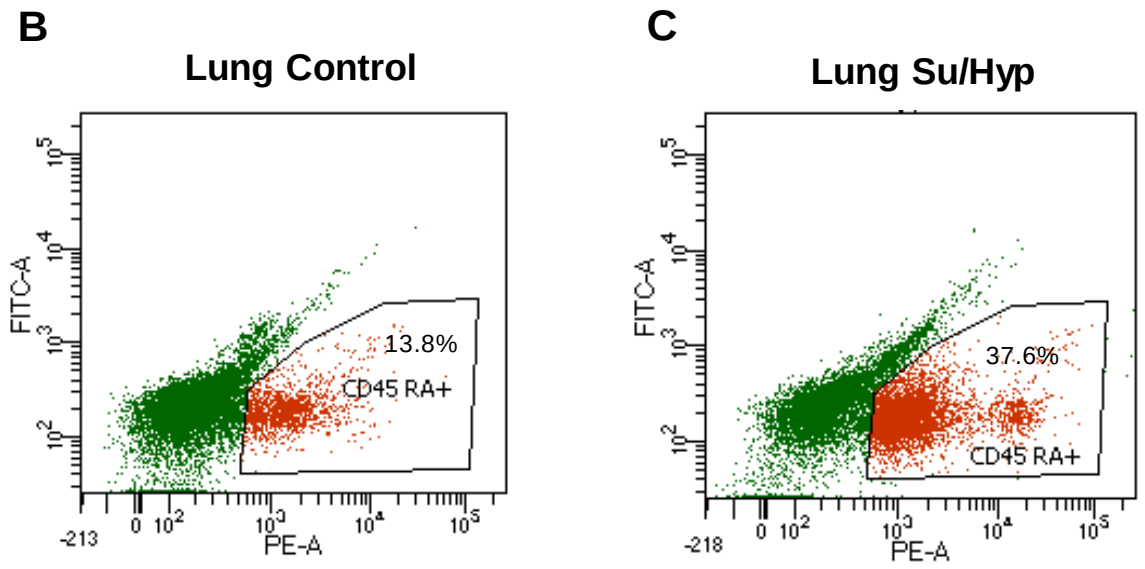


Fig. 10: Local B-cell accumulation in PH. (A) Pulmonary B-cell accumulation in the monocrotaline (MCT) and sugen/hypoxia (SuHx) model of PH, evident by infiltration of CD45RA positive cells, compared to healthy control rats. Representative dot plots from control (B) and Su/Hyp treated rats (C) Each bar represents mean ± SEM of n = 4-7 experiments, respectively; *p < 0.05 versus control.

In addition, B-cell accumulation was determined systemically in blood, lymph nodes, spleen and bone marrow, to see if the inflammatory component in PH is restricted to the lung or if it extents to other tissues. Flow cytometric analyses revealed a trend towards elevated CD45RA positive cells in all analyzed tissues, except bone marrow, in both the MCT and the SuHx model relative to healthy controls, although statistical analyses failed to reach the level of significance. In bone marrow, in contrast, the percentage of CD45RA positive cells was slightly lower in MCT and SuHx rats as compared to control (fig. 11). The results show that not only Ig levels, but also B-cell numbers are increased in experimental PH. Furthermore, in experimental PH, inflammation seems to extend beyond the lung, to various other tissues.

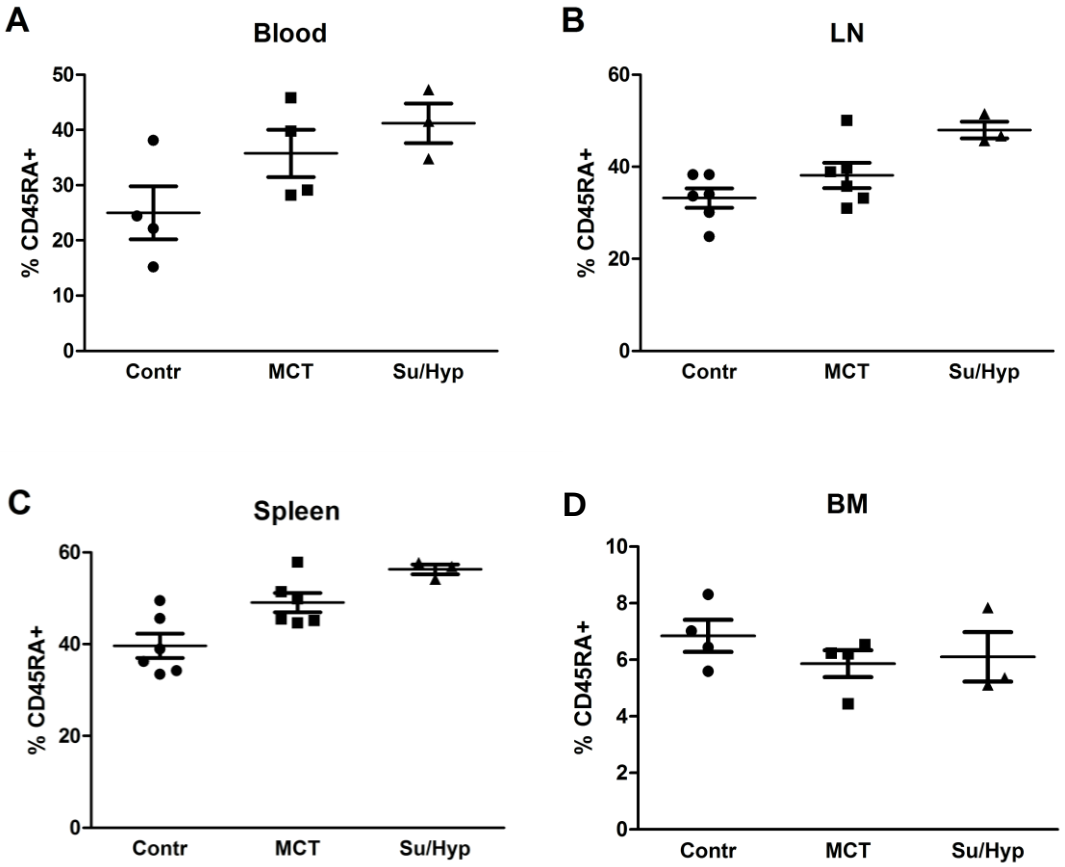


Fig. 11: Systemic B-cell accumulation in PH. B-cell accumulation in the monocrotaline (MCT) and sugen/hypoxia (SuHx) model of PH, evident as abundance of CD45RA positive cells as compared to healthy control rats, measured in blood (A), lymph node (LN; B), spleen (C) and bone marrow (BM; D). Each bar represents mean ± SEM of n = 3-6 experiments, respectively.

4.4 Mast cells secrete IL-6

The cytokine IL-6 is a key player in PH, and additionally has been reported to be secreted by mast cells and to be involved in B-cell stimulation. Here we wanted to investigate the role of mast cells as a potential source of IL-6 in PH. To this end, we tested by immune fluorescence staining for co-localization of mast cells and IL-6 in lung slides of MCT treated rats, (fig. 12A). The results were quantified as shown in fig. 12B. A remarkably high percentage of 87.9% of all mast cells, as evident by tryptase staining, co-localized with IL-6. On the other hand, 72.6% of all IL-6 positive cells also stained positive for mast cell tryptase, thus identifying mast cells as a major source of IL-6 in PH. ELISA measurements confirmed an elevation in IL-6 levels in lung homogenate of MCT treated rats as compared to control rats (fig. 12C). However, in MCT rats treated with the mast cell stabilizer ketotifen, IL-6 was almost completely lost, further corroborating mast cells as the primary source of IL-6 in PH following MCT treatment in rats.

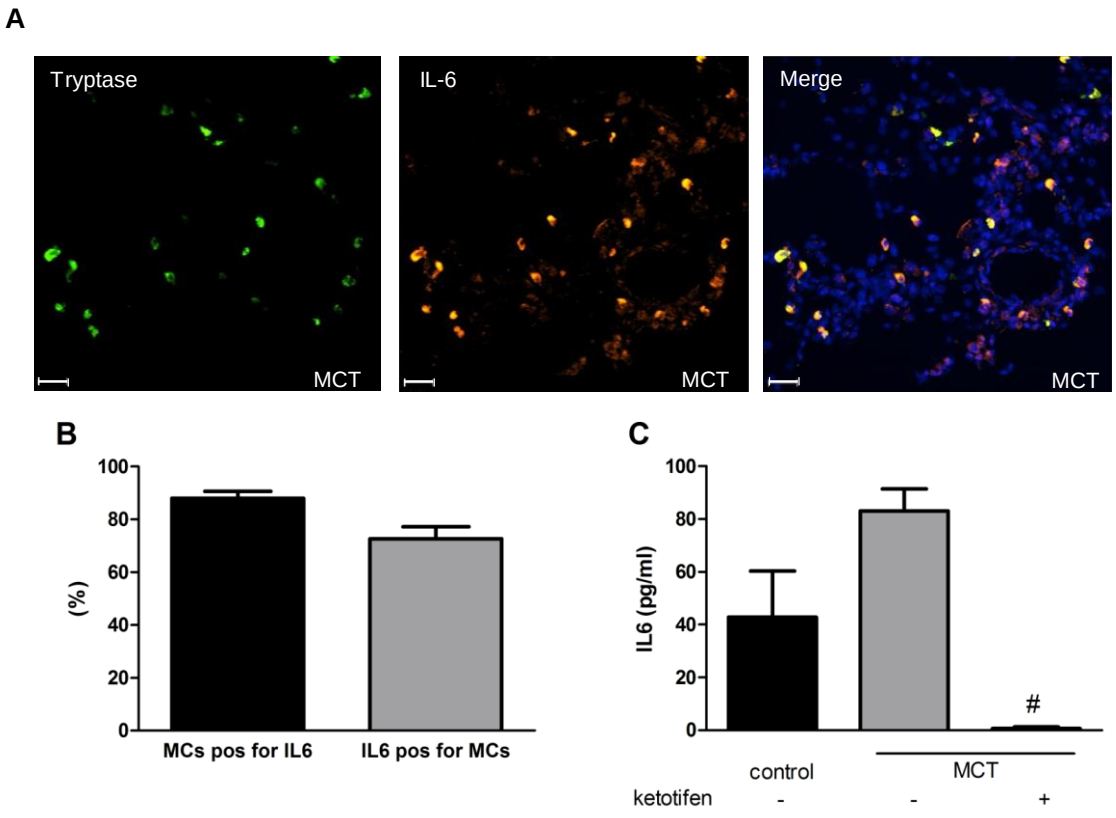


Fig. 12: Mast cell – IL-6 co-staining. A) Lung slides of monocrotaline (MCT) treated rats were stained for mast cells by a primary anti-tryptase antibody, and for IL-6 with a primary anti-IL-6 antibody. Scale Bar: 30µm B) Quantification of tryptase positve cells co-stained with IL-6 and IL-6 positive cells co-stained with tryptase in percent. C) IL-6 ELISA measurements of lung homogenate of control, MCT alone and MCT plus ketotifen treated rats, results are expressed in pg/ml. Each bar represents mean ± SEM of n = 4-6 experiments, respectively.

4.5 Effect of IL-6 depletion on mast cell and B-cell accumulation

Next we wanted to confirm that mast cell numbers are increased in experimental PH and furthermore, wanted to investigate, the effect of IL-6 on mast cell accumulation. In line with previous reports (27, 66) we found pulmonary mast cell numbers to be dramatically increased in MCT treated as compared to untreated control rats. Interestingly, mast cell accumulation was at a similar level in IL-6 depleted MCT treated rats as compared to MCT alone (fig. 13), therefore, suggesting that IL-6 is not needed for mast cell accumulation in PH. Subsequently we wanted to investigate the effect of both IL-6 and mast cell deficiency on B-cell accumulation in experimental PH, in order to test our hypothesis that mast cells via IL-6 secretion play an important role in pulmonary B-cell accumulation. Since B-cells were accumulating in large clusters consistent with bronchus associated lymphoid tissue (BALT), we took the BALT area size as a quantitative outcome parameter (fig. 14A). BALT size was significantly increased in MCT rats compared to control. There was a trend to reduced BALT area size, in both mast cell deficient and IL-6 depleted MCT rats relative to naïve MCT rats, although it failed to reach the level of significance (fig 14B). The general tendency to reduced pulmonary B-cell numbers in both mast cell and IL-6 deficient rats, indicate the presence of a potential mast cell – B-cell axis in PH, fueled by the cytokine IL-6.

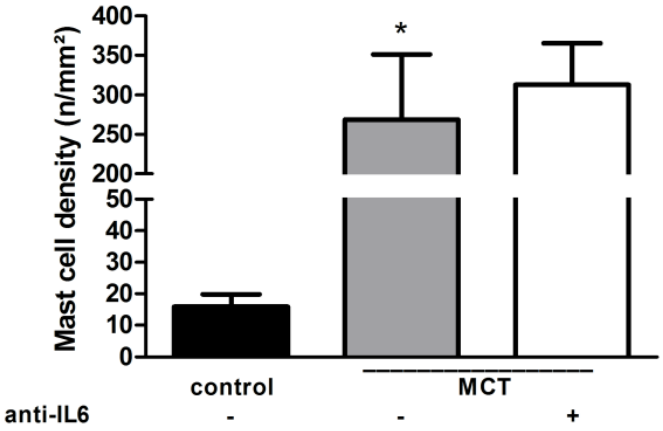


Fig. 13: Quantification of mast cells after IL-6 depletion. Mast cells were stained by toluidine blue and counted in lung slides of control, MCT and IL-6 depleted MCT rats, the result is expressed as mast cell number per mm^2. Each bar represents mean ± SEM of n = 6 experiments, respectively, *p < 0.05 versus control.

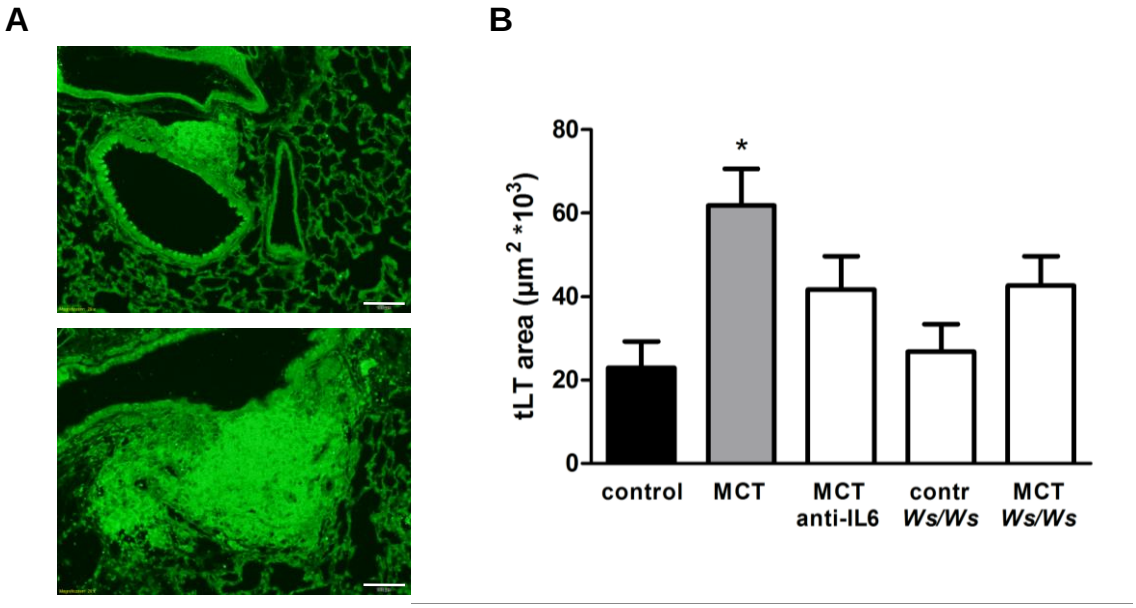


Fig. 14: Quantification of BALT size in lungs of MCT-treated rats in the absence or presence of either mast cell deficiency or IL-6 depletion, respectively. A) Representative slides show B-cells and BALT stained by an anti CD45RA antibody in lung slides of healthy control and MCT treated rats, Scale Bar = 100 µm B) After CD45RA staining, BALT area size in μm^2 was measured in lung slides of control, MCT alone, mast cell deficient *Ws/Ws* and in IL-6 depleted MCT rats. Each bar represents mean ± SEM of n = 5-9 experiments, respectively, *p < 0.05 versus control.

4.6 A functional role for IL-6 in PH

Based on our previous results, suggesting 1) a connection between innate and adaptive immunity in PH, 2) indicating IL-6 as a potential link between innate and adaptive immunity and a stimulator of B-cells in PH and 3) previous reports describing IL-6 as a key mediator in PH (44), we wanted to probe for a general functional role of IL-6 in the development of PH. IL-6 was depleted by daily administration of an anti-IL-6 antibody. Both RVSP and vascular remodeling, measured as medial wall thickness in small and medium sized pulmonary vessels, were increased significantly in the MCT treated rats relative to the healthy control group. IL-6 depletion resulted in a significant reduction in RVSP and vascular remodeling, therefore confirming a functional role for IL-6 in PH (fig. 15).

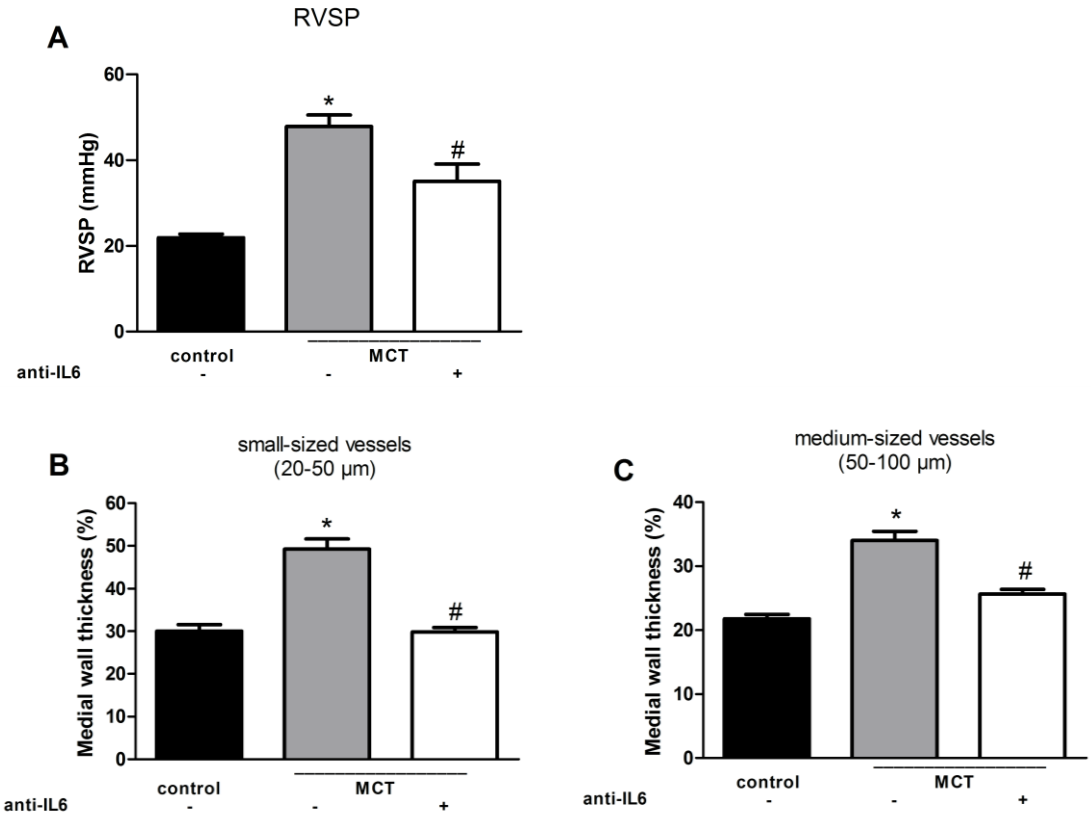
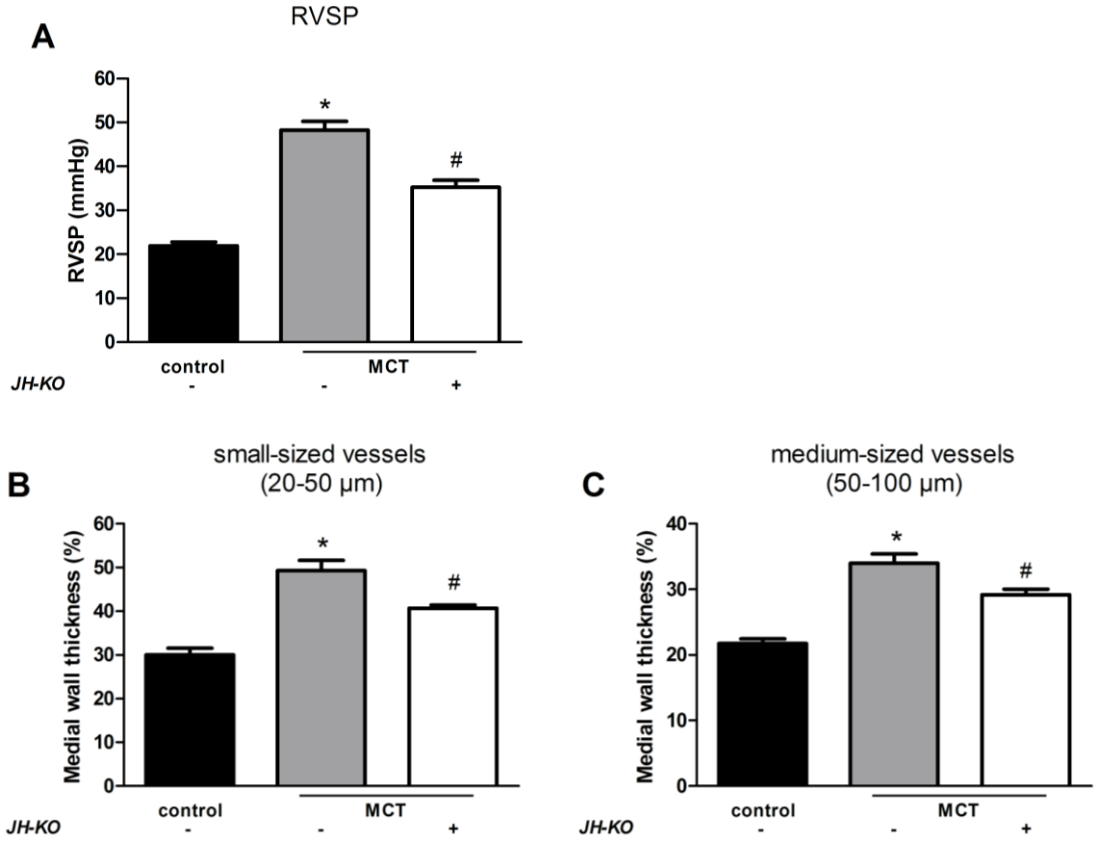


Fig. 15: Functional role of IL-6 in PH. Right ventricular systolic pressure (RVSP; A) and medial wall thickness in percent, in small (B) and medium (C) sized vessels were determined in healthy control, MCT alone and in IL-6 depleted MCT treated rats. Each bar represents mean ± SEM of n = 6 experiments, respectively; *p < 0.05 versus control, #p < 0.05 versus MCT.

4.7 A functional role for B-cells in PH

After giving evidence for a mast cell – B-cell axis, with the cytokine IL-6 as the potential driver of this axis, we aimed to further substantiate our notion of a functional role of B-cells in the development of PH. The question was approached, by either the use of B-cell deficient JH-KO rats or by depleting B-cells by an anti-CD20 antibody, in both the MCT and the SuHx model of PH. Both MCT and SuHx treatment resulted in a significant increase in RVSP and vascular remodeling, demonstrating the development of PH. Both the use of B-cell deficient and B-cell depleted rats, significantly reduced RVSP and vascular remodeling in both the MCT and the SuHx model, confirming a functional role of B-cells in the pathophysiology of PH (fig. 16/17).



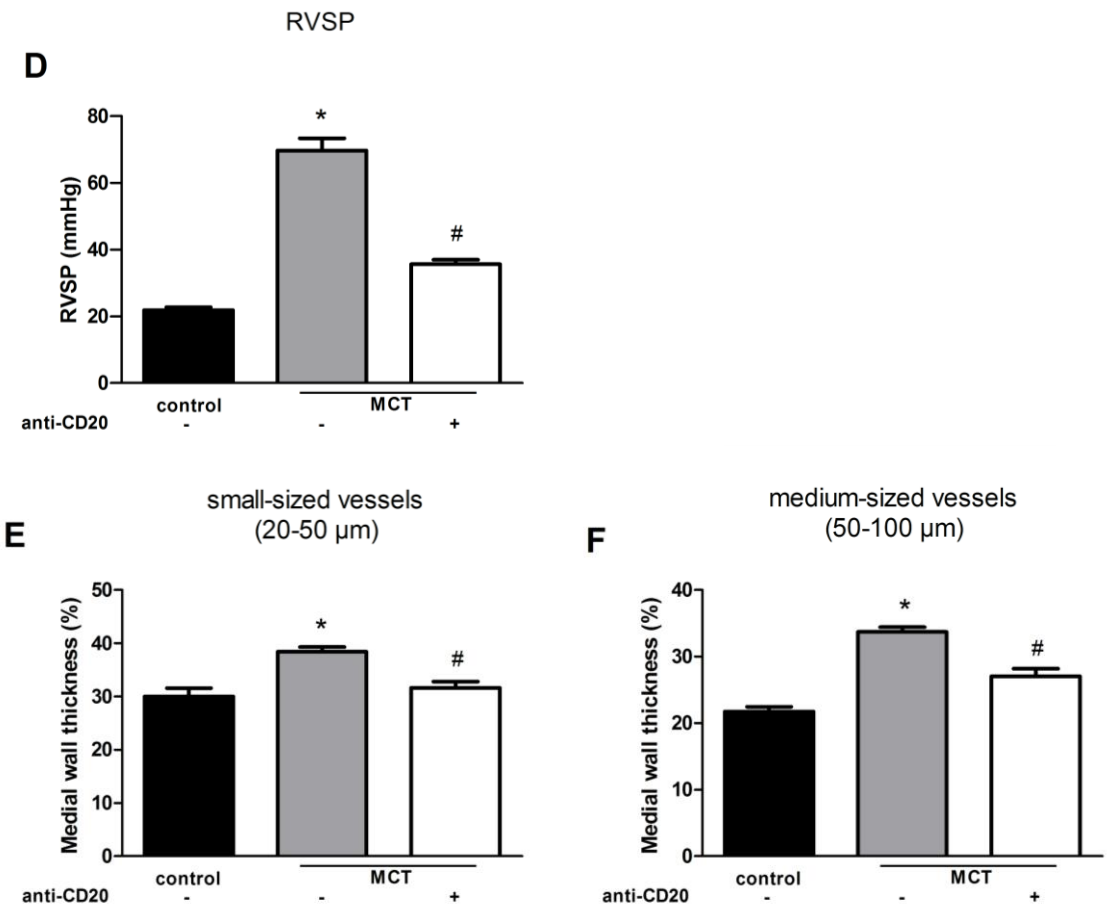
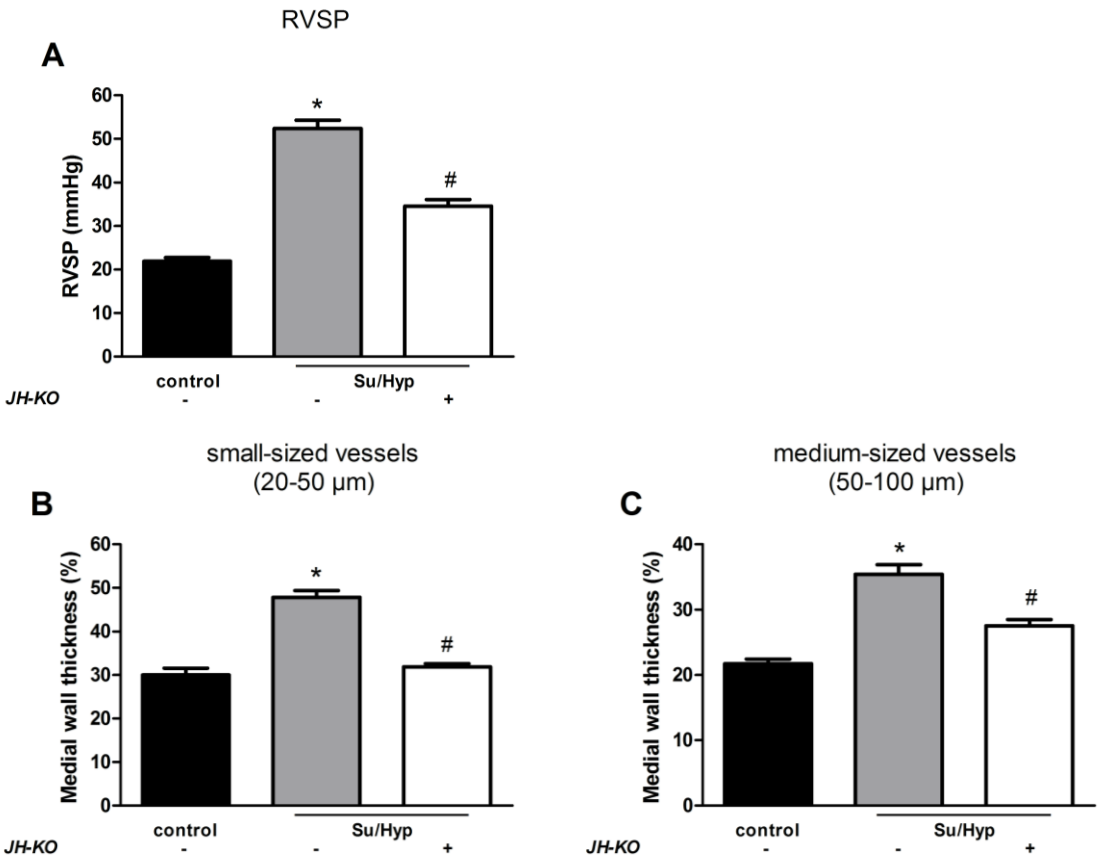


Figure 16: Functional assessment of B-cells in the MCT model. Right ventricular systolic pressure (RVSP) and medial wall thickness in percent, in small and medium sized vessels were determined in healthy control, MCT in wild type and MCT in B-cell deficient JH-KO rats (A-C) as well as in healthy control, MCT treated with an isotype matched control antibody and MCT anti-CD20 treated rats (D-F). Each bar represents mean ± SEM of n = 6-10 experiments, respectively; *p < 0.05 versus control, #p < 0.05 versus MCT alone.


RVSP
A
RVSP (mmHg)
60
50
40
30
20
10
0
*
#
control
Su/Hyp
JH-KO
-
-
+
small-sized vessels
(20-50 µm)
B
Medial wall thickness (%)
60
50
40
30
20
10
0
*
#
control
Su/Hyp
JH-KO
-
-
+
medium-sized vessels
(50-100 µm)
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Medial wall thickness (%)
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control
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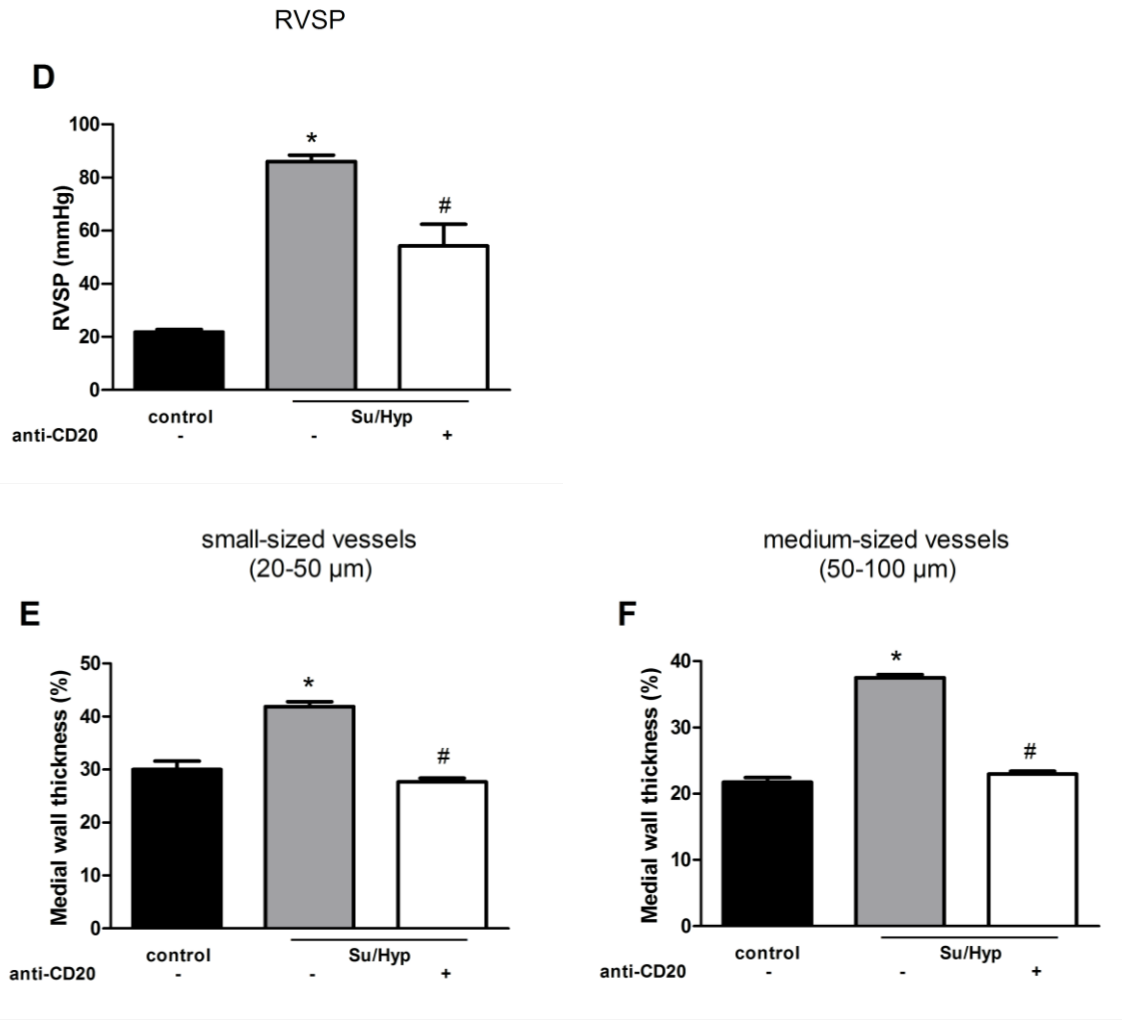

Figure 17: Functional assessment of B-cells in the sugen/hypoxia (Su/Hyp) model. Right ventricular systolic pressure (RVSP) and medial wall thickness in percent, in small and medium sized vessels were determined in healthy control, Su/Hyp in wild type and Su/Hyp in B-cell deficient JH-KO rats (A-C) as well as in healthy control, Su/Hyp treated with an isotype matched control antibody and Su/Hyp treated with an anti-CD20 antibody (D-F). Each bar represents mean ± SEM of n = 6-10 experiments, respectively; *p < 0.05 versus control, #p < 0.05 versus Su/Hyp alone.

4.8 Autoantibodies in PH

Lastly we aimed to test for the hypothesis that autoantibodies are responsible for the pathogenic role of B-cells in PH, and that treatment with the mast cell stabilizer ketotifen would reduce the abundance/formation of autoantibodies. For this purpose, we incubated lung slides of healthy control rats with plasma of control, MCT alone and MCT plus ketotifen treated rats. Subsequently these slides were incubated with an Alexa555 conjugated secondary anti-rat IgG antibody (fig. 18A). The results were expressed as fluorescence intensity. In slides treated with plasma from MCT rats, the fluorescence intensity was significantly elevated as compared to healthy control rats (fig. 18B). On the other side, in MCT rats treated with ketotifen, the fluorescence intensity was significantly attenuated again, further substantiating a connection between mast cells and B-cells in PH. ELISA measurements of IgG levels in plasma confirmed these results. IgG levels were elevated in MCT treated rats compared to controls, while IgG levels were attenuated again in MCT plus ketotifen treated rats, although these differences failed to reach the level of significance (fig.18C).

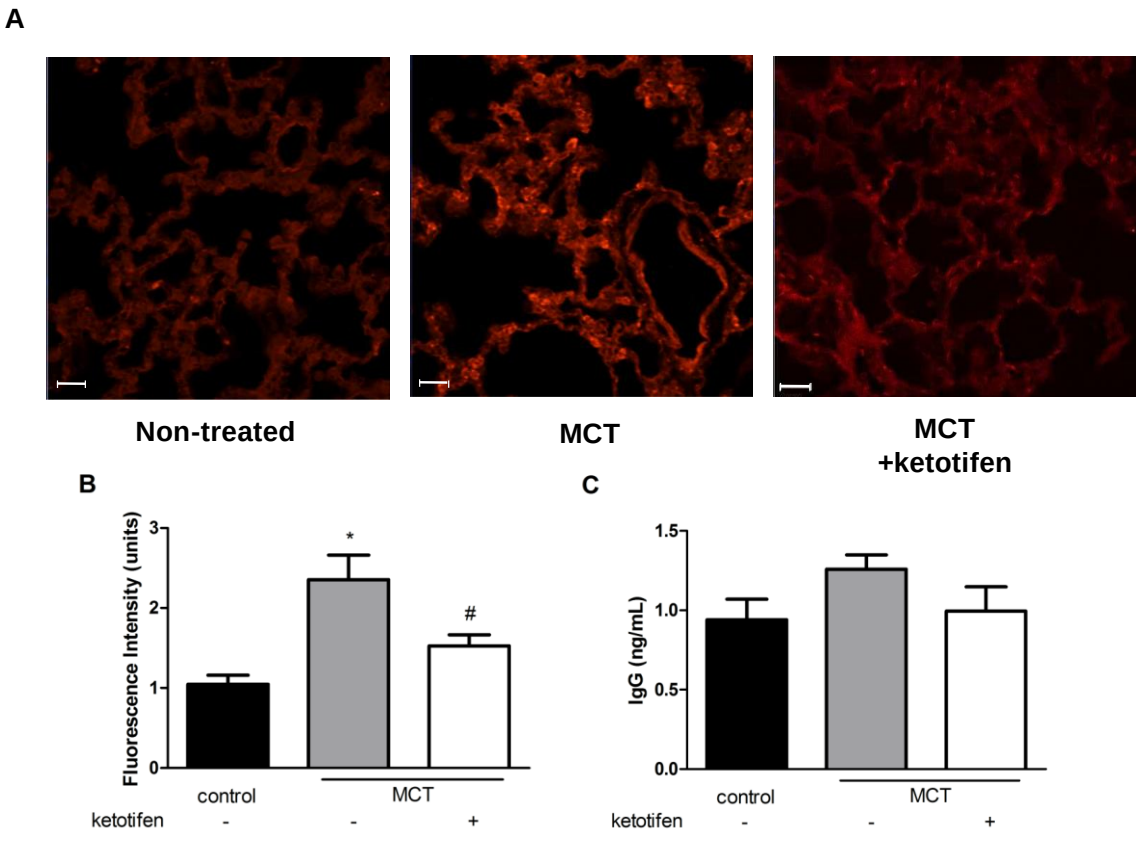

Fig. 18: Autoantibodies in PH. A) The presence of autoantibodies in plasma of control, MCT alone and MCT plus ketotifen treated rats was examined by incubation of naïve lung slides and subsequent detection of autoantibodies with a secondary anti-IgG antibody. Representative slides show results for non-treated, MCT and MCT+ketotifen groups. Scale Bar = 30 µm B) Fluorescence intensity was measured in lung slides incubated with plasma of control, MCT alone and MCT plus ketotifen treated rats. C) IgG ELISA measurements, results are expressed in ng/ml, in control, MCT alone and MCT plus ketotifen treated rats. Each bar represents mean ± SEM of n = 4 experiments, respectively; *p < 0.05 versus control, #p < 0.05 versus MCT

5. DISCUSSION

Pulmonary hypertension is a devastating disease significantly lowering the quality and expectancy of life of patients and is still associated with a poor prognosis. Despite considerable progress in the elucidation of underlying mechanisms and the development of new drugs against PAH there are still no definite treatments available, which could actually reverse the disease process and thus cure PH. In the here presented work we give novel evidence for an important involvement of both the innate and adaptive immune system in the formation of PH. We demonstrate a novel mast cell – B-cell interaction that is driven by the cytokine interleukin-6 (IL-6) and critically contributes to vascular remodeling and PH.

5.1 Mast cells interact with B-cells in PH

Remarkably, Paul Ehrlich who described mast cells for the first time in his doctoral thesis in 1878 (22, 37) noted that mast cells are abundant in ‘‘brown induration of the lung’’ (38), i.e. in haemosiderosis following mitral stenosis, i.e. in what today would be commonly described as PH-LHD (97). Notably Paul Ehrlich had made his discovery 13 years before Ernst von Romberg, in 1891, for the first time described pulmonary hypertension in a histological specimen (109). Almost one century passed by, until the attention was again directed towards mast cells in pulmonary hypertension. In 1962 increased pulmonary mast cell infiltration was again reported in an MCT rat model (132), followed in 1969 by documentation of mast cell abundance in patients with mitral stenosis and chronic left heart failure (59) and subsequently also in patients with IPAH (60). However, the specific role of mast cells in PH has always been disputed and it was not until recently that some light was cast on the pathophysiological role of mast cells in PH. In our own group we could previously confirm an elevation of mast cell numbers in the pulmonary vasculature of the MCT model of experimental PAH, our aortic banding model mimicking PH-LHD, as well as in lung samples of iPAH patients (27, 66). Furthermore, for the first time we were able to establish an important functional role for mast cells, by either pharmacological treatment with the mast cell stabilizer ketotifen or by a genetic approach using mast cell deficient *Ws*/*Ws* rats. Both

approaches significantly attenuated vascular remodeling and PH (66). The transferability of these results into the clinical setting has been indicated by first clinical trials. Farha and colleagues tested the mast cell stabilizer cromolyn in a subgroup of 9 patients and detected a decrease in LTE4 as well as a decrease in the vascular endothelial growth factor (VEGF) and the circulating proangiogenic progenitor cells, CD34+CD133+ (41). In a later study, by the same group, the effects of the c-kit inhibitor imatinib in PAH patients, were tested. Imatinib, which largely reduces mast cell numbers (40), has been shown to significantly reduce PVR, concomitant with an increase in cardiac output, both in a phase 2 and phase 3 study, although strong adverse effects have been reported (51, 65). However, it should be mentioned that the rational for imatinib was to target the PDGF receptor and c-kit and not mast cells.

Encouraged by these previous results we wanted to take a deeper insight into the mechanisms by which mast cells contribute to the formation of PH. For this purpose, we performed a gene array analysis, and specifically searched for mast cell dependent genes that are differentially regulated in vascular remodeling and PH. We decided to use the aortic banding model of PH-LHD, since, 1.) group 2 PH is the group of PH with the highest prevalence in Western countries, 2.) group 2 PH is hugely understudied and so far, no therapies targeting the lung are available and 3.) since the inflammatory effect of the actual stimulus, i.e. aortic banding, is low in this model compared to other experimental models such as the MCT model of PAH (35). The resulting changes in immune and inflammatory status can be considered to reflect changes in inflammation and immunity in the process of PH and vascular remodeling rather than a response to the initial trigger. Out of 31000 genes that were analyzed, only 32 were significantly regulated (≥4-fold change with $p<0.05$) in aortic banding rats as compared to the control group. Subsequently out of these 32 genes, three were significantly counter regulated again, with more then 2-fold change and with a significance of $p<0.05$, in aortic banding rats by treatment with the mast cell stabilizer ketotifen, as compared to untreated aortic banding rats. Surprisingly all three genes were encoding for constituents of immunoglobulins, namely; IgJ, IgG-2a and Igha, therefore for the first time pointing towards a putative mast cell B-cell interaction in vascular remodeling and PH. The notion of mast cells as modulators of the adaptive

immune system outside the context of IgE production and allergy is relatively new (84). Tkaczyk and colleagues showed that unstimulated mast cells induce B-cell proliferation and IgM secretion, in a ratio of mast cells to B-cells as low as 1/10000. Notably mast cells and B-cells were spatially separated from each other by using transwells in these experiments, therefore the authors concluded that B-cells must be stimulated by a yet unidentified soluble factor (139). A subsequent study found that mast cells have the ability to induce antigen independent B-cell proliferation, by secreting membrane vesicles or exosomes again without direct cell – cell contact (123). Similarly, Merluzzi and colleagues reported that mast cells promote B-cell survival and activation as well as further proliferation and differentiation into immunoglobulin secreting plasma cells. In contrast to the previous two studies, the authors here reported that B-cells are stimulated by both direct cell – cell contact and by the secretion of soluble factors, in particular the of IL-6 (87).

In our 9-week time course study of our aortic banding model, we detected a sudden increase in mast cell numbers within the first few days, corresponding with previously reported results, in MCT treated rats. In a previous collaboration with Dr. Schermuly´s group we had shown that treatment with the mast cell stabilizer sodium cromolyn, starting from day one after MCT, alleviated medial thickening, right ventricular hypertrophy and PH, and reduced perivascular mast cell accumulation. On the other hand, cromolyn given from day 21 after MCT, failed to show any beneficial effects (27), thus supporting the notion that mast cell infiltration is an early event in PH. The increase in mast cell numbers in the aortic banding model was followed by a secondary increase in IgG levels, starting at approximately day 10 of the time course study. By the end of the experiment at day 64, IgG levels had more than tripled. The results are in line with our gene array analysis, confirming an increase in IgG levels on the proteomic level. Furthermore, the increase in IgG levels, which happened several days after mast cell infiltration had peaked, substantiates the view that mast cells might play a role in B-cell proliferation and differentiation.

By flow cytometry analysis we could next demonstrate a significant increase in the pulmonary abundance of B-cells, in two different models of PAH, namely the SuHx and the MCT model, compared to control. Thus extending the concept of an increase in B-cell/Ig levels from the PH-LHD model to other experimental models of PH. In line with our results, increased pulmonary B-cell accumulation has previously been reported in both the SuHx and MCT model of PAH (20, 42), in broiler chickens susceptible to PAH (57) as well as in patients with IPAH (100, 142) and in patients with secondary forms of PH (21). In addition, our measurements revealed a distinct increase in B-cell numbers in blood, lymph nodes and spleen, but not in bone marrow. The latter finding could be explained by the fact that due to stimulation, B-cells are increasingly released into the blood stream upon their production (2). Therefore, B-cell numbers show a tendency to be decreased in bone marrow, while they are increased in all other analyzed tissues. Interestingly, similar increases in cellular abundance in blood and lymph nodes has previously likewise been reported for mast cells in an MCT rat model (132). Our results and these previous findings thus give rise for speculation of a systemic involvement of the immune system in PH and at the same time supporting our notion of a mast cell - B-cell axis, in PH. So far, only very few publications have looked at systemic effects in PH, e.g. a connection between PAH and insulin resistance has been reported (147, 154). With nearly 47%, a significantly increased prevalence in insulin resistance was found in a cohort of female PAH patients, independent of age and degree of obesity, compared to only 22% in the control population. Interestingly, both insulin resistance and PAH are linked to an underlying inflammatory pathology (154). Furthermore, a susceptibility to mitochondrial defects and metabolic abnormalities in skeletal muscle cells have been found in PAH patients (82). Both are examples substantiating the provocative view of PAH as a systemic disease, however, more research in this area is clearly needed.

5.2 IL-6 drives mast cell – B-cell interaction in PH

Mast cells secrete a multitude of biologically active mediators, which provides them with an effective tool set to modulate both innate and adaptive immune responses. Mast cell mediators can be categorized into two groups; 1.) preformed mediators stored in the mast cell granules, which can be released immediately upon activation, such as heparin, histamine, proteoglycans and neutral proteases, and a few cytokines and 2.) mediators which are newly synthesized in response to stimulation such as most cytokines, chemokines, lipid mediators, and growth and angiogenic factors (89). One mediator which frequently stands out as a key player in PH, is synthesized by mast cells (71), and has been reported to activate B-cells (87) and to contribute to increased immunoglobulin secretion and autoantibody production (64) is the cytokine IL-6. IL-6 levels are elevated in both experimental models of PH (10) as well as in PAH patients (68). Furthermore, IL-6 levels are a strong prognostic marker in PAH patients, predicting mortality better than the 6-minute walk test and hemodynamic measurements (126).Therefore, we speculated that IL-6 might also play a key role in driving mast cell – B-cell interaction. Indeed, our results show a strong co-localization between mast cells and IL-6 in immune fluorescence stainings of MCT rat lungs, suggesting mast cells as a primary source for IL-6 secretion in experimental PH. Elevated IL-6 levels in our MCT model were confirmed by ELISA measurements. Notably IL-6 levels were almost completely undetectable in MCT rats treated with the mast cell stabilizer ketotifen, thus confirming a paramount role for mast cells as IL-6 secreting cells.

Mast cell staining with toluidine blue confirmed previously reported findings of a significant accumulation of mast cells in the lung in experimental PH (27, 66). In IL-6 depleted animals, mast cell numbers remained elevated, thus indicating that in experimental PH, mast cell infiltration and proliferation is independent of IL-6. In order to test for a potential mast cell – B-cell axis, in a next step we investigated the effect of mast cell deficiency and IL-6 depletion on pulmonary B-cell accumulation in experimental PAH. Interestingly, in immune fluorescence stainings of PH lungs we detected only very few single B-cells; instead, B-cells were organized in clusters, which shared great similarity with bronchus-associated lymphoid tissue as illustrated in fig. 16 below. Only recently the presence of BALTs

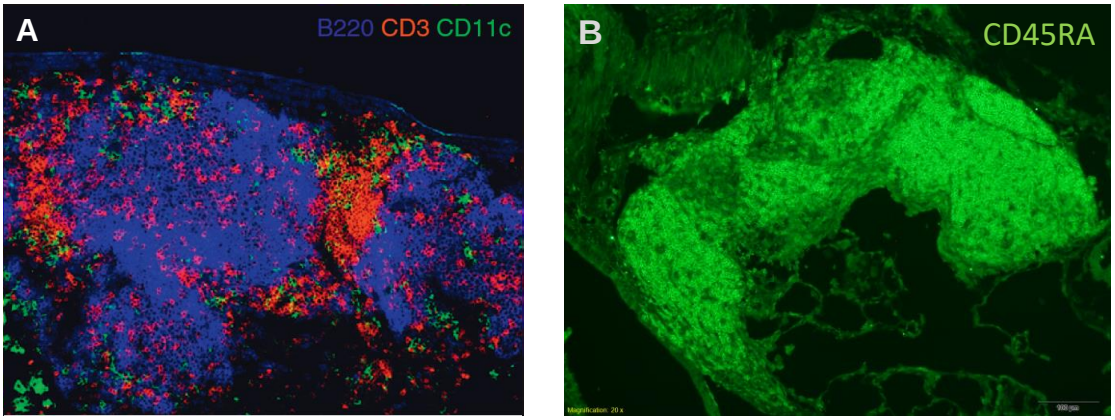

Fig. 19: A) Organization of murine BALT. Representative lung section from a mouse infected with influenza virus. Sections were probed with antibodies for B220 (blue), CD3 (red), and CD11c (green) to visualize B-cells cells, T-cells cells, and Dendritic cells, respectively. Reprinted from Randall et al. *Adv Immunol* 107: 187–241, 2010, Copyright © Elsevier Inc., with permission B) Representative lung section from a rat treated with MCT; section was probed with an antibody for CD45RA to visualize B-cell accumulation; own results.

in the lungs of IPAH patients has been described (100). BALTs can be categorized as tertiary lymphoid tissue (tLT). Similar to secondary lymphoid tissues such as lymph nodes and spleen, tLTs help to facilitate optimal interaction of immune cells and to drive adaptive immune responses. In humans or mice tLTs are formed ectopically as a reaction to acute or chronic inflammation (24). However, in some other species such as rat, they have also been reported in healthy individuals (99). In line with the latter data, we detected BALTs in histological slides of healthy control rats in our present study. The presence of BALTs has been reported before in other chronic inflammatory diseases, for example in patients with chronic obstructive pulmonary disease (25) or rheumatoid arthritis (106). B-cells are usually the predominant cell type in BALTs and often BALTs consist almost exclusively of B-cells (105). Since single B-cells were scant in our immune fluorescence staining, we assessed the area of BALTs in the histological samples as a quantitative measure of B-cell abundance. As reported before by Colvin and colleagues (20), BALT area size was found significantly increased in MCT treated animals as compared to healthy controls. Notably, both mast cell deficiency and IL-6 depletion markedly attenuated BALT area size. Interestingly, IL-6 was originally described as B-cell stimulating factor (76) and a connection between IL-6 depletion and a reduction in B-cell numbers (5) as well as a reduction in germinal

center formation (33) have previously been reported in the context of myasthenia gravis, a chronic autoimune diease. Together with our above discussed results of a strong co-localization between mast cells and IL-6, this data substantiates the view of mast cell derived IL-6 as a critical mediator driving B-cell activation and proliferation.

Finally, we aimed to establish a functional role for IL-6 in experimental PH. Depletion of IL-6 by an anit-IL-6 antibody significantly reduced vascular remodeling and PH, demonstrating that IL-6 is an essential component in the onset of PH. Previous studies have shown before that overexpression of IL-6 suffices to induce vascular remodeling and PH (52, 128). Furthermore, the overexpression of IL-6 amplifies hypoxia-induced vascular remodeling and PH (52). Conversely in a hypoxia model of PAH, IL-6 deficiency significantly reduced vascular remodeling and PH (128). In our own experiments we could not only demonstrate an important role for IL-6 in PH, we also identified IL-6 as a potential mediator between innate and adaptive immunity in PH, with mast cells as its main source.

5.3 A functional role for B-cells and a case for autoimmunity

B-cells are an integral part of adaptive immunity, commonly acting as the second line of defense in fighting infections and can be characterized by their antigen specificity and ability to serve as the memory of the immune system. The hypothesis of a functional role of B-cells in the pathophysiology of PH might seem counterintuitive at first. Especially since anti-inflammatory therapies, such as corticosteroids, have not been successful and therefore not been indicated in treating PH. However, as described above B-cell infiltration has been reported in both experimental models of PH as well as in PAH patients (9, 20 49, 65). Moreover, gene array analysis in peripheral blood B-cells revealed differential gene expression indicative of B-cell activation in IPAH patients compared to healthy controls (144). In order to establish a functional role for B-cells in the formation of PH, we used both a genetic and a pharmacological approach. As genetic approach we used so called *JH*-KO rats, which by zinc-finger nuclease targeted deletion of the *JH* locus are deficient of B-cells and immunoglobulins (86).

As pharmacological approach we depleted B-cells by an anti-CD20 treatment which has previously been reported to deplete approximately 98% of mature B-cells (56), supposedly by B-cell apoptosis (119). Both approaches significantly attenuated vascular remodeling and PH in both the SuHx and the MCT model of PH, therefore providing proof-of-principle for a functional role of B-cells in PH. A similar approach has recently been applied by Norbert Voelkel's group in an allergic model of PH, induced by a combination of SU5416 and OVA sensitization. In this model anti-CD20 antibody mediated B-cell depletion significantly improved vascular remodeling and hemodynamics (91). It might be expected that B-cells play a role in an allergic model of PH, however, it is remarkable that also in our SuHx model, which is not an inherently inflammatory model, B-cells seem to play an important role. This results give room for speculation, for a much broader relevance of B-cells in PH.

On a mechanistic level, autoantibodies increasingly appear as important mechanism promoting disease progression in PH. A study examining the sera of 40 PAH patients found circulating autoantibodies with a frequency of 62.4% (149). Indeed, autoantibodies directed against various targets, including anti–endothelin receptor type A, anti–angiotensin receptor type-1 (8), endothelial cells (34), fibroblasts (133), fibrillin-1 (92) and vascular smooth muscle cells (15) have been reported in secondary PAH as well as in IPAH patients. Anti-endothelial cell antibodies were shown to cause cell apoptosis (12), while anti-vascular smooth muscle cell antibodies were shown to induce cell contraction (15). Notably, the passive transfer of IgGs from MCT treated rats into healthy control rats is sufficient to elicit PH (20), providing essential proof for the pathogenic role and potential of autoantibodies in PH disease. Taken together these findings strongly support the notion that autoimmunity may play a critical role at least in some forms of clinical PH.

The criteria that classify a disease as an autoimmune disease include the identification of a target antigen, the presence of antibodies and/or T cells in the target organ, and the transfer of the disease to animals by cells or antibodies (110). Importantly, all these criteria can be applied to PH. Our own results of the present study confirm the presence of autoantibodies in experimental PH, in that immune

fluorescence staining of naïve lung slices for binding of plasma-derived autoantibodies revealed a significant increase in fluorescence intensity in plasma of MCT treated rats as compared to control animals. However, in contrast to Colvin and colleagues who reported preferential adventitial and small vessel staining (20), autoantibody staining in our present study was rather uniform, therefore making it difficult to match the staining to a definite histological structure. In order to substantiate the hypothesis of a mast cell – B-cell axis in PH, we also tested for autoantibody staining using plasma from MCT rats which had been treated with the mast cell stabilizer ketotifen. Interestingly, fluorescence intensity in this group was significantly attenuated as compared to MCT alone. In line with the notion of a mast-cell dependent autoimmunity in experimental PH, ELISA measurements confirmed an elevation in IgG plasma levels in MCT treated rats as compared to control animals which was reduced again in MCT rats treated with the mast cell stabilizer ketotifen, further substantiating the notion of a mast cell – B-cell axis in PH.

5.4 Concluding remarks

In conclusion we provide here for the first time evidence for a functionally relevant link between mast cells and B-cells, mediated via the cytokine IL-6, in the pathophysiology of PH. Our findings further indicate that this role of inflammation and autoimmunity might not be restricted to certain cases of PAH, but could play a broader role in various forms of PH including PH-LHD, as demonstrated by our gene array analysis, which was performed in the aortic banding model. Confirmation of the here presented results in human samples would allow for a swift translation into the clinical setting by targeted immunotherapy as a novel treatment strategy, including the use of clinically approved interventions, such as mast cell stabilizers (ketotifen or cromolyn), anti-IL-6 receptor antibodies (tocilizumab), or anti-CD20 antibodies (rituximab). Tocilizumab is currently approved for the treatment of rheumatoid arthritis (134), an autoimmune disease commonly associated with pulmonary hypertension (117). A recent case report of a patient presenting with mixed connective tissue disease and PAH describes significant improvements in key PAH parameters such as mPAP and 6-minute

walk test after three months of tocilizumab treatment. Interestingly, tocilizumab treatment also led to a substantial decrease in circulating IgGs from 6451 mg/dL pretreatment to 2238 mg/dL posttreatment (44), supporting the view of a potential link between IL-6 and B-cells in PH. The anti-CD20 antibody rituximab is a widely used drug for the treatment of lymphomas, leukemias, transplant rejection, and autoimmune diseases, including again rheumatoid arthritis (138). Regarding the treatment of PAH, only very few reports are available, although a clinical phase two trial is currently recruiting patients to test rituximab for the treatment of systemic sclerosis-associated pulmonary arterial hypertension (NCT01086540). A case report describing a patient with systemic lupus erythematosus and PAH observed significant improvements in hemodynamic parameters after rituximab treatment for one month (61). Overall the here presented results identify novel promising targets for immunotherapy for the treatment of PAH and other forms of PH, which have not been traditionally associated with inflammation, and next need to be validated in human studies, to be tested for their efficacy and safety.

6. SUMMARY

Pulmonary Hypertension (PH) is a progressive cardiovascular disease, characterized by an increase in mean pulmonary arterial pressure concomitant with severe pulmonary vascular remodeling and obliteration of small and medium sized arteries and endothelial dysfunction, ultimately leading to death by right heart failure. Although a contribution of the immune system to the pathogenesis of PH has been discussed for several decades, the role of autoimmunity in PH and its underlying mechanisms are still poorly understood. Previously, pulmonary infiltration of mast cells in PH has been reported by several groups, including our own. Furthermore, our group could demonstrate the functional relevance of mast cells in lung vascular remodeling and PH. Based on these results we performed a gene array analysis and probed for mast cell dependent genes which are differentially regulated in experimental PH. Surprisingly, the only mast cell dependent genes significantly regulated were genes encoding for constituents of immunoglobulins. In subsequent experiments we were indeed able to detect an increase in pulmonary IgG and B-cell levels in the aortic banding model of PH due to left heart disease as well as in the monocrotaline and the sugen-hypoxia model of pulmonary arterial hypertension. IL-6 is a potential candidate to link mast cells and B-cells in PH, based on its well-known pathogenic role in PH and based on previous reports showing mast cells to secrete IL-6, and IL-6 to stimulate B-cells. In our experiments we detected a high degree of co-localization between mast cells and IL-6, thus identifying mast cells as primary source of IL-6 in experimental PH. Furthermore, we could show a connection between IL-6 depletion and B-cell accumulation, to the effect that IL-6 depletion reduced the area size of tertiary lymphoid tissue, as observed in immunofluorescence. A functional role for B-cells in the disease process was demonstrated by use of both B-cell deficient *JH*-KO rats and by depleting B-cells using an anti-CD20 antibody. Both approaches significantly attenuated vascular remodeling and PH. Lastly our data indicate the presence of autoantibodies in PH, and are thus hinting towards a potential autoimmune disease mechanism. Overall our data yield novel insights into the role of the immune system in PH, in that they provide evidence for a mast cell – B-cell axis which is driven by IL-6. Provided our findings can be confirmed in human

samples, they could be rapidly translated into the clinical scenario by implementation of targeted immune therapy with drugs already on the market such as Rituximab (anti-CD20 antibody), or Tocilizumab (anti-IL-6 receptor antibody).

7. ZUSAMMENFASSUNG

Die Pulmonale Hypertonie (PH) ist eine stetig fortschreitende kardiovaskuläre Erkrankung die durch einen Anstieg des mittleren pulmonalarteriellen Drucks, einhergehend mit pulmonalem Gefäßumbau und Obliteration der kleinen und mittleren Arterien und endothelialer Dysfunktion, charakterisiert ist, und schlussendlich zum Tod durch Rechtsherzversagen führt. Obgleich eine Beteiligung des Immunsystems an der Entstehung der PH bereits seit mehreren Jahrzehnten diskutiert wird, sind die Rolle der Autoimmunität und die ihr zugrundeliegenden Mechanismen bislang nur unzureichend verstanden. Die Infiltration von Mastzellen in die Lunge bei PH wurde bereits früher von mehreren Gruppen beschrieben, darunter auch der unsrigen. Darüber hinaus konnten wir in vorangegangenen Untersuchungen bereits eine allgemeine funktionelle Beteiligung der Mastzellen am vaskulären Gefäßumbau und der PH nachweisen. Auf der Grundlage dieser Ergebnisse haben wir nun in der vorliegenden Arbeit Gene-Array-Analysen durchgeführt, um Gene zu identifizieren, welche Mastzell-abhängig in der PH reguliert werden. Bemerkenswerterweise waren die einzigen Mastzell-abhängigen Gene, welche signifikant reguliert wurden, Gene, die für Bestandteile von Immunglobulinen kodieren. Tatsächlich zeigten unsere nachfolgenden Experimente einen Anstieg an pulmonalen IgGs und B-Zellen sowohl im Aortenbandingmodell der PH infolge von Linksherzversagens, als auch im Monocrotaline und im Sugen-Hypoxiemodell der pulmonalarteriellen Hypertonie. IL-6 ist ein vielversprechender Kandidat, um als Bindeglied zwischen Mastzellen und B-Zellen in der PH zu fungieren. Dies leitet sich aus der gut dokumentierten pathogenen Rolle von IL-6 in der PH sowie aus Studien ab, welche zeigen konnten, dass Mastzellen IL-6 sezernieren und dass IL-6 B-Zellen zur Proliferation und Aktivierung zu stimulieren vermag. In unseren Untersuchungen konnten wir eine ausgeprägte Co-Lokalisation von Mastzellen und IL-6 nachweisen, und damit Mastzellen als die primäre Quelle von IL-6 in der PH identifizieren. Weiterhin konnten wir eine Verbindung zwischen der Depletion von IL-6 und der verminderten Akkumulation von B-Zellen nachweisen, dahingehend, dass nach Depletion von IL-6 eine Reduktion in der Fläche des tertiär lymphoiden Gewebes beobachtet werden konnte. Eine funktionelle Rolle

von B-Zellen im Krankheitsverlauf konnten wir schließlich entweder durch Verwendung B-Zell defizienter *JH*-KO Ratten oder durch Depletion von B-Zellen mittels eines anti-CD20 Antikörpers demonstrieren insofern als beide Methoden den vaskulären Gefäßumbau und die PH signifikant zu vermindern vermochten. Schließlich weisen unsere Befunde auf das Vorhandensein von Autoantikörpern in der PH hin, und zeigen so einen potentiellen Mechanismus der Autoimmunität auf. Insgesamt vermitteln unsere Daten neue Einblicke in die Rolle des Immunsystems in der PH und liefern Hinweise auf eine Mastzell – B-Zell Achse, die durch IL-6 vermittelt wird. Vorausgesetzt unsere Daten können in humanen Proben bestätigt werden, wäre eine zügige Übertragung der Ergebnisse in den klinischen Alltag durchaus realistisch, zumal entsprechend immunmodulatorische Medikamente wie z.B. Rituximab (anti-CD20 Antikörper) oder Tocilizumab (anti-IL-6 Rezeptor Antikörper) bereits heute zugelassen sind, und entsprechend umgehend zur gezielten Immuntherapie bei PH eingesetzt werden könnten.

8. PUBLICATIONS AND AWARDS

8.1 Publications in peer reviewed journals

Breitling S, Hui Z, Zabini D, Hu Y, Hoffmann J, Goldenberg NM, Buelow R, Dos Santos C, Kuebler WM. The mast cell – B-cell axis in lung vascular remodeling and pulmonary hypertension. Am J Physiol Lung Cell Mol Physiol. 2017 (submitted)

Breitling S, Krauszman A, Parihar R, Walther T, Friedberg MK, Kuebler WM. Dose-dependent, therapeutic potential of angiotensin-(1-7) for the treatment of pulmonary arterial hypertension. Pulm Circ. 2015: 5(4):649-57

Breitling S, Ravindran K, Goldenberg NM, Kuebler WM, The pathophysiology of pulmonary hypertension in left heart disease. Am J Physiol Lung Cell Mol Physiol. 2015:309(9):L924-41

Okumura K, Kato H, Honjo O, Breitling S, Kuebler WM, Sun M, Friedber MK. Carvedilol improves biventricular fibrosis and function in experimental pulmonary hypertension. J Mol Med (Berl). 2015:93(6):663-74

8.2 Presentations at international conferences

B cells promote pulmonary hypertension and vascular remodeling in the rat model: Symposium on Cardiac and Vascular Stiffness, Graz, Austria, 2014

Dose-Dependent, therapeutic potential of angiotensin-(1-7) for the treatment of pulmonary hypertension: ATS, San Diego, USA, 2014

B cells promote pulmonary hypertension and vascular remodeling in the rat model: ATS, Philadelphia, USA, 2013

The mast cell – B cell axis in lung vascular remodeling and pulmonary hypertension: 8th international PH symposium, Athens, Greece, 2013

8.3 Awards and scholarships

Travel Scholarship from the Ludwig Boltzmann Institute for Lung Vascular Research, Graz, Austria, 2014

Shortlisted for the Bayer Pulmonary Hypertension Award (1 of 10 shortlisted candidates), 2013

International Trainee Scholarship Award from the American Thoracic Society (ATS), 2013

9. LIST OF ABBREVIATIONS

ALK1	Activin-Like Kinase-type 1
BALT	Bronchus Associated Lymphatic Tissue
BCR	B-cell receptor
BMPR2	Bone Morphogenetic Protein Receptor 2
Bregs	regulatory B-cells
BSA	Bovine Serum Albumin
Bw	Body weight
cAMP	cyclic Adenosine Monophosphate
CD	Cluster of Differentiation
cGMP	cyclic Guanosine Monophosphate
CTEPH	Chronic ThromboEmbolic Pulmonary Hypertension
CTMC	Connective Tissue Mast Cells
DNA	Deoxyribonucleic Acid
ENG	endoglin
ET	Endothelin
FcεRI	high-affinity immunoglobulin E, F_C-epsilon receptor I
FDA	Food and Drug Administration
Ig	Immunoglobulin
IL	Interleukin
IPAH	Idiopathic Pulmonary Arterial Hypertension
LPS	Lipopolysaccharide
LVEF	Left Ventricular Ejection Fraction
MCT	Monocrotaline
MHC	Major Histocompatibility Complex
MMC	Mucosal Mast Cells
NIH	National Institute of Health
mPAP	mean Pulmonary Arterial Pressure
NO	Nitric Oxide

OVA	Ovalbumin
PAH	Pulmonary Arterial Hypertension
PCWP	pulmonary capillary wedge pressure
PDE5	Phosphodiesterase type 5
PH	Pulmonary Hypertension
PH-LHD	Pulmonary Hypertension due to Left Heart Disease
PVR	Pulmonary Vascular Resistance
RAG	Recombination-Activation Gene
RNA	Ribonucleic Acid
RVSP	Right Ventricular Systolic Pressure
SuHx	Sugen-Hypoxia
TGF	Transforming Growth Factor
TNF	Tumor Necrosis Factor
tLT	tertiary Lymphoid Tissue
WHO	World Health Organization

10. STATUTORY DECLARATION

I hereby declare that this dissertation is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this dissertation. Due acknowledgement has been made in the text to all other materials used.

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