Aus der Abteilung für Transplantations- und Infektionsimmunologie
der Medizinischen Fakultät
der Universität des Saarlandes, Homburg/Saar
Leitung: Univ.-Prof. Dr. rer. nat. Martina Sester

Neuroimmune interactions in allergic airway diseases: Studies in mouse models and humans

Dissertation zur Erlangung des Grades eines Doktors
der Naturwissenschaften
der Medizinischen Fakultät
der Universität des Saarlandes
2017

Vorgelegt von:

Duc Dung Le

geb. am: 25.06.1981 in Nghe An

For my family

Content

List of original papers

This thesis is based on the following papers, which are referred to in the text by Roman numerals (I - IV):

I **Le DD**, Schmit D, Heck S, Omlor AJ, Sester M, Herr C, Schick B, Daubeuf F, Fähndrich S, Bals R, Frossard N, Al Kadah B* and Dinh QT*. Increase of Mast Cell-Nerve Association and Neuropeptide Receptor Expression on Mast Cells in Perennial Allergic Rhinitis. *Neuroimmunomodulation. 2016 Dec 29. DOI: 10.1159/000453068*

II **Le DD**, Rochlitzer S, Fischer A, Heck S, Tschernig T, Sester M, Bals R, Welte T, Braun A and Dinh QT. Allergic airway inflammation induces the migration of dendritic cells into airway sensory ganglia. *Respir Res. 2014 Jun 30;15:73.*

III **Le DD***, Funck U*, Wronski S, Heck S, Tschernig T, Bischoff M, Sester M, Herr C, Bals R, Welte T, Braun A and Dinh QT. Steroid Treatment Reduces Allergic Airway Inflammation and Does Not Alter the Increased Numbers of Dendritic Cells and Calcitonin Gene-Related Peptide-Expressing Neurons in Airway Sensory Ganglia. *Neuroimmunomodulation. 2016;23(1):18-26.*

IV: Schmit D*, **Le DD***, Heck H, Bischoff M, Tschernig T, Herr C, Beisswenger C, Kobelt P, Lepper MP, Chung KF, Bals R and Dinh QT. Allergic airway inflammation induces migration of mast cell populations into the mouse airway. *Cell Tissue Res (2017). doi:10.1007/s00441-017-2597-9*

* These authors contributed equally to the study

Additional original papers and manuscripts that were published during the PhD study but not included in the thesis:

1. Omlor AJ, **Le DD**, Schlicker J, Hannig M, Ewen R, Heck S, Herr C, Kraegeloh A, Hein C, Kautenburger R, Kickelbick G, Bals R, Nguyen J, Dinh QT. Local Effects on Airway Inflammation and Systemic Uptake of 5 nm PEGylated and Citrated Gold Nanoparticles in Asthmatic Mice. *Small. 2016 Dec 23. doi: 10.1002/smll.201603070*

2. Heck S, Al-Shobash S, Rapp D, **Le DD**, Omlor A, Bekhit A, Flaig M, Al-Kadah B, Herian W, Bals R, Wagenpfeil S and Dinh QT. High probability of co-morbidities in bronchial asthma in Germany. *Accepted for publication in npj Primary Care Respiratory Medicine*

3. **Le DD,** Schmit D, Heck S, Schick B, Bals R, Frossard N, Al Kadah B anh Dinh QT. Increased expression of MrgX1 receptor in human nasal mucosa by perennial allergic rhinitis. *(in preparation)*

1 Summary

Allergic airway diseases, such as allergic rhinitis (AR) and allergic asthma, are major public health problems in Western Europe and industrial countries, and the prevalence of these problems has increased dramatically over the past few decades. Allergic inflammation arises due to a complex interaction between the immune and nervous systems. Bidirectional neuroimmune interactions during allergic inflammation has been proposed, and it is believed to play an important role in allergic diseases. Communication between immune cells and nerves can occur through either direct contact or via soluble mediators and receptors expressed by immune cells and nerves. We investigated the interaction of mast cells (MCs) and dendritic cells (DC) with nerves in allergic airway diseases in human and mouse models.

Mast cells (MCs) and nerves play an important role in allergic rhinitis (AR), but little is known about their interactions in AR. We found increased crosstalk of MCs with nerves in AR by showing the elevated associations between MCs, especially MCs expressing tryptase-chymase (MC_{tc}), and nerves fibres. Additionally, the neuropeptide receptors NK1R, NK2R and CGRPR were also found to be expressed on MCs. The number of MCs expressing NK1R and NK2R but not CGRPR was significantly increased in AR. Interestingly, MC_{tc} mostly expressed these neuropeptide receptors. Furthermore, tachykinergic nerve fibres were found to express PAR2 and TrkA as receptors for MC mediators. These results suggested that the interactions of MCs, especially of MC_{tc} with airway nerves, may play an essential role in the pathophysiology of allergic rhinitis (Paper I)

Neuroimmune interactions can occur not only in target organs innervated by nerve fibres but also with their neuropeptides and receptors. Immune cells have been shown to interact directly with neurons in airway ganglia (Paper II). Immune cells with DC-phenotypes were found to be closely located to vagal sensory neurons in the

ganglia jugular–nodosum complex (JNC), which innervate the lower airways. The number of DC increased significantly in allergic airway inflammation induced by house dust mites (HDM). The proliferation analysis suggested that DC migrated into the ganglia during allergic airway inflammation. Furthermore, the number of neurons expressing Calcitonin Gene-Related Peptide (CGRP), which can induce the migration of DCs, was also found to be increased in HDM-treated mice.

Fluticasone propionate (FP) has been commonly used for treating bronchial asthma and chronic obstructive pulmonary disease (COPD). FP treatment was found to suppress allergic airway inflammation. However, this treatment did not have any effects on the numbers of DCs and neurons expressing CGRP in JNC (Paper III).

In another study (Paper IV), we investigated the distribution and proliferation of MC populations in different lung compartments, along with the association of mast cells with nerve endings, using a house dust mite (HDM) model for allergic airway inflammation. HDM treatment caused an increased migration of MCs into bronchi, alveolar parenchyma and airway vessels. The number of tryptase-chymase expressing MC (MC_{tc}) increased significantly in the bronchi and alveolar parenchyma but not in the vascular tissues through allergic airway inflammation. Our analysis revealed an anatomical connection between MCs and bronchial nerve fibres. Under morphological aspects this connection was not changed after HDM treatment.

Altogether, the data support the hypothesis that mast cell populations may contribute to allergic airway inflammation and that the immune-nerve interaction occurred either in the affected organs itself and also in the peripheral airway ganglia. Neuro-immune interactions may play a crucial role in the pathophysiology of allergic airway inflammation, such as allergic rhinitis and allergic asthma. Understanding these underlying mechanisms may provide novel therapeutic targets for the treatment of allergic disorders.

2 Zusammenfassung

Allergie ist eine Überempfindlichkeitsreaktion des Immunsystems gegen harmlose Proteine, auch als Allergene bekannt, wie Pollen, Tierhaare und Hausstaubmilben. Allergische Atemwegserkrankungen, wie allergische Rhinitis und allergisches Asthma, sind wegen der steigenden Prävalenz in den letzten Jahrzehnten in Europa und in Industrieländern zu einer Volkskrankheit geworden. Allergische Entzündung kann nach den gegenwärtigen Forschungsergebnissen weder als eine rein immunologische noch als eine ausschließlich neurogene Entzündung angesehen werden. Bidirektionale neuroimmune Interaktionen wurden erforscht und spielen vermutlich eine wichtige Rolle bei allergischen Erkrankungen. Es wurde gezeigt, dass neuroimmune Interaktionen über den direkten Kontakt oder über die Mediatoren und Rezeptoren der Immunzellen und Nerven vermittelt werden. In den dieser Arbeit zugrunde liegenden Studien wurden Interaktionen von Mastzellen und dendritischen Zellen mit der Atemwegsinnervation bei allergischen Erkrankungen der Maus und Mensch untersucht.

Mastzellen (MCs) und Atemwegsinnervation spielen eine wichtige Rolle in allergischer Rhinitis (AR), jedoch ist über ihre Interaktion noch wenig bekannt. In einer Untersuchung wurde erhöhte Kontakte von Nerven mit Mastzellen, inbesondere Mastzellen mit Tryptase und Chymase (MC_{tc}) bei AR beobachtet. Zusätzlich wurde auch die Expression der Neuropeptidrezeptoren NK1R, NK2R und CGRPR auf MCs nachgewiesen. Die Anzahl der Mastzellen, die NK1R und NK2R, aber nicht CGRPR exprimieren, war in AR signifikant erhöht. Interessanterweise, exprimierten ein großer Teil der MC_{tc} diese Neuropeptidrezeptoren. Umgekehrt konnte gezeigt werden, dass tachykinerge Nervenfasern auch Rezeptoren für Mediatoren von MCs wie PAR2 und TrkA exprimieren. Diese Ergebnisse deuten daraufhin an, dass die Interaktion von

MCs, insbesondere von $MC_{tc,}$ mit Atemwegsinnervation eine wesentliche Rolle bei der Pathophysiologie der allergischen Rhinitis spielen können (Paper I).

Die neuroimmunen Interaktionen finden nicht nur in den Zielorganen sondern auch in den weit entfernten Atemwegsganglien statt. Die Immunzellen können auch direkt mit dem Atemwegsneuron in Wechselwirkung treten (Paper II). Es wurde festgestellt, dass Immunzellen mit charakteristischen Phänotypen für dendritische Zellen (DCs) direkt an vagalsensiblen Neuronen der Atemwegsganglien des Jugular-Nodosum-Komplex (JNC) lokalisiert sind. Die Anzahl der DCs hat sich bei der Hausstaubmilben (HDM)-induzierten allergischen Atemwegsntzündung deutlich erhöht. Untersuchungen mittels Proliferationsanalyse schlossen eine Proliferation der DCs in den Ganglien während der allergischen Atemwegsentzündung aus, so dass eine Migration von DCs in den Atemwegsganglien bei HDM-behandelten Mäusen angenommen wird.

Fluticasonpropionat (FP) wird häufig für die Therapie von Asthma bronchiale und chronisch obstruktive Lungenkrankheit (COPD) verordnet. FP Behandlung unterdrückte die HDM induzierte allergische Atemwegsentzündung im Mausmodell, hatte jedoch keine Auswirkungen auf die Anzahl von DCs und Neuronen, die CGRP in JNC exprimieren (Paper III).

In weiteren Studien wurden die Verteilung, die Proliferation und das Überleben von MC-Populationen in verschiedenen Kompartimenten der Lunge und die Assoziation von Mastzellen mit den Nervenfasern in einen HDM-Mausmodell für allergische Atemwegsentzündung untersucht. Behandlung mit HDM führte zu einer gesteigerten Migration von MCs zu den Bronchien, dem alveolaren Parenchym und Atemwegsgefäßen. Die Anzahl der Tryptase-Chymase exprimierten MCs (MC_{tc}) erhöhte sich signifikant in den Bronchien und dem Alveolarparenchym, jedoch nicht im Bereich um die Gefäße während der allergischen Atemwegsentzündung. Kontakte

von MCs und bronchialen Nervenfasern waren zahlreich, vermehrte Kontakte wurden bei allergischer Atemwegsentzündung aber nicht gefunden. Die Ergebnisse dieser Arbeiten zeigen, dass die Immunzellen, wie die antigenpräsentierenden Zellen DC und Mastzellen, direkt mit den Nervenfasern oder indirekt mit den Atemwegsneuronen in den Ganglien in Kontakt stehen, so dass neuroimmune Interaktionen in den betroffenen Organen und auch in den Atemwegsganglien stattfinden können. Ein besseres Verständnis der neuroimmunen Kommunikation kann zu einem besseren Verständnis der Pathophysiologie der allergischen Atemwegsentzündung führen und somit auch neue therapeutische Möglichkeit eröffnen.

3 Introduction and aims of the studies

3.1 Respiratory system

The cells and tissues within the body require oxygen to stay alive and to function. The respiratory system provides oxygenated blood to the body tissues and removes carbon dioxide. Anatomically, the respiratory system can be divided into two main components: the upper and lower airways [1]. The upper airway extends from the sinonasal area to the larynx. Its functions are conducting ambient air directly into the trachea, warming, filtering, and humidifying the inspired air and protecting the lower airway from foreign materials [2;3]. The lower airway extends from the trachea to the lungs. The inspired air is conducted in the lower airway through the many branches of the respiratory tree to the alveoli, which are the small air sacs at the end of the respiratory bronchioles where gas exchange takes place [4;5]. Continuous exposure to airborne allergens, such as pollen and house dust mites may lead to allergic diseases of the airways, including allergic rhinitis and allergic asthma [1].

3.2 Allergic rhinitis

Allergic rhinitis is a common inflammatory disease of the nasal mucosa with a high prevalence in developed countries. According to World Allergy Organization (WAO), over 400 million people of all ages suffer from allergic rhinitis worldwide [6;7]. Allergic rhinitis occurs when the immune system mistakenly identifies a typically harmless substance, such as pollen, as an intruder [7-10]. Allergic rhinitis can be categorised into two types: seasonal allergic rhinitis and perennial allergic rhinitis. Seasonal allergic rhinitis is caused by outdoor allergens, such as pollen, while perennial allergic rhinitis is caused by indoor allergens, such a pet dander and house dust mites [6-9;11].

The typical symptoms of allergic rhinitis, including sneezing, itching and nasal congestion, arise as a result of inflammation caused by an IgE-mediated immune

response in which the specific allergens bind to the IgE on the surface of basophils and mast cells [8-10;12]. This binding leads to the activation of mast cells, which results in the release of various mediators, including histamine, proteases, cytokines and other compounds. The end result of this mediator release of mast cells is an immediate hypersensitivity [8;13-16]. Moreover, mast cell mediators can also induce the infiltration of other immune cells as well as activating the nervous system. Immune cells and nerves in turn release further inflammatory mediators and neuropeptides that can impair the symptoms of allergic rhinitis or can conversely affect the functions of mast cells [12;17-20].

3.3 Allergic asthma

Asthma is designated as a serious public health problem that affects approximately 300 million people of all ages in the world and prevalence has markedly risen in the last few decades [21;22]. Allergic bronchial asthma is a chronic inflammatory respiratory disease characterised by airway hyperresponsiveness, mucous secretion, airflow obstruction and the structural changes in the airways (airway remodelling) [23-26]. Allergic asthma is triggered by inhaling a harmless substance, such as pollen, house dust mites, pet dander or mould. Environmental triggers can concurrently act on airway epithelial cells and airway afferent nerves [25;27-29]. The activation of epithelial cells initiates the responses of the immune system by releasing cytokines and chemokines, which activate or induce the migration, proliferation and differentiation of immune cells [23;30]. The activation of airway afferent nerves may lead to the release of their own neuropeptides as well as stimulate reflex responses that lead to the release of the bronchoconstrictor acetylcholine. The neuropeptides can either influence immune cells or contribute to the symptoms of asthma [29;31-35]

3.4 Allergy

Allergic disease is one major public health problem that affects approximately 25% of people in Western Europe and industrial countries, and the prevalence increased dramatically over the past few decades [36;37]. The term allergy was used for the first time in the year 1906 by Clemens von Pirquet in the “Münchener Medizinische Wochenschrift” to describe the unusual tendency of some individuals to develop signs and symptoms of reactivity when they were exposed to certain substances [36;38].

Allergy is a hypersensitive adaptive immune response to non-infectious environmental substances called allergens that are usually harmless, such as house dust mite, pollen, and animal dander [25;26;36]. The exaggerated response of the immune system may cause damage to the host. Allergy often arises as allergic rhinitis, allergic asthma, dermatitis, and anaphylaxis [26;36]. Based on the pathophysiological mechanisms, allergy can be divided into four types: type I, type II, type III and type IV [39-41].

Type I or immediate hypersensitivity, is an IgE-mediated allergic reaction. The binding of the allergen to the crosslinking of the IgE-Fc-receptor triggers the release of histamine and other proinflammatory mediators from mast cells and basophils [26;36;40]. These mediators cause a range of allergic symptoms. The reaction usually takes several minutes to develop after exposure to the allergen. Examples include allergic asthma, allergic rhinitis, allergic conjunctivitis and anaphylaxis [36;39-41].

Type II or cytotoxic hypersensitivity reactions are primarily mediated by the antibodies of the IgM and IgG classes and their complement. The binding of antibodies to antigens on the surface of cells or other tissue components leads to cell lysis or extracellular tissue damage via complement activation or antibody-dependent

cell-mediated cytotoxicity (ADCC). Examples include transfusion reactions, erythroblastosis fetalis, autoimmune haemolytic anaemia and transplant rejection [39-41].

Type III or immune complex reactions are immune complex-mediated allergic reactions. Antigen-antibody complexes trigger the complement cascade and generate components, including C5a, C3a, C3b. These complement components attract polymorphonuclear leukocytes (PMN), which then release lysosomal enzymes that lead to inflammatory responses. Antigen-antibody complexes can be deposited systemically or locally and cause systemic or local inflammation. Examples include systemic lupus erythematosus and serum sickness [39-41].

Type IV or delayed-type hypersensitivity is a cell-mediated allergic reaction. The activation of the CD4+ helper T cells leads to the release of cytokines that activate cytotoxic (CD8+) T cells and other immune cells, including macrophages and monocytes, which mediate direct cellular and tissue damage. Examples include contact dermatitis and granulomatous diseases [39-41].

3.5 Pathomechanism of allergy (Type I hypersensitivity)

Most allergic diseases, including allergic asthma and allergic rhinitis, are type I hypersensitivities. The process of allergic inflammation can be divided into two stages: sensitization and allergic reactions [40;41] (Figure 1).

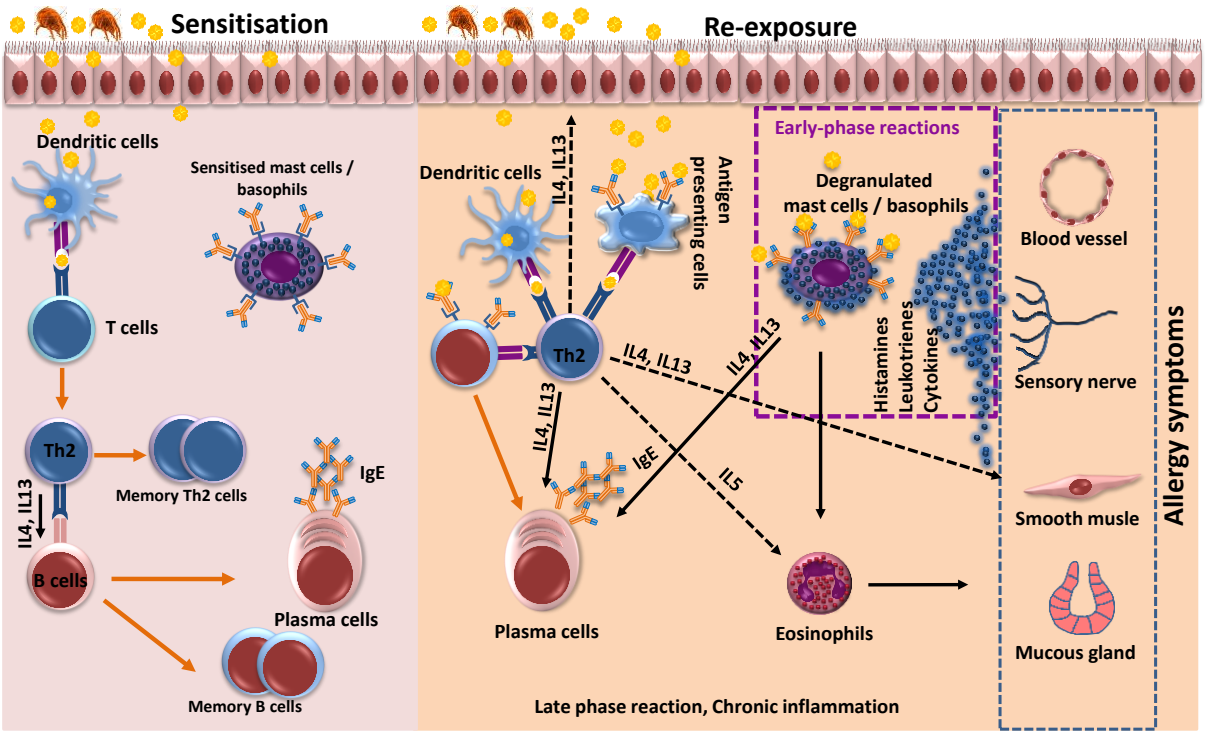


Figure 1: Mechanisms of type I hypersensitivity. The allergic immune response begins with sensitization. Following entry via mucosal surfaces, allergens are taken up by local antigen presenting cells, such as dendritic cells. APC then process the allergens and they are presented to naïve T-lymphocytes in regional lymph nodes. T cells preferentially differentiate to a Th2 phenotype. Cytokines, such as IL4, IL13, and co-stimulatory signals from Th2 cells, stimulate B cells to transform memory-activated B cells and antibody secreting cells called plasma cells, which produce allergen-specific immunoglobulin E (IgE). IgE then sensitizes mast cells and basophils by binding to Fce receptors (FceRs) on their cell surfaces. Upon re-exposure, the cross-linking of the IgE–receptor complexes on the surface of sensitized mast cells and basophils leads to the degranulation and release of inflammatory mediators, including histamine, leukotrienes, and prostaglandins, that cause immediate reactions. In late and chronic reactions, the allergens are presented to Th2 by antigen-presenting cells, such as B cells and dendritic cells. These cells lead to the activation, the proliferation and the release of pro-inflammatory cytokines, such as IL-4, IL-5 and IL-13 of Th2 cells. Mast cell-derived mediators and Th2 cytokines, such as IL-5, lead to the recruitment of eosinophils, which can also release inflammatory mediators.

3.5.1 Sensitization

The allergic immune response begins with sensitization. When the allergen, such as house dust mites, pollen or pet dander, are inhaled, they will be taken up by antigen

presenting cells (APC), such as dendritic cells (DC) and macrophages [28;36;40;42]. The APC then process the allergens into small antigenic peptides (antigen), which are displayed on the cell surface by major histocompatibility complex class II (MHC II) molecules and presented to the T cell receptors on the naïve T-lymphocytes in regional lymph nodes. That presentation leads to the activation and differentiation of $CD4^+$ naïve T-cells to T helper type 2 (Th2) cells [36;40;42;43]. Cytokines, such as IL4, IL13, and co-stimulatory signals from Th2 cells, stimulate B cells to transform to antibody secreting cells called plasma cells, which produce allergen-specific immunoglobulin E (IgE). The secreted IgE antibodies bind through its Fc portion to the high affinity receptor FcεRI on the cell surface of various cells, such as mast cells and basophils [14;25;36;40].

3.5.2 Allergic reactions

The allergic immune response to allergen by re-exposure can be classified into three temporal phases. The early phase reactions occur within seconds to minutes after re-exposure to the allergen. The late-phase reactions are induced within several hours. The chronic allergic inflammation is a constant inflammatory response that occurs if the allergen-exposure persists [36;40].

3.5.2.1 Early phase reactions

Upon re-exposure, the allergens are captured by mast cell/basophil-bound-IgE antibodies. The cross-linking of the FcεRI via IgE-antigen complexes activates the intracellular signalling pathways that lead to the degranulation of mast cells or basophils with the release of inflammatory mediators, including histamine, leukotrienes and prostaglandins. These actions cause a range of allergy symptoms, such as oedema caused by the increased vascular permeability and vasodilation,

sneezing, airway mucus secretion, bronchoconstriction, urticaria, vomiting and diarrhoea [26;28;36;40].

3.5.2.2 Late phase reactions

The late phase reactions occur approximately 4 to 6 hours after the allergen exposure. The mast cell-derived mediators cause the vascular permeability leading to recruitment of leukocytes into the tissue. The mediators, such as IL4 and IL5, act as chemoattractants that promote the infiltration and activation of inflammatory cells such as Th2 cells, eosinophils, basophils and other leukocytes [24;28;36]. After activation, these immune cells release an amount of inflammatory mediators. Eosinophils produce and store a variety of mediators, including cytotoxic proteins, lipid mediators, and cytokines that lead to epithelial damage. The Th2-lymphocyte-derived cytokines IL-4 and IL-5 induce IgE production, eosinophil survival and inflammatory cell recruitment [28;36]. The late phase response was associated with various symptoms, including shortness of breath, coughing, continuous mucus production, constant blockage of the nasal passages, swelling and oedema [36;39;40].

3.5.2.3 Chronic allergic inflammation

Continuous allergen exposure causes persistent inflammation, which is characterized by the presence of large numbers of inflammatory cells as well as the alteration of structural cells, including goblet cell hyperplasia, airway wall fibrosis, smooth muscle thickening and increased vascularity [36;39;40].

3.6 Neuroimmune interactions in allergic inflammation

Allergic inflammation is due to a complex interaction between the immune and nervous systems. Bidirectional neuroimmune interplay during allergic inflammation has been proposed and is believed to be involved in allergic disorders [32;44].

Previous studies supported this assumption by showing the structural and functional associations of immune cells and nerves in several allergic diseases [18;45;46]. The communication between immune cells and nerves can occur by either direct contact (membrane-to-membrane contact) or via soluble mediators released by immune cells and nerves [18;47-49]. The activation of afferent nerves by environmental triggers, such as HDM, pollen and pet dander, may lead to a release of neuropeptides and to the reflex responses of the autonomic nervous system [29;50]. Neuropeptides, including SP and CGRP, can act on either structural cells or immune cells, including dendritic cells and mast cells, by inducing the proliferation, migration and mediator expression or degranulation that contribute to symptomatic disease [29;31;33]. Conversely, immune cell-derived mediators, such as proteases and cytokines, can mediate signals from the immune system to the nervous system and stimulate the synthesis of neuropeptides, which also contribute to symptoms of allergy [17;51-53]. Neuroimmune interactions are believed to play an important role in the pathophysiology of allergic diseases and have become a focus of allergy research.

3.7 Airway innervation

Airways are richly innervated by autonomic and sensory nerve fibres, which regulate many aspects of airway function, including airway and vascular tone, mucus secretion, vascular permeability, and the migration and activation of immune cells [29;54]. According to traditional classification, the nerve supply of the airways is divided into a sensory and autonomic efferent system, including sympathetic and parasympathetic innervation [29;32;54;55].

3.7.1 Upper airway innervation

3.7.1.1 Sensory innervation of the upper airway

The functions of sensory nerves are relaying the signals from organs to the central nervous system so that suitable responses in the motor outputs can occur. The sensory nerve fibres innervating the nasal mucosa originate from the neurons located in the trigeminal ganglion and have been found to innervate blood vessels, glands and the epithelium of the nasal mucosa [20;55;56]. Sensory neuropeptides, such as tachykinins (substance P (SP)) and CGRP, have also been found in nasal-specific trigeminal neurons and nerve fibres projecting to the nasal mucosa [55-57]. Through mechanoreceptors and chemosensitive nerve endings, sensory nerve fibres can protect the lower respiratory tract against inhaled harmful particles and chemicals through nasal protective reflexes [8;20]. Stimuli, such as chemicals, cigarette smoke and pollen, can activate sensory neurons and lead to the release of proinflammatory neuropeptides, such as SP and CGRP [56;57]. These neuropeptides can induce vasodilatation of blood vessels, mucous secretion and the activation of various immune cells [20;55-58].

3.7.1.2 Autonomic innervation of upper airway

The autonomic nervous system controls the functions of the nose, including nasal resistance, the air-conditioning action, mucous secretion, mucus blanket and the function of the cilia through sympathetic and parasympathetic nerve fibres. The functions of the autonomic nerve systems can become unbalanced during nasal inflammation, which may lead to an increase in nasal congestion and nasal secretions [20;55;59;60]. Autonomic nerve fibres in the nose originate from the superior salivatory nucleus in the brain stem (parasympathetic) and superior cervical ganglion (sympathetic) via the pterygopalatine ganglion [55;60].

Several sympathetic nerve fibres were found around nasal blood vessels, whereas, the parasympathetic nerve fibres abundantly innervate mucus glands [20;55]. Stimulation of sympathetic nerve fibres may lead to the release of noradrenaline (NA) which causes the contraction of blood vessels in nasal mucosa. Another neuropeptide of sympathetic nerve fibres is the neuropeptide tyrosine (NPY). NPY is able to regulate the vasoconstriction of the blood vessels [19;55;60]. Nasal secretions of mucus glands are controlled by parasympathetic nerve fibres through neuropeptides, such as acetylcholine (ACh), whereas other parasympathetic neuropeptides, including vasoactive intestinal peptide (VIP), stimulate the vasodilatation of blood vessels [12;20;55;60;61].

3.7.2 Lower airway innervation

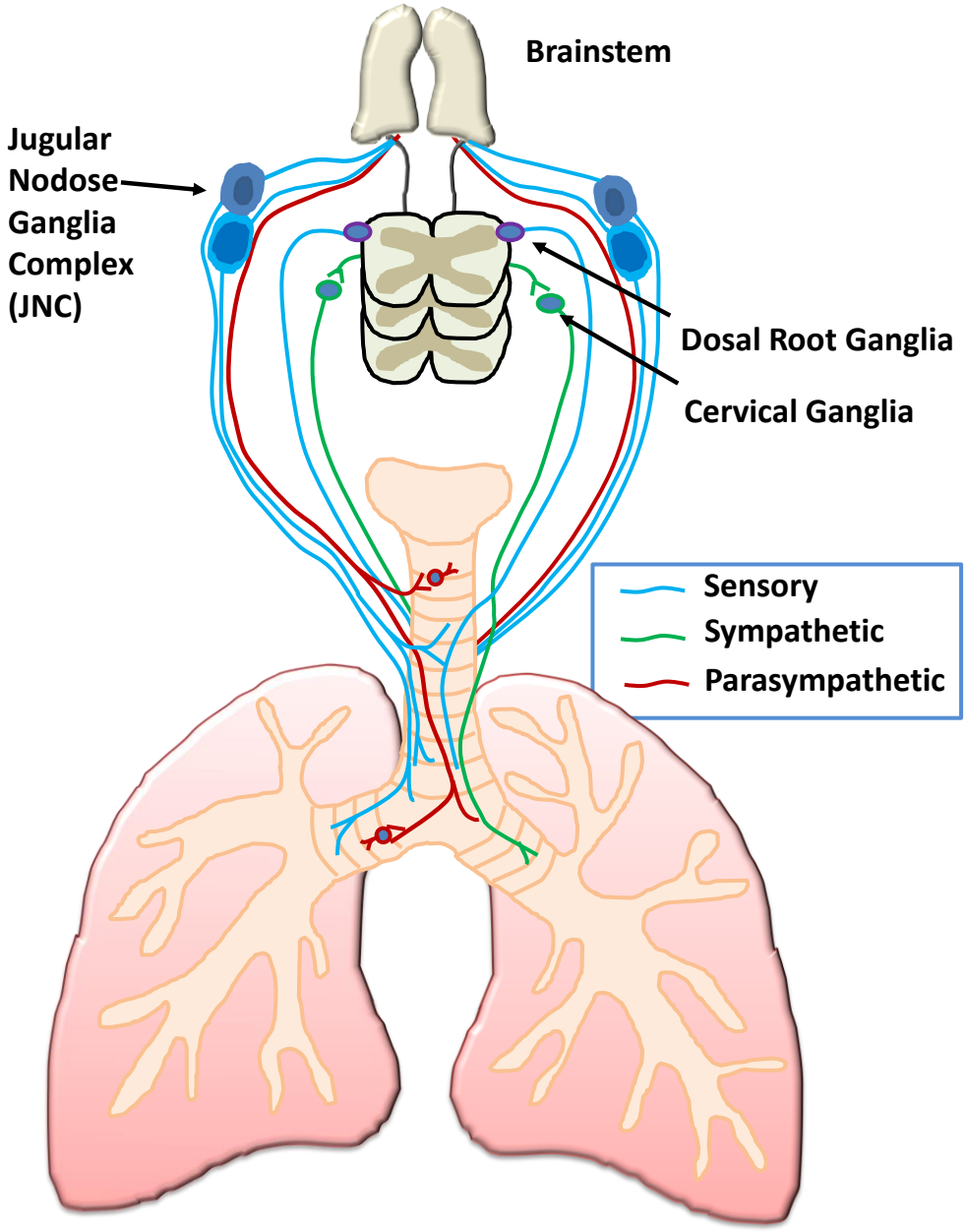


Figure 2: Lower airway innervation. The sensory innervation of the lower airway originates from the jugular nodose and dorsal root ganglia. Parasympathetic ganglia located within the airway wall are connected with the motor nuclei of the brainstem by vagus nerves. Sympathetic neurons, which are derived from the cervical and thoracic spinal cord, are located in the cervical and thoracic ganglia, and the sympathetic postganglionic fibres reach from these ganglia to the lungs [62-65]

3.7.2.1 Sensory innervation of the lower airway

The largest portion of sensory innervation to the mammalian lungs is carried in the vagus nerve. The neuron cell bodies are localised in two distinct vagal sensory ganglia, including the nodose ganglion and the jugular ganglion, which provide input to the nucleus in the brain stem. These ganglia have also been called the nodose and jugular ganglia complex (JNC) in the mouse [35;54;55;63;65-67]. The smaller part of sensory nerves innervating the lower airways originates from the dorsal root ganglia [68]. Based on the functional properties, the sensory nerve endings were categorised in three classes: rapidly adapting stretch receptors (RARs), slowly adapting stretch receptors (SARs), and C-fibre endings [32;54].

Stimuli, such as allergens, cigarette smoke and proinflammatory mediators, can activate airway sensory nerves that lead to changes in the motoric outputs and the release of neuropeptides, such as tachykinins (SP, NKA) and CGRP, which causes various respiratory defence reflexes, such as hyperaemia, oedema, mucus hypersecretion, bronchoconstriction, changes in blood pressure and cough [29;32;54;63;64;67]. Moreover, sensory neuropeptides can also influence the function of various immune cells in the lungs, including dendritic cells [49;69-72] (Figure 2).

3.7.2.2 Autonomic innervation of the lower airway

Autonomic nervous innervation is derived from the parasympathetic and sympathetic nerves to the lower airway and plays a major role in regulating airway smooth muscle and vascular tone [29;32;34;54;55;73]. There are two clusters of neuronal cell bodies involved in the autonomic nerve pathway. One is located in the brain stem or spinal cord and is called a nucleus. It is connected by nerve fibres to the other autonomic ganglia [33;54;55;73].

Parasympathetic ganglia located within the airway wall are connected with motor nuclei of the brainstem by vagus nerves. The ganglia of the sympathetic nerves are located outside the spinal cord, and postganglionic fibres reach from these ganglia to the lungs [33;55;73].

The autonomic innervation in mammalian lower airways is composed mostly of parasympathetic nerves terminating in bronchial smooth muscle and submucosal glands [33;54;55;73]. Parasympathetic nerves in the human lungs contain neurotransmitters, such as acetylcholine (Ach), vasoactive intestinal peptide (VIP) and the molecular nitric oxide (NO). Neuropeptides, such as SP and CGRP, were also found on airway parasympathetic nerve fibres of other species. Stimulation of parasympathetic nerves may lead to the release of Ach that causes bronchoconstriction, mucus secretion and bronchial vasodilation [29;31;33;55;73;74]. Sympathetic nerves were sparsely found around bronchial blood vessels and submucosal glands [54;55;73]. The neurotransmitter release by airway sympathetic nerves are noradrenalin (NA), neuropeptide Y (NPY), VIP and NO [29;31;54;55;73]. Relatively little is known about the functions of airways sympathetic nerves. It could be that the sympathetic nervous system regulates the airways via noradrenalin receptors (α- and ß-adrenergic receptors), which were found in bronchial smooth muscle from the trachea to the terminal bronchioles [31;32;54;75]. Due to the release of several neuropeptides, sympathetic nerves may play an important role in the physiological and pathophysiological conditions of the airways (Figure 2).

3.8 Tachykinins

The tachykinins (also called neurokinins) are a family of closely related neuropeptides, which represent one of the largest peptide families described in the animal organism [31;76]. The tachykinins are derived from two distinct genes: the preprotachykinin-I gene (PPT-I or PPT-A) encodes for substance P (SP), neurokinin

A (NKA), neuropeptide K (NPK), and neuropeptide γ (NPγ), while the PPT-II gene (or PPT-B) encodes for neurokinin B (NKB). The best known members of the family are SP, NKA and NKB [67;76;77]. Tachykinins have been shown to be in a distinct subpopulation of primary afferent nerves, which are sensitive to capsaicin. The release of tachykinins can be evoked by different stimuli, such as histamine, bradykinin, prostaglandins, and leukotrienes [31;76;77].

The tachykinins are known to have various effects on allergic inflammation [35;56;57;76]. SP and NKA have been found to induce the constriction of airway smooth muscle, the secretion of the submucosal gland and vascular permeability [29;31;33;50;76;77]. Additionally, SP and NKA have the capacity to regulate the function and migration of various immune cells, such as mast cells, B cells, T cells, eosinophils and neutrophils [78-80]. Tachykinins act on specific membrane receptors that belong to the family of G-protein-coupled receptors [76;77]. Three well-characterised tachykinin receptors are the Neurokinin 1 receptor (NK1R), NK2R, and NK3R. All three receptors can be activated by tachykinin Calcitonin Gene-Related Peptide (CGRP). However, according to the order of tachykinin potency, three receptors are recognized: SP > NKA > NKB for NK1R, NKA > NKB > SP for NK2R and NKB > NKA > SP for NK3R [76;77]. Additionally, the newly discovered Mas-related gene X2 (MrgX2) receptor belongs to a mas-related gene family, which has been reported to contain receptors of SP [81-84].

3.9 Calcitonin Gene-Related Peptide (CGRP)

CGRP is a member of the calcitonin family of peptides, consists of 37 amino acids and is a product of calcitonin/CGRP pre-mRNA alternative splicing [77;85;86]. CGRP is expressed predominantly by the sensory nervous system and is often co-localized along with tachykinins [85;87]. This neuropeptide was found to be expressed in nerve fibres that project to many organs, including the respiratory system [31;77;86;88].

CGRP acts through the high affinity G-protein coupled receptor called calcitonin receptor-like receptor (CRLR) and a receptor activity modifying protein 1 (Ramp1) [32;77;87].

CGRP is reported to have multiple effects in allergic diseases, such as the regulation of bronchoconstriction of airway smooth muscle and vasodilation of vessels [29;77;86;87]. Furthermore, CGRP has been described to play an immunomodulatory role in the regulation of immune cells, such as dendritic cells and mast cells [47;69;70;89]. With respect to its pro-inflammatory role, CGRP has the capacity to act as a chemoattractant factor for different immune cells, such as $CD4^+$ T-lymphocytes, $CD8^+$ T-lymphocytes, eosinophils and DCs, and it is able to induce the proliferation of airway epithelial cells [29;49;69;72;90;91]. On the other hand, CGRP has been reported to have anti-inflammatory functions, such as suppression of IL-2 production and proliferation in murine T cells and induction of TNF-α secretion in murine bone marrow-derived dendritic cells (BMDC) by inhibition of the toll-like receptor (TLR) ligand [92-95].

3.10 Mast cells

Mast cells (MCs) are key effector cells in allergic diseases [15;96-98]. When MCs are activated, they degranulate and release a number of biologically active molecules from their secretory granules. These molecules include histamine, prostaglandins (PGs), leukotrienes (LTs), nerve growth factor (NGF) and various MC-specific proteases, such as tryptase and chymase [14;16;52;99;100]. These mediators can have effects on various cell types, such as immune, epithelium, endothelium and airway smooth muscle cells in different compartments of the airway [98;101-103].

Based on the type of neutral protease content, human mast cells can be classified in two subpopulations, including MC_t, which contains only tryptase, and MC_{tc}, which

contains chymase in addition to tryptase [98;104;105]. In contrast, rodents have been shown to express a number of chymases, such as mast cell protease (mMCP)-1, mMCP-2, mMCP-4 and mMCP-5 with distinct proteolytic properties. With respect to their biological effects, mMCP-4 expressed by mouse MCs have been suggested to be a functional homologue of human chymase [105-108].

Activation of mast cells can be modulated by various mediators, including neuropeptides [109]. Sensory neuropeptides, such as tachykinins (SP and NKA), act via neurokinin 1 receptor (NK1R), neurokinin 2 receptor (NK2R) and MrgX2, which is a newly discovered receptor belonging to a mas-related gene family (MrgX receptors), whereas CGRP activates the CGRP receptor (CGRPR) [16-18;81;110;111]. Reciprocally, a variety of molecules, including histamine, serotonin and nerve growth factor (NGF), which are synthesized and released by mast cells, can influence neuronal activity as well as the release of neuropeptides [56;112-114]. Additionally, protease-activated receptor 2 (PAR2) and the histamine receptor have also been found on trigeminal neurons in animals [115;116]. MCs exhibit variable functional aspects of both the nervous and immune systems [18;88;113]. MCs were found to be associated with nerve fibres in animal and human tissue under physiological and pathophysiological conditions [117-119]. However, little is known about the mast cell-nerve interaction in allergic airway inflammation, such as allergic rhinitis and allergic asthma .

3.11 Dendritic cells

Dendritic cells (DCs) are phagocytic cells that are localised in many organs in the skin, in the mucosa of the intestines, the upper airways, the lungs and the brain [42;43;120;121]. As professional antigen-presenting cells, DCs play a key role in the induction of allergic airway inflammation [42;43;122]. They capture the antigen,

process it and subsequently present it to naïve T lymphocytes on the MHC class II molecules (MHC II) in local lymph nodes, which leads to the initiation of Th2-immune allergic inflammatory processes [43;123;124]. It has been shown that the maturation and differentiation of DCs can be modulated by various cytokines as well as neuropeptides, such as calcitonin gene-related peptide (CGRP) [69;76;125]. In contrast, DCs can activate neurons via secretion of neurotrophins leading to the production of neuropeptides that cause neurogenic airway inflammation [126;127]. Previously, DCs were found to have a frequent anatomic association with CGRP-containing sensory nerve fibres of the airways and skin [46;128]. However, DCs in airway sensory ganglia have been not explored under normal and allergic airway inflammation yet.

3.12 Aims and Hypotheses

Paper I: Mast cells (MCs) and trigeminal nerves play an important role in allergic rhinitis (AR), but little is known about mast cell-nerve interaction in the nasal mucosa. This study aimed to investigate the mast cell-nerve association and neuropeptide receptor expression on mast cells in the human nasal mucosa during allergic rhinitis.

Paper II: Neuroimmune crosstalk between dendritic cells (DCs) and airway nerves in the lung has recently been reported. However, the presence of DCs in airway sensory ganglia under normal and allergic conditions has not been explored. This study therefore aimed to investigate the localisation, distribution and proliferation of DCs and CGRP immunoreactive (IR)-neurons in vagal sensory jugular-nodose ganglia under allergic airway inflammation by using a chronic house dust mite (HDM) mouse model.

Paper III: DCs were found to be localised in JNC under physiological conditions, and their number was significantly increased during allergic airway inflammation by

migration from outside the ganglia. However, the impact of fluticasone propionate on neuropeptide expression and on the migration of DCs in airway sensory ganglia has been not explored so far. The present study aimed to investigate the anti-inflammatory effects of steroid treatment on the presence and distribution of DCs and CGRP immunoreactive neurons in vagal sensory jugular nodose ganglia under allergic airway inflammation.

Paper IV: Mast cells (MCs) and airway nerves play an important role in allergic asthma. However, little is known about MCs and their interaction with airway nerves during allergic airway inflammation. This study aimed to investigate the distribution and proliferation of MC populations in different lung compartments, along with the association of mast cell with nerve endings, using a house dust mite *(*HDM) model for allergic airway inflammation.

4 Materials and Methods

4.1 Patients and nasal biopsies

Fourteen subjects were recruited for this study. Subjects with a positive history of allergy symptoms and subjects with a positive prick test for common aeroallergens (grass or birch pollen, hazel, *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, cat or dog dander) as well as a positive radioallergosorbent test (RAST test) were identified as patients with allergic rhinitis. The allergic rhinitis group contained eight patients with perennial allergic rhinitis. All patients had symptoms of allergic rhinitis, such as sneezing, itching, nasal blockage, and rhinorrhea at the time of nasal surgery. The control group contained six healthy subjects without any allergic diseases or symptoms of allergic rhinitis at the time of nasal surgery. All subjects were prohibited from taking any anti-inflammatory drugs for at least four weeks prior to the nasal surgery. Biopsy specimens of the nasal mucosa were collected from 2013 to 2014 and taken from the turbinate in nasal surgery to reduce the size of the turbinate due to airflow limitations with causes, such as deviation of the septum or nasal concha hyperplasia. All specimens were divided into two portions, one for cryosections and the other for PCR. All samples were then examined blindly and independent of the clinical data. This study was approved by the local ethics committee, and all participants provided written consent.

4.2 Animals

Female wild-type BALB/c-mice (6-8 weeks old) were purchased from Charles River and Janvier-Labs. The animals were held in regular 12 h dark/light cycles at a temperature of 22°C and received laboratory food and tap water ad libitum. The animals were acclimatised for at least 2 weeks prior to the study. All animal experiments were performed in strict concordance with the German animal protection law and approved by the appropriate governmental authority.

4.3 HDM-mouse models for allergic airway inflammation

BALB/c mice (n = 10) were exposed for 5 consecutive days per week within a total period of 7 weeks by intranasal instillation of HDM extract (Greer Inc., USA) with a dose of 25 µg protein in 50 µl of saline. The control group (n = 10) was treated intranasally with 50 µl of saline. For the proliferation study animals received an intraperitoneal (i.p.) injection of 1 mg of 5-ethynyl-2'-deoxyuridine (EdU) (in DMSO) (Invitrogen) at a volume of 200 µl 24 h before sacrifice. Analyses were performed 24 h after the last allergen challenge (Figure 3).

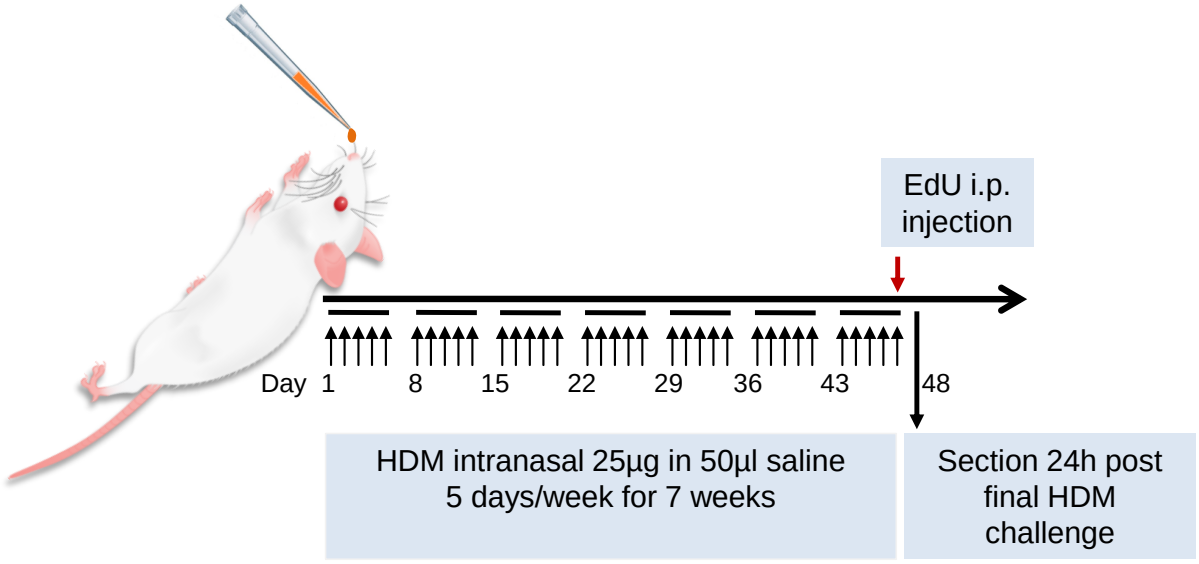


Figure 3: HDM treatment protocol: The animals were systemically sensitised by intranasal application of house dust mites 5 days a week for 7 weeks. 5-ethynyl-2'-deoxyuridine (EdU) was injected (i.p.) one day before analysis

4.4 Preparation of samples

After dissection, the human nasal biopsies as well as the mouse samples were immediately placed in Zamboni solution (Morphisto – Evolutionsforschung und Anwendung GmbH, Frankfurt am Main, Germany). After an overnight fixation, specimens were rinsed in 0.1 M phosphate buffered saline (PBS) for 24 hours and cryoprotected overnight with 30% sucrose in 0.1 M PBS. All steps in the specimen processing were performed at 4°C. The biopsy specimens were embedded in

optimum cutting temperature (O.C.T) medium (Tissue-Tek, Sakura) and frozen in liquid nitrogen. Serial 8-µm sections were prepared using a cryostat (Leica CM 1950, Nussloch, Germany), placed on APES (3-aminopropyltriethoxysilane) coated glass slides, dried at room temperature for 30 min and then stored in the freezer at -80°C.

4.5 Preparation of bronchoalveolar lavage fluid (BALF)

The animals were sacrificed 24 hours following the last airway challenge. Mice were anesthetized with an intraperitoneal injection of 0.5 – 0.7 ml/kg of a compound of Ketamine (90 - 100 mg /kg bodyweight) and Rompun 6 - 8 mg/kg bodyweight) and the tracheae were cannulated with an 18 gauge needle (Dispomed). Bronchoalveolar lavage (BAL) was performed by instillation of 1 ml ice-cold PBS containing protease inhibitors. The total number of cells was counted using a Casy® cell counter or Neubauer chamber. The BALF was centrifuged (320 x g, 10 min, 4°C), and the supernatants were removed. The cell pellets were resuspended in 1 ml PBS and 100 µl of BALF for each mouse were used for the preparation of cytospots with cytospin (Tharmacspin). The cytospot-slides were fixed with ice-cold 100% methanol and stained with Diff-Quik (Medion Diagnostics), and the differential cell counts were evaluated.

4.6 Histological staining

Most cells are transparent and colourless. Therefore, the histological stains are frequently used to make the cells or structures more visible. The techniques can either be a non-specific stain, which stains most of the cells, or a specific stain, which stains selectively chemical groupings or molecules of cells or tissues. Staining techniques usually work with two steps. First, some components of the cells are highlighted by a bright colour by using a dye. The following counterstain stains the rest of the cell in a different colour to make the stained structure more easily visible.

Acidic dyes colour cationic or basic cell components, including proteins and other components in the cytoplasm. Basic dyes stain anionic or acidic cell components, such as nucleic acids [129].

4.6.1 Hematoxylin and eosin (H&E) staining

The H&E stain is the most common staining technique used in histology, and it is usually used for recognizing various tissue types and morphological changes by highlighting the structures and cell populations. H&E stain are combined from two dyes, hematoxylin and eosin, to stain different tissue elements. Eosin reacts like an acidic dye. Therefore, it stains basic or acidophilic structures, including the cell walls, cytoplasm and extracellular fibres in various shades of red, pink and orange. Haematoxylin is a basic dye. It colours acidic or basophilic structures, such as the cell nucleus and organelles, that contain RNA a purplish blue [129].

Procedure

- Dry cryosections at room temperature for 15 minutes
- Place in Hematoxylin for 3 min
- Rinse in running tap water
- Differentiate with 1 % ethanoic acid for 10 sec
- Rinse in running tap water
- Stain with eosin for 1 min
- Dehydrate in ascending ethanol solutions (70 %, 96 %, 99 %)
- Clear in xylene for 3 minutes
- Mount coverslip onto glass slides with Entellan (Merck)

Results

Nuclei should be stained blue, and cytoplasm should be stained pink to red (Figure 4)

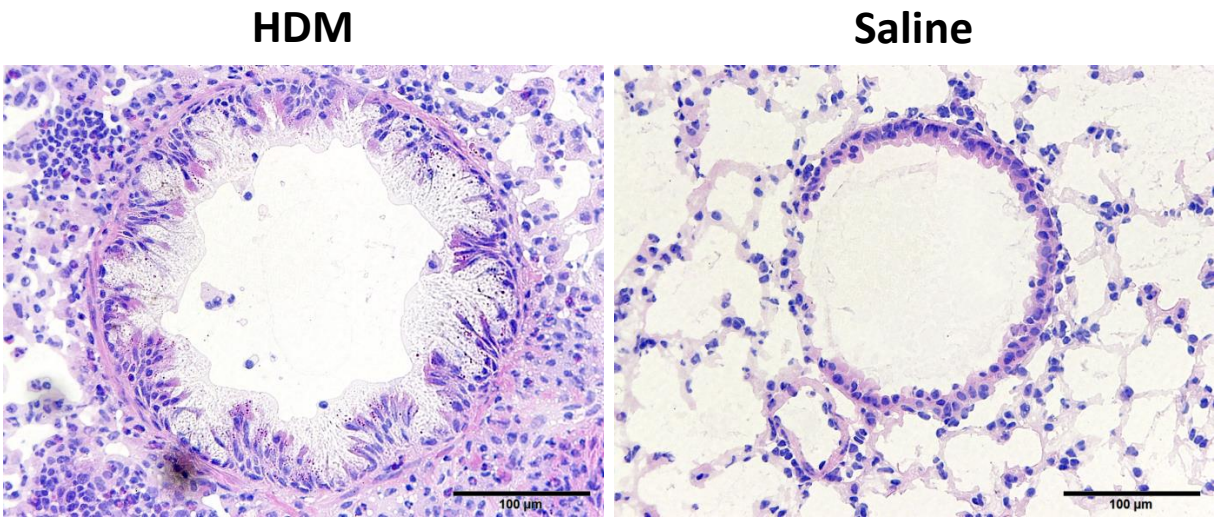


Figure 4: H&E staining of the lungs from mice treated with HDM and saline.
The lung sections from HDM-sensitised and challenged mice showed infiltration of mononuclear cells. Scale bars: 100 µm

4.6.2 Periodic acid–Schiff (PAS) staining

Periodic Acid–Schiff (PAS) staining is used to detect polysaccharides, including glycogen, and mucosubstances, such as glycolipids, glycoproteins and mucins, in cells and tissues.

Procedure

- Dry cryosections at room temperature for 15 minutes
- Rehydrate sections in PBS for 5 minutes
- Oxidize in 0.1% periodic acid solution for 5 minutes
- Wash in lukewarm tap water for 1 minute
- Rinse in distilled water for 5 minutes
- Place in Schiff reagent (Roth) for 20 minutes
- Rinse in distilled water for 5 minutes
- Counterstain in hematoxylin (Sigma-Aldrich) for 1 minute
- Wash in tap water for 5 minutes
- Dehydrate in ascending ethanol solutions (75%, 80%, 99%)

- Clear in xylene for 3 minutes
- Mount coverslip onto a labelled glass slide with Entellan (Merck)

Results

Glycogen, mucin and some basement membranes should be stained red to purple, and the nuclei should be stained blue (Figure 5).

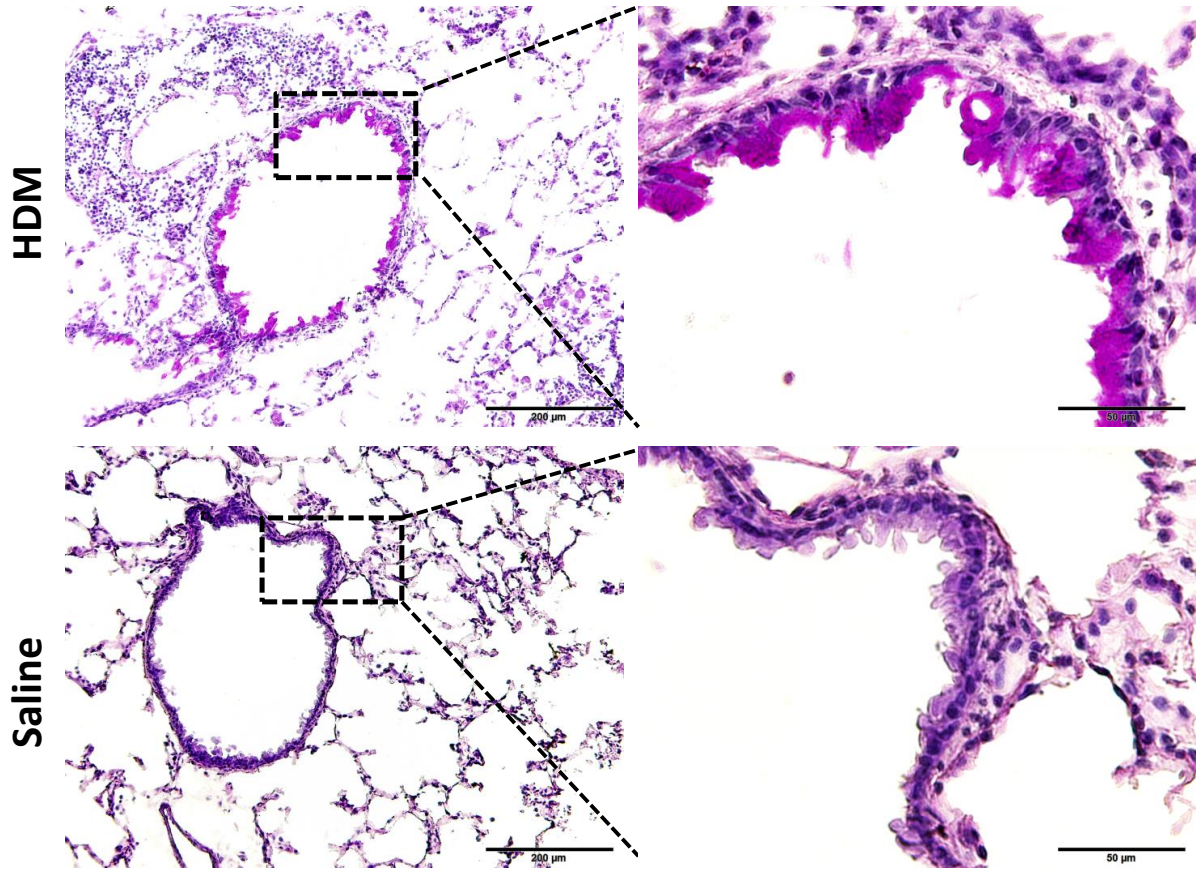


Figure 5: PAS staining of lung tissues from mice treated with HDM and saline.
C and D are larger magnifications of A and B. The lung sections from HDM-sensitised and challenged mice showed more mucus-expressing cells (purple) than saline-treated animals. Scale bars: 200 µm in A and B, 50 µm in C and D

4.6.3 Diff-Quik staining, BALF and NALF differential cell count analysis

Diff-Quick is a modification of the Romanowsky Stain technique, which is used in the differential staining of basophilic and acidophilic material. Diff-Quick is a polychromatic stain combined from Eosin Y (an anionic dye) and thiazine dyes (cationic dyes) that consists of Methylene Blue and Azure A. When applied to

immune cells, the dyes produce multiple colours based on the ionic charge of the stain and the various cell components. The eosin ions are negatively charged and stain the granules in the cytoplasm a bright orange to pink colour. The methylene blue ions are positively charged and colour the acid cell component nucleoli and cytoplasm in varying shades of blue [130]. In these studies, Diff-Quik staining was used to stain the cells in BALF.

Procedure

Diff-Quik staining set of Medion Diagnostics was used, and the stain was performed according to the manufacturer's recommendations with modifications as described below.

- Dry slides at room temperature for 15 minutes
- Dip slides in Stain Solution I for 45 seconds
- Dip slides in Stain Solution II for 45 seconds
- Rinse slides in distilled water
- Mount coverslip onto glass slides with Entellan (Merck)

Results

Nuclei should be stained dark-blue to blue-violet with blue cytoplasm blue, and granules that are red to red-orange (Figure 6).

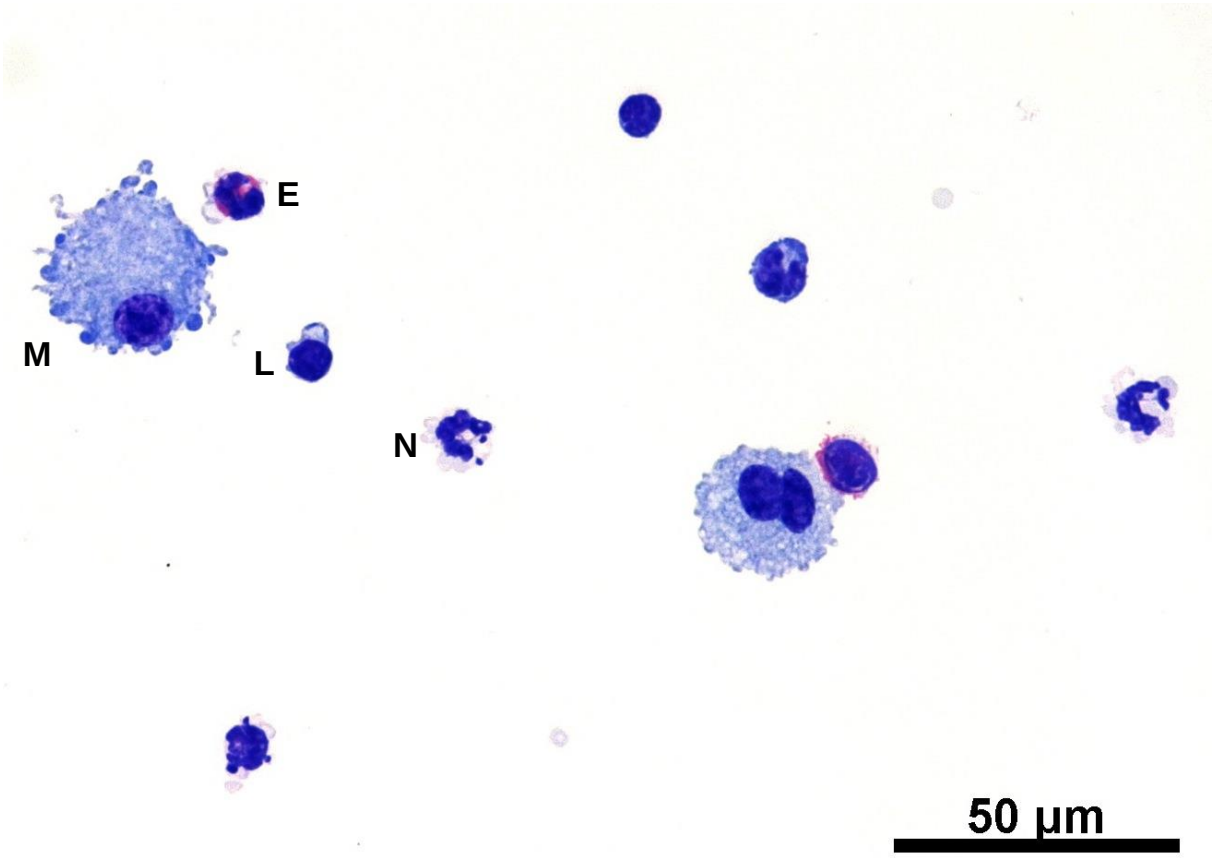


Figure 6: Diff-Quik Staining of BALF cells from mice treated with HDM treated: M: macrophages, L: lymphocytes, N: neutrophils, E: eosinophils. Scale bars: 50 µm

4.6.4 BALF and NALF differential cell count analysis

After the Diff-Quick staining, the differential cell counts were evaluated according to morphological characteristics of the immune cells using a light microscope. More than 300 cells from each sample were counted.

4.7 Indirect immunofluorescence stain

Immunofluorescence (IF) is an imaging technique that is based on the use of specific antibodies to label a target antigen in cells or tissue sections with a fluorescent dye. There are two major types of immunofluorescence stain: direct and indirect immunofluorescence. Direct immunofluorescence uses a fluorescent dye-conjugated primary antibody to detect the target antigen. Indirect IF uses of an unlabelled primary antibody, which binds to the target of interest, and then a fluorescent dye

conjugated anti-immunoglobulin antibody (called secondary antibody), which recognizes and binds to the constant portion of primary antibody, that indirectly localizes the target for detection with a fluorescence microscope. An indirect immunofluorescence stain was used in these studies (Figure 7).

Procedure

Cryosections were dried at room temperature for 15 minutes and then rehydrated in PBS for 5 min. To reduce non-specific antibody binding, the sections were incubated for 15 min at room temperature in 5% normal serum of the host species of the secondary antibody diluted in 0.1 M PBS. The sections were incubated with primary antibodies or the appropriate isotype control antibodies for 1 h at room temperature and then overnight at 4°C. After rinsing with 0.1 M PBS twice, the sections were incubated with secondary fluorescein-conjugated antibodies for 2 h at room temperature. For counterstaining, the sections were incubated with 100 µl of DAPI (0.5 µg/ml, Carl Roth, Germany) for 15 min at room temperature. Finally, the sections were washed twice with 0.1 M PBS and once with double distilled water, then mounted with fluorescent mounting medium Fluoroshield™ (Sigma-Aldrich) or Prolong Gold (Invitrogen) and covered with coverslips.

The slices were visualised with epifluorescence microscopes (Axioskop 2 plus, Axio Imager M2 (Carl Zeiss) and Olympus BX5) or a confocal laser scanning microscope, LSM 510 META (Carl Zeiss, Jena, Germany). The confocal images were processed using Imaris 4.5.2 (Bitplane, Zurich, Switzerland).

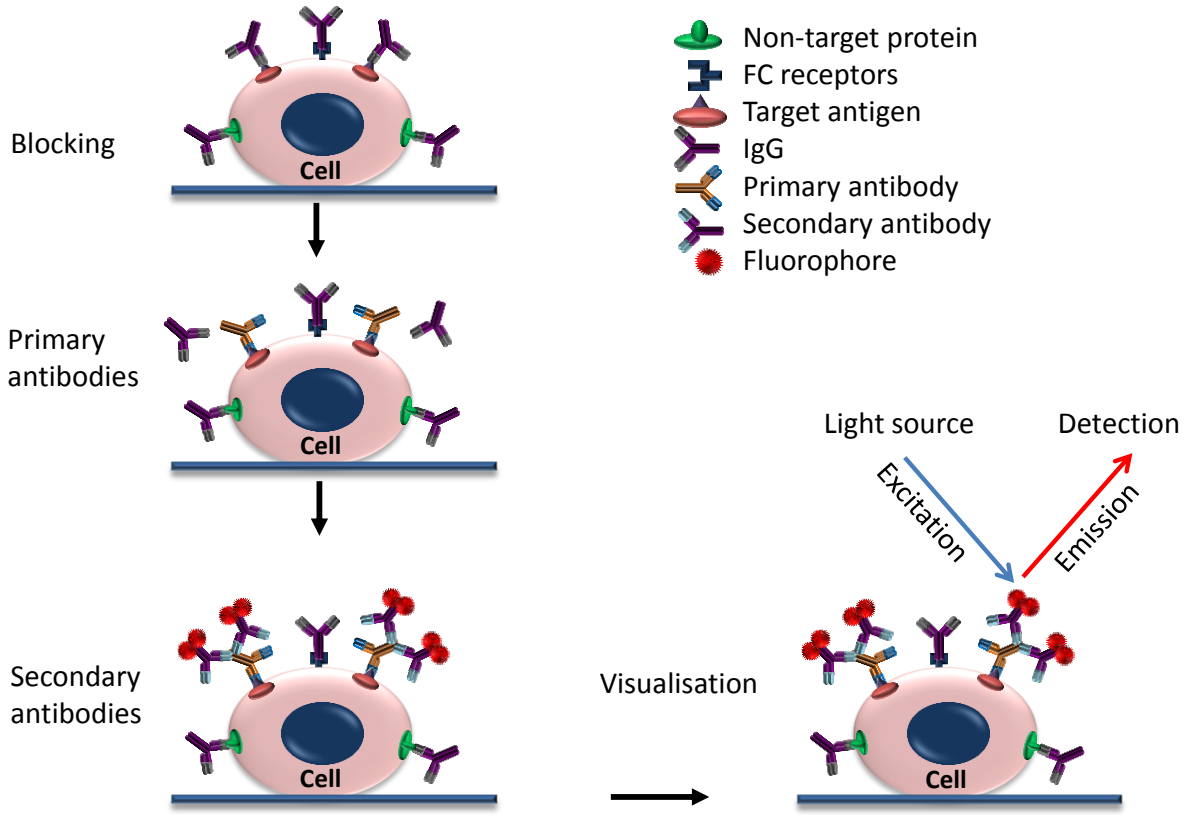


Figure 7: Principles of an indirect immunofluorescence stain: In the blocking step, the IgG in the serum of the host species of the secondary antibody will bind unspecifically to the target antigen as well as the non-target antigen. The primary antibody has a high affinity to the target antigen and will replace the normal IgG and bind specifically to the epitope on the target antigen. The fluorescent dye conjugated secondary antibodies will bind specifically to the Fc-portion of the primary antibody.

4.8 In vivo proliferation study with EdU (5-ethynyl-2'-deoxyuridine)

The Click-iT™ (Invitrogen) method is a technique for labelling DNA in vivo that allows researchers to image the replicated DNA in the context of well-preserved cellular and chromatin ultrastructure. Click-iT™ reactions using azides and alkynes as specific binding moieties in a two-step procedure that first labels and then subsequently detects the molecule of interest. In these studies, the 5-ethynyl-2'-deoxyuridine (EdU), which contains the alkyne, was used for the labelling step (Figure 8).

EdU is a nucleoside analogue of thymidine and is incorporated into DNA during active DNA synthesis. Controls and HDM-treated animals received an i.p. injection of 1 mg of EdU (in DMSO) (Invitrogen) at a volume of 200 µl 24 h before sacrifice. Incorporated EdU was detected by using the Click-iT™ Cell reaction buffer kit and Alexa Fluor 594 azide (Invitrogen) according to the manufacturer's protocol. The cryosections were rehydrated for 5 min in PBS and then blocked with 5 % normal serum. The sections were incubated with 200 µl of the prepared Click-iT reaction cocktail for 30 min and then incubated with primary and secondary antibodies as described above.

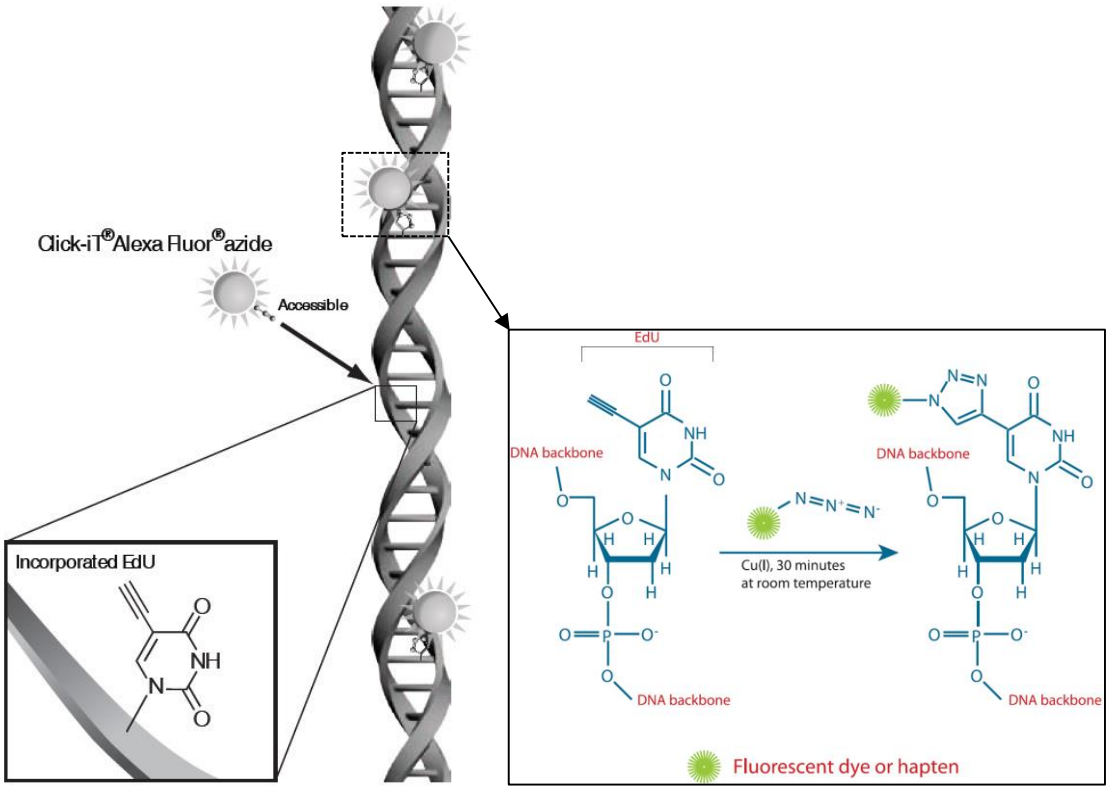


Figure 8: Click-iT technology overview provided by the manufacturer (Invitrogen). Click-iT™ reactions use EdU as a specific binding moiety to label replicated DNA. Incorporated EdU was detected using an azide tagged with an Alexa Fluor™ dye. The copper-catalysed click reaction links an alkyne with an azide to form a stable inert product (this figure was adapted from Invitrogen materials).

4.9 Total RNA extraction, cDNA synthesis and PCR

4.9.1 Total RNA extraction

In these studies, total RNA was isolated using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. To remove genomic DNA, additional DNase digestion during RNA isolation was performed. The quality of total RNA was assessed with a NanoDrop 8000 (Thermo Scientific).

4.9.2 cDNA synthesis

Reverse Transcription (cDNA synthesis) was performed using an Omniscript RT kit and random primers (Qiagen, Hilden, Germany), according to the following protocol:

1x cDNA reaction

10x Buffer RT	2 µl
dNTP Mix (5 mM each dNTP)	2 µl
Oligo-dT primer (10 µM)	2 µl
RNase inhibitor (10 units/µl)	1 µl
Omniscript Reverse Transcriptase	1 µl
Total RNA	variable
H_2O	variable
Total Volume	20 µl

4.9.3 Real-time PCR

Real-time PCR was conducted using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Germany) with a SensiMix SYBR & Fluorescein Kit from Bioline (Bioline Germany), and the reactions were conducted based on the manufacturer's manual. All samples were run in duplicate and RNA levels were normalized to the level of β-actin. The expression of target genes was calculated relative to the control group using the ΔΔCt method. Images of the PCR Midori Green Advance-stained agarose gels were acquired with the Gel Doc XR+ System (Bio-Rad).

5 Papers

5.1 Paper I

Title: Increase of mast cell–nerve association and neuropeptide receptors expression on mast cells in perennial allergic rhinitis

Authors: Duc Dung Le, David Schmit, Sebastian Heck, Albert Joachim Omlor, Martina Sester, Christian Herr, Bernhard Schick, François Daubeuf, Sebastian Fähndrich, Robert Bals, Nelly Frossard, Basel Al Kadah* and Quoc Thai Dinh*

***Neuroimmunomodulation*. 2016 Dec 29. [Epub ahead of print]**

* These authors contributed equally to the study

Original Paper

NeuroImmunoModulation

Neuroimmunomodulation
DOI: 10.1159/000453068

Received: August 8, 2016
Accepted after revision: November 4, 2016
Published online: December 29, 2016

Increase of Mast Cell-Nerve Association and Neuropeptide Receptor Expression on Mast Cells in Perennial Allergic Rhinitis

Duc Dung Le[a] David Schmit[a] Sebastian Heck[a] Albert Joachim Omlor[a] Martina Sester[b] Christian Herr[c] Bernhard Schick[d] François Daubeuf[e] Sebastian Fähndrich[c] Robert Bals[c] Nelly Frossard[e] Basel Al Kadah[d] Quoc Thai Dinh[a, c]

Departments of [a]Experimental Pneumology and Allergology, [b]Transplant and Infection Immunology, [c]Internal Medicine, Pneumology, Allergology and Critical Care Medicine, and [d]Otorhinolaryngology, Saarland University Hospital, Homburg, Germany; [e]Laboratoire d'Innovation Thérapeutique, UMR7200, CNRS-Université de Strasbourg and LabEx Medalis, Faculté de Pharmacie, Illkirch, France

Keywords
Mast cell-nerve interaction · Chymase · Allergic rhinitis · Tachykinins · Calcitonin gene-related peptide

Abstract
Objectives: Mast cells (MCs) and nerves play an important role in allergic rhinitis (AR), but little is known about their crosstalk in AR. The aim of this study was to investigate MC-nerve interaction in the human nasal mucosa during AR. ***Methods:*** The association between MCs and nerves, the expression of neuropeptide receptors (neurokinin 1 receptor [NK1R], neurokinin 2 receptor [NK2R], calcitonin gene-related peptide receptor [CGRPR], and MrgX2) on MCs, and protease-activated receptor 2 (PAR2) and tyrosine receptor kinase A (TrkA) on nerve fibres in the human nasal mucosa were investigated with immunofluorescence and real-time PCR. ***Results:*** The association between MCs and nerves was found to be significantly increased, although the numbers of MCs and nerve fibres were unchanged during AR. MCs expressing tryptase-chymase (MC_{tc}) were frequently associated with nerve fibres and these contacts increased significantly in AR. Neuropeptide receptors NK1R, NK2R, and CGRPR were firstly found to be largely localised on MCs. The number of MCs expressing NK1R and NK2R, but not CGRPR, was significantly increased in AR. Interestingly, MC_{tc} mostly expressed these neuropeptide receptors. The newly discovered tachykinin receptor MrgX2 was not expressed on nasal MCs, but was expressed on gland cells and increased in AR. Additionally, tachykinergic nerve fibres were found to express PAR2 or TrkA as receptors for MCs. ***Conclusions:*** This study revealed for the first time an increase of MC-nerve association and neuropeptide receptor expression on MCs during AR as well as nerve fibres containing receptors for MCs. These results suggest that targeting or controlling airway sensory nerve function as a modulator of MCs may prevent allergic airway inflammation such as AR.

Basel Al Kadah and Quoc Thai Dinh contributed equally to this work.

KARGER

© 2016 S. Karger AG, Basel
1021–7401/16/0000–0000$39.50/0

E-Mail karger@karger.com
www.karger.com/nim

Prof. Quoc Thai Dinh, MD
Department of Experimental Pneumology and Allergology
Saarland University Hospital and Saarland University Faculty of Medicine
Kirrberger Strasse, Geb. 61.4, DE–66421 Homburg/Saar (Germany)
E-Mail Thai.Dinh@uniklinikum-saarland.de

Background

Allergic rhinitis (AR) is a common chronic inflammatory disease of the upper airways and is characterized by nasal congestion, rhinorrhea, sneezing, and an itchy nose [1]. Activation of the central and peripheral nervous system plays a crucial role in the pathophysiology of these processes [2, 3]. Trigeminal sensory nerves innervating the nasal mucosa transmit signals to the central nervous system, causing sensations such as itching [4, 5].

Airway sensory nerves can be activated by a variety of factors, including external physical and chemical irritants, as well as by products of allergic reactions, such as mast cell (MC) mediators [2, 5, 6]. The subsequent sensory axonal responses lead to the release of neuropeptides, such as calcitonin gene-related peptide (CGRP) and/or tachykinins substance P (SP), and neurokinin A (NKA), in the nasal mucosa [7, 8]. In a vicious cycle, these neuropeptides induce vasodilatation, increased vascular permeability, glandular activation, leukocyte recruitment, and differentiation of immune cells, including MCs [2, 9–11].

MCs are key effector cells in allergic diseases, such as AR [12]. When MCs are activated, they degranulate and thereby release a number of biologically active molecules from their secretory granules. These include histamine, prostaglandins, leukotrienes, nerve growth factor (NGF), and various MC-specific proteases, such as tryptase and chymase [13–17]. Based on the type of neutral protease content, human MCs can be classified in 2 subpopulations, namely, MC_t that contain only tryptase and MC_{tc} that contain chymase in addition to tryptase [18, 19].

Activation of MCs can be modulated by various mediators including neuropeptides [20]. Sensory neuropeptides, such as tachykinins (SP and NKA), act via neurokinin 1 receptor (NK1R), neurokinin 2 receptor (NK2R), and MrgX2, a newly discovered receptor belonging to the mas-related gene family (MrgX receptors), whereas CGRP activates the CGRP receptor (CGRPR) [21–23]. Reciprocally, a variety of molecules including histamine, serotonin, and NGF, which are synthesized and released by MCs, can influence neuronal activity as well as the release of neuropeptides [24–26]. Additionally, protease-activated receptor 2 (PAR2) and the histamine receptor have also been found on trigeminal neurons in animals [27, 28]. MCs exhibit variable functional aspects of both the nervous and immune systems [29, 30]. MCs were found to be associated with nerve fibres in animal and human tissue under physiological and pathophysiological conditions such as intestinal villi and atopic dermatitis [30–33]. However, little is known about MC-nerve interaction in the nasal mucosa of AR.

This study aimed to investigate the MC-nerve association and the neuropeptide receptor expression on MC subpopulations in the nasal mucosa of patients with AR as neuropeptides released from peripheral airway nerves have the capacity to activate immune cells. Therefore, the proportion of MC subpopulations, the MC-nerve association, the expression of neuropeptide receptors such as neurokinin-receptors NKR, mas-related gene family MrgX2 receptor, CGRPR on MCs, and receptors of MC-derived mediators on nerves like PAR2 and tyrosine receptor kinase A (TrkA) on nerve fibres were analysed.

Table 1. Characteristics of patients and controls

	AR	Controls
Total	8	6
Age, years[a]	42 (15–63)	50.5 (35–61)
Female/male	3/5	2/4
AR symptoms[b]	8/8	0/6
Prick tests positive	8/8	0/6
Common allergens	Dp, Df, Dd, Cd	no
Total IgE, IU/mL[a]	535.0 (225.2–744.3) $p = 0.001$	43.2 (32.4–51.6)

Values are given as *n* unless otherwise indicated. Dp, *Dermatophagoides pteronyssinus*; Df, *Dermatophagoides farinae*; Dd, dog dander; Cd, cat dander. [a] Data are given as means (range). [b] Rhinorrhea, nasal itching, sneezing, and nasal obstruction.

Methods

Patients and Nasal Biopsies

In total, 14 subjects were recruited for this study. Subjects with a positive history of allergy symptoms and those with a positive prick test for common aeroallergens (grass or birch pollen, hazel, *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, cat or dog dander) and a positive radioallergosorbent test (RAST test) were identified as patients with AR. The AR group contained 8 patients with perennial AR. All patients had symptoms of AR such as sneezing, itching, nasal blockage, and rhinorrhea at the time of nasal surgery (Table 1). The control group contained 6 healthy subjects without history of allergies and any allergic diseases or symptoms of AR at the time of nasal surgery, and had negative skin prick-tests to a panel of common aeroallergens (Table 1). All subjects were prohibited from taking any anti-inflammatory drugs for at least 4 weeks prior to the nasal surgery. Biopsy specimens of the nasal mucosa were collected from 2013 to 2014 and taken from the inferior turbinate in nasal surgery to reduce the size of the turbinate due to airflow limitations with causes such as deviation of the septum or nasal concha hyperplasia. Control tissues were obtained

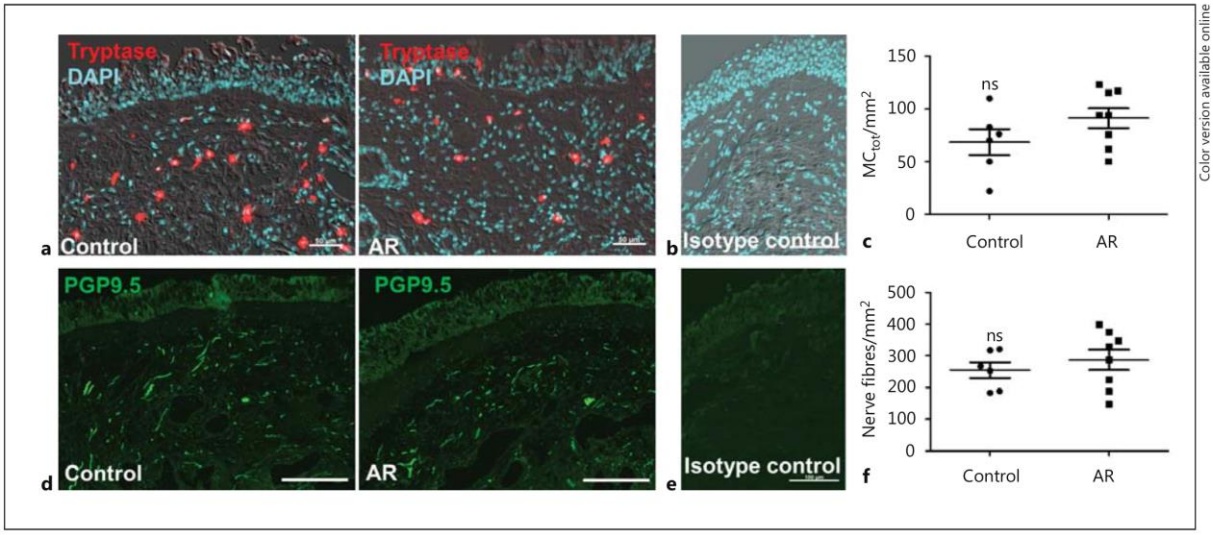


Fig. 1. Density of MCs and nerve fibres in the human nasal mucosa. **a** Differential interference contrast-fluorescence combined microphotographs of MCs (red, stained with antibody against tryptase; color in online version only) in the human nasal mucosa of controls and AR patients. **b** Isotype control for tryptase. **c** The densities of MCs in the human nasal mucosa; results are expressed as the number of cells per mm^2. **d** Innervation in the human nasal mucosa. **e** Isotype control for PGP9.5. **f** Quantification of nerve fibres in the human nasal mucosa; results are expressed as the number of nerve fibres per mm^2. ns, not significant. Scale bars: 50 μm (**a**), 100 μm (**b**, **e**), 200 μm (**d**).

from the same region of the nasal mucosa. All specimens were divided into 2 portions, one for cryosections and the other for PCR. All samples were then examined blindly and independently of the clinical data. This study was approved by the Ethics Committee of the Medical Association (Aerztekammer) of Saarland, and all participants provided written consent.

Preparation of Samples

After dissection, the human nasal biopsies were immediately placed in Zamboni solution (Morphisto – Evolutionsforschung und Anwendung GmbH, Frankfurt am Main, Germany). After overnight fixation, specimens were rinsed in 0.1 M phosphate-buffered saline (PBS) for 24 h and cryoprotected overnight with 30% sucrose in 0.1 M PBS. All of the steps in the specimen processing were performed at 4°C. The biopsy specimens were embedded in optimum cutting temperature medium (Tissue-Tek, Sakura) and frozen in liquid nitrogen. Serial 8-μm sections were prepared using a cryostat (Leica CM 1950; Leica Biosystems, Nussloch, Germany), placed on APES (3-aminopropyltriethoxysilane)-coated glass slides, dried at room temperature for 30 min, and then stored in a freezer at –80°C.

Immunofluorescence Staining

The immunofluorescence staining has been described previously [34]. Briefly, cryosections were dried at room temperature for 15 min and then rehydrated for 5 min in PBS. To reduce nonspecific antibody binding, the sections were incubated for 15 min at room temperature in 5% normal serum of the host species of the secondary antibody diluted in 0.1 M PBS. The sections were incubated with primary antibodies or the appropriate isotype control antibodies (listed in online suppl. Table S2; for all online suppl. material, see www.karger.com/doi/10.1159/000453068) for 1 h at room temperature and then overnight at 4°C. After rinsing with 0.1 M PBS twice, the sections were incubated with secondary fluorescein-conjugated antibodies (listed in online suppl. Table S1) for 2 h at room temperature. For counterstaining, the sections were incubated with 100 μL of DAPI (0.5 μg/mL, Carl Roth, Karlsruhe, Germany) for 15 min at room temperature. Finally, the sections were washed twice with 0.1 M PBS and once with double-distilled water, mounted with fluorescent mounting medium Fluoroshield™ (Sigma-Aldrich), and covered with coverslips. Staining was performed identically for the control and patient groups

Quantitative Immunohistochemical Analysis

For quantification, 16 sections of each biopsy were analysed. Digital images were generated randomly with epifluorescence microscopes (Axio Imager M2, Carl Zeiss, Goettingen, Germany). The quantification of the total MCs (MC$_{tot}$, tryptase – positive by immunostaining); MC$_{tc}$ (tryptase and chymase – positive by immunostaining); MC-nerve fibre contacts; and MCs positive for NK1R-, NK2R-, and CGRPR-immunoreactivity in human nasal mucosa were performed manually in the randomized blinded images using AxioVision software (Carl Zeiss). On average, an area of 8 mm^2 (6–10 mm^2) of the human nasal mucosa tissue per biopsy specimen was investigated for each examination. The MC$_t$ number was calculated by subtracting the number of MC$_{tc}$ from the number of MC$_{tot}$. The analysed tissue areas were measured by computerized image analysis using the AxioVision virtual mi-

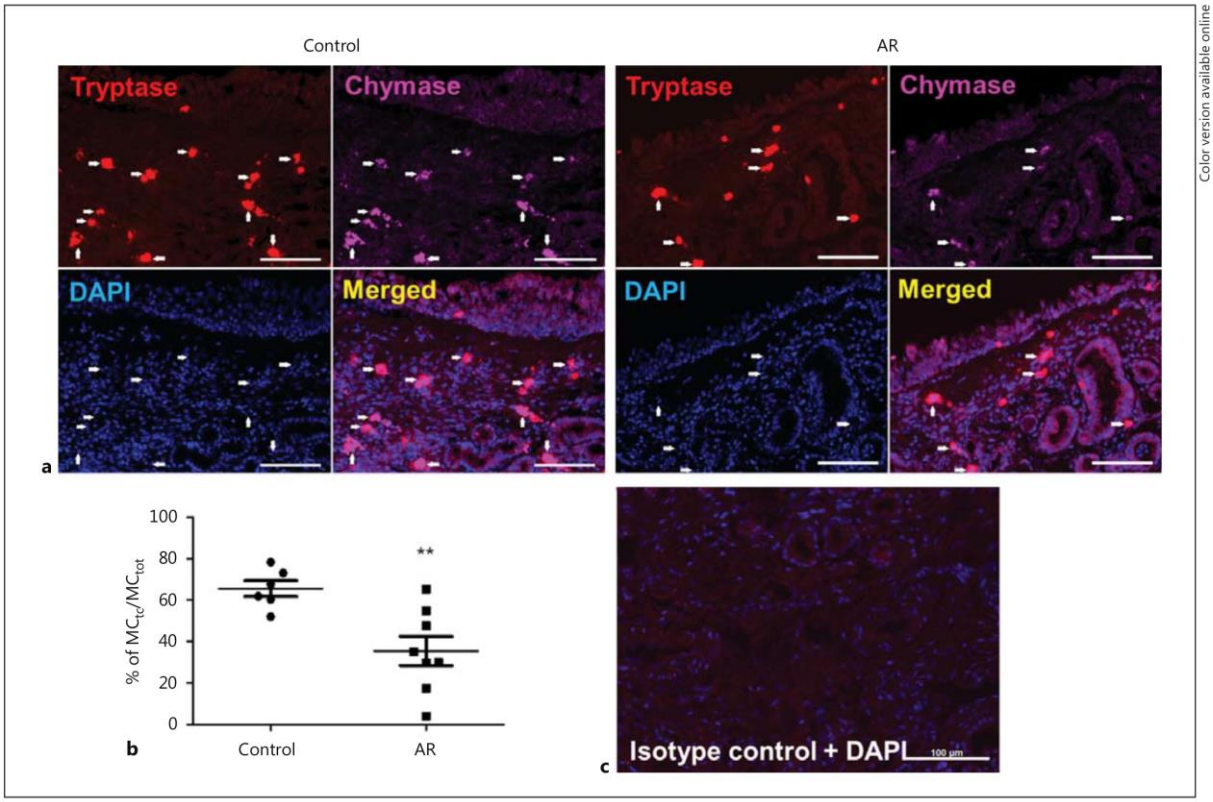


Fig. 2. Proportions of MC_{tc} in the human nasal mucosa. **a** Fluorescence staining of the nasal mucosa of controls and AR patients showing tryptase (red; color in online version only), chymase (magenta), and tryptase-chymase double positive-mast cells (arrows), indicating 2 subpopulations of MCs: tryptase positive (MC_t) and tryptase-chymase positive (MC_{tc}). **b** The proportion of MC_{tc} in the human nasal mucosa of controls and patients with AR. The results are expressed as the percentage of MC_{tc} to the number of MC_{tot} (MC_t + MC_{tc}). **c** Negative control of tryptase and chymase. ** $p \leq 0.01$. Scale bars: 100 µm.

croscopy software (Carl Zeiss). The number of nerve fibres (PGP 9.5 – positive by immunostaining) was evaluated automatically with ImageJ software (National Institutes of Health). All quantifications were carried out on blinded sections and controlled by a second investigator.

Total RNA Extraction and Quantitative Real-Time PCR

Total RNA was isolated from the human nasal mucosa using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. To remove genomic DNA, an additional DNase digestion during RNA isolation was performed. The quality of total RNA was assessed with NanoDrop 8000 (Thermo Scientific).

Reverse transcription (cDNA synthesis) was performed using an Omniscript RT Kit (Qiagen). Real-time PCR was undertaken on CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Germany) using the SensiMix SYBR & Fluorescein Kit from Bioline (Bioline Germany) and reactions were conducted based on the manufacturer's manual. All samples were run in duplicate and RNA levels were normalized to the level of β-actin. The expression of target genes was calculated relative to the control group using the ΔΔCt method. Primers used for PCR are listed in online supplementary Table S2.

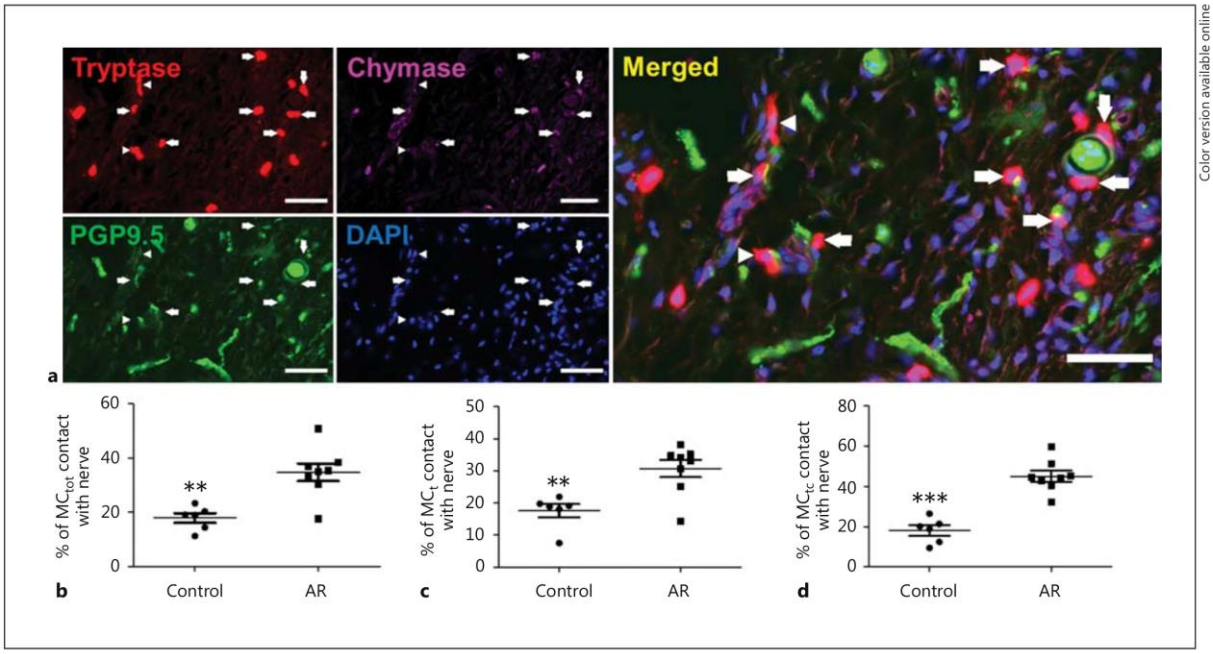
Statistical Analysis

Data are given as means ± SEM. The differences between 2 normally distributed groups were analysed with an unpaired *t* test with GraphPad Prism 5.0. $p \leq 0.05$ was considered significant (* $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Results

Unaltered Density of MCs and Nerve Fibres in AR

To determine the density of MCs, sections of the human nasal mucosa were stained with an antibody against



Color version available online

Fig. 3. Association between MCs and nerves. **a** Microphotograph showing MCs (red, stained with antibody against tryptase; color in online version only) and nerve fibres (green, stained with antibody against PGP 9.5) in the nasal mucosa. Arrows show the association between MC_{tc} and nerve fibres, while arrowheads show contacts between MC_t and nerve fibres. **b** The proportion of MC_{tot} in contact with nerve fibres in the human nasal mucosa. The results are expressed as the percentage of MC_{tot} in contact with nerves to the number of MC_{tot}. Proportions of MC_t (**c**) and MC_{tc} (**d**) in contact with nerves in the human nasal mucosa. The results are expressed as the percentage of MC_t and MC_{tc} associating with nerves to the numbers of MC_t and $MC_{tc,}$ respectively. ** $p \leq 0.01$, *** $p \leq 0.001$. Scale bars: 50 μm.

tryptase. The number of MCs was counted and expressed as the number of cells per mm^2. The densities of MCs in the nasal mucosa were unchanged in patients with AR in comparison to the control group (AR: 91.36 ± 9.50 MCs/mm^2, $n = 8$ vs. control: 68.62 ± 12.20 MCs/mm^2, $n = 6$, $p = 0.160$). However, an increased distribution of MCs in the epithelium was observed in patients with AR. The number of nerve fibres (fibres per square millimetre) was found to be unaltered in the human nasal mucosa of patients with AR in comparison to the control group (AR: 287.7 ± 32.49, $n = 8$ vs. control: 255.5 ± 24.53, $n = 6$, $p = 0.4720$) (Fig. 1).

Reduction of MC_{tc} in AR

With triple immunofluorescence staining, 2 MC populations, MC_t and MC_{tc}, were found in the human nasal mucosa. The proportion of MC_{tc} (MC_{tc}/MC_{tot}) in nasal mucosa was significantly decreased in patients with AR (MC_{tc}/MC_{tot}; AR: 35.51 ± 7.07%, $n = 8$ vs. control: 65.55 ± 3.86%, $n = 6$, $p = 0.0055$) (Fig. 2).

Increased MC-Nerve Associations in AR

The proportion of MC_{tot} in contact with nerves was significantly increased in patients with AR (AR: 34.71 ± 3.24%, $n = 8$ vs. control: 17.90 ± 1.77%, $n = 6$, $p = 0.0014$). The associations between MC_t and nerve fibres were found to be increased significantly in patients with AR (AR: 34.20 ± 2.99%, $n = 8$ vs. control: 17.77 ± 2.05%, $n = 6$, $p = 0.0012$). Similarly, a significant increase in the proportion of MC_{tc} in contact with nerves was also observed in subjects with AR (AR: 45.58 ± 2.72%, $n = 8$ vs. control: 18.24 ± 2.54%, $n = 6$, $p < 0.0001$) (Fig. 3).

Expression of Tachykinin- and CGRPRs in Nasal MCs

Concerning the presence of NK1R in MCs, triple immunofluorescence staining for tryptase, chymase, and NK1R showed the expression of NK1R in both MC subpopulations, MC_t and MC_{tc}. The number of MC_{tot} (MC_t + MC_{tc}) expressing NK1R was also found to be elevated in the AR group ($NK1R^+MC_{tot}/MC_{tot}$; AR: 9.31 ± 1.97%,

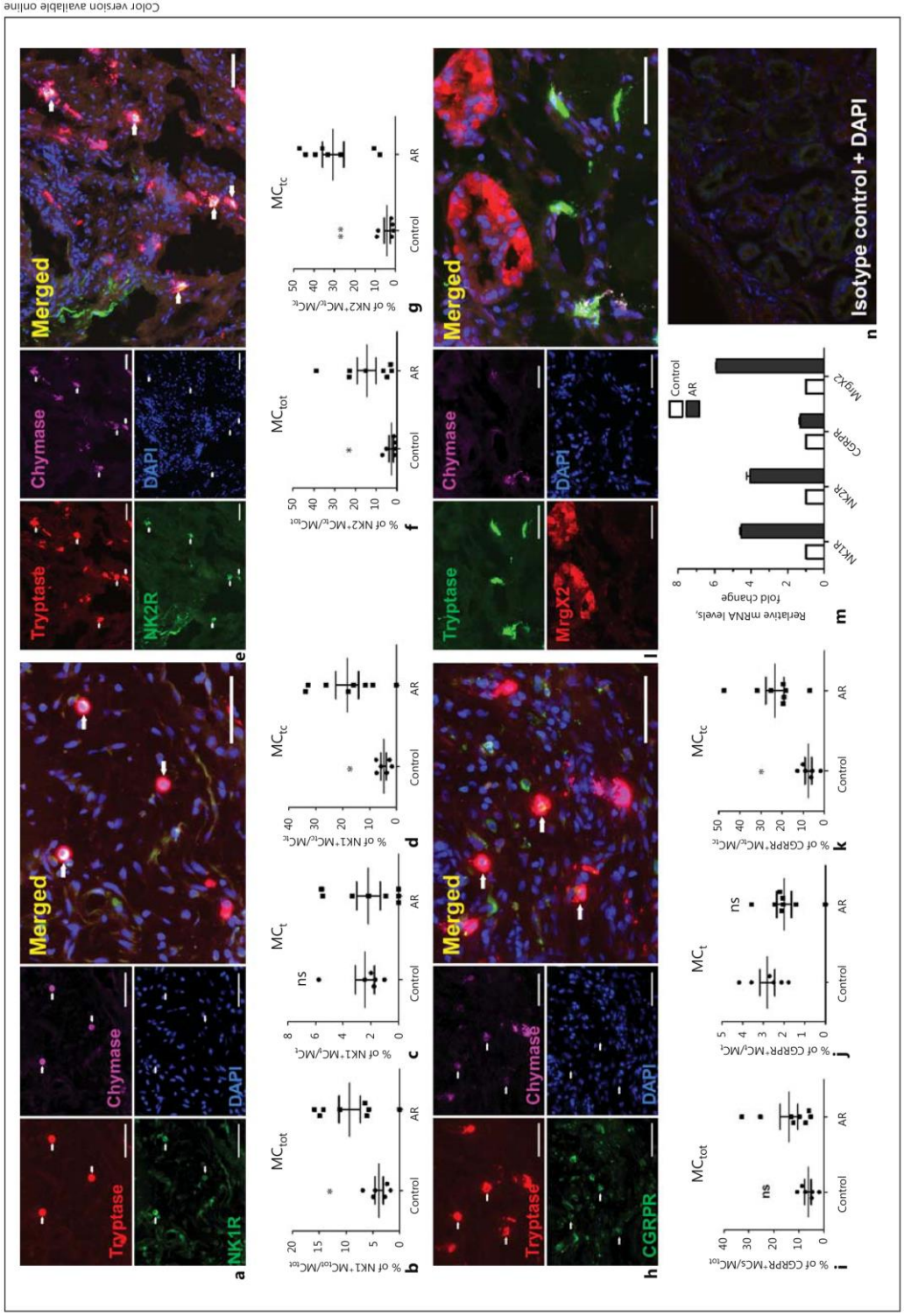


Fig. 4. Expression of tachykinins, CGRP, and MrgX2 receptors on mast cells. Fluorescence staining for tryptase (red; color in online version only), chymase (magenta), and NK1R (**a**), NK2R (**e**), CGRPR (**h**) (green). Arrows show the mast cells positive for NK1R, NK2, and CGRPR. The proportion of MC_{tot} positive with NK1R (**b**), NK2R (**f**) CGRPR (**i**) in controls and AR patients. Percentages of NK1R- (**c**) and CGRPR-positive (**j**) MC_t. The proportion of MC_{tc} immunoreactive for NK1R (**d**), NK2 (**g**), and CGRPR (**k**). **l** MrgX2 (red) was expressed on gland cells, but not on mast cells (green) in the human nasal mucosa. **m** Quantitative real-time PCR analyses of NK1R, NK2R, CGRPR, and MrgX2 in the human nasal mucosa. **n** Negative control for primary rabbit antibodies. ns, not significant. * $p \leq 0.05$, ** $p \leq 0.01$. Scale bars: 50 μm.

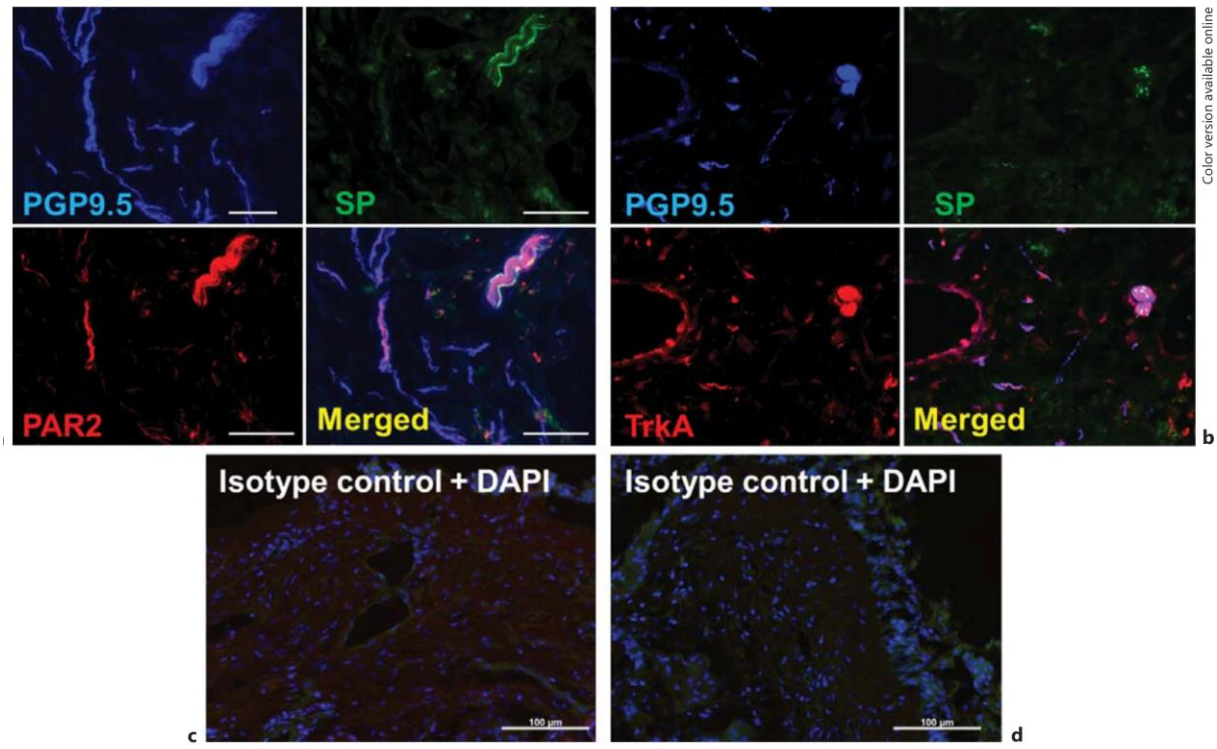


Fig. 5. Expression of PAR2 and TrkA on nerve fibres in the human nasal mucosa. Microphotograph of human nasal mucosa showing the expression of SP and PAR2 receptors (**a**) and TrkA receptors (**b**) on nerves. Isotype controls for PAR2 (**c**) and TrKA (**d**). Scale bars: 50 μm (**a**), 100 μm (**c**, **d**).

$n = 8$ vs. control: 3.85 ± 0.79%, $n = 6$, $p = 0.0425$) (Fig. 4a–d). The proportion of MC_t expressing NK1R ($NK1R^+MC_t$) did not differ among the AR and control groups ($NK1R^+MC_t/MC_t$; AR: 2.19 ± 0.84%, $n = 8$ vs. control: 2.45 ± 0.68%, $n = 6$, $p = 0.8252$). In contrast to the MC_t subpopulation, the number of MC_{tc} expressing NK1R was significantly increased in patients suffering from AR ($NK1R^+MC_{tc}/MC_{tc}$; AR: 18.39 ± 4.21%, $n = 8$ vs. control: 4.76 ± 1.02%, $n = 6$, $p = 0.0184$).

With respect to the expression of NK2R in MCs, NK2R immunoreactivity was only found in MC_{tc}. The proportion of MC_{tc} expressing NK2R ($NK2R^+MC_{tc}$) to the numbers of MC_{tc} and MC_{tot} was significantly increased in patients with AR ($NK2R^+MC_{tc}/MC_{tc}$; AR: 30.66 ± 5.20%, $n = 8$ vs. control: 4.09 ± 1.53%, $n = 6$, $p = 0.001$; $NK2R^+MC_{tc}/MC_{tot}$: AR: 14.62 ± 4.52%, $n = 8$ vs. control: 2.79 ± 1.10%, $n = 6$, $p = 0.0475$) (Fig. 4b–g).

CGRPRs were found to be frequently expressed in MC_{tc} compared to the MC_t population. The number of MC_{tot} expressing CGRPR was not changed in atopic subjects in comparison to the control ($CGRPR^+MC_{tot}/MC_{tot}$; AR: 14.55 ± 3.47%, $n = 8$ vs. control: 6.53 ± 1.28%, $n = 6$, $p = 0.0804$). The proportion of MC_{tc} expressing CGRPR ($CGRPR^+MC_{tc}$) was significantly increased in the AR group ($CGRPR^+MC_{tc}/MC_{tc}$; AR: 23.58 ± 4.21%, $n = 8$ vs. control: 7.78 ± 1.57%, $n = 6$, $p = 0.0092$), whereas the proportion of CGRPR expressing MC_t was unchanged ($CGRPR^+MC_t/MC_t$; AR: 2.84 ± 0.25%, $n = 8$ vs. control: 2.95 ± 0.30%, $n = 6$, $p = 0.7742$) (Fig. 4i–k).

The newly discovered tachykinin receptor MrgX2 was found to be expressed on gland cells, but not on MCs in the human nasal mucosa (Fig. 4l).

Real-time PCR analysis showed that the mRNA expression levels of NK1R, NK2R, MrgX2, and CGRPR in the nasal mucosa of patients with AR was higher than in the control group (Fig. 4m).

Expression of Tachykinins and, PAR2 and TrkA Receptors on Nerve Fibres in the Human Nasal Mucosa

Immunofluorescence staining showed a small proportion of nerve fibres expressing SP in the nasal mucosa of patients with AR and the controls. The expression of the PAR2 receptor and the tyrosine receptor kinase TrkA was found on a small number of nerve fibres of both groups. PAR2 and TrkA receptors were often colocalized with SP-positive nerve fibres (Fig. 5).

Discussion

Accumulated evidence from in vivo and in vitro studies shows a crosstalk between the immune and nervous systems under physiological and pathophysiological conditions. In the present study, we found an increased anatomical association between MCs and nerves, an increased expression of neuropeptide receptors on MCs, and, vice versa, a substantial expression of tachykinins, PAR2-, and TrkA-receptors on nerve fibres innervating the human nasal mucosa, indicating an important role of MC-nerve interaction in AR. Like the previous findings, the density of MCs in the nasal mucosa of patients with AR was found in our study to be unchanged in comparison to the controls [8, 35, 36].

With respect to MC density, in patients with AR after local provocation with grass pollen, MC_{tc} were found to be lower in comparison to the controls [35]. The reasons for the reduction of MC_{tc} in the nasal mucosa of patients with AR are still unknown. This may be explained by the increased release of MC_{tc} chymase during allergic inflammation, which leads to reduced detection by histological staining. In this respect, MC_{tc} were observed to have a lower density in their chymase expression. Otherwise, the MC_{tc} subpopulations in atopic subjects with AR may be natively lower than nonatopic subjects.

The role of human MC_{tc} in allergic diseases remains poorly understood and controversially discussed. MC chymase is a chymotrypsin-like serine protease stored within granules that hydrolyse a variety of substrates, e.g., angiotensin I, metalloproteases, lipoproteins, and procollagen [37]. MC chymase has the capacity to convert angiotensin I to angiotensin II to induce cell proliferation, angiogenesis, and hypertension [38, 39], which seems to be associated with AR [40]. Additionally, angiogenesis is a characteristic feature of upper airway structural remodelling, and increased airway angiogenesis was found in patients with rhinitis without asthma [41]. Chymase has been reported to increase not only vascular [42] and epithelial permeability [43], but also the migration of immune cells, including neutrophils and eosinophils [44]. MC chymase was revealed to be a potent secretagogue for airway serous gland cells [45]. Conversely, previous studies suggested that positive chymase MCs may play a protective role in human allergic airway diseases. It has been shown that MC_{tc} may improve lung function in severe asthma patients [46]. Other studies have shown that MC protease 4 (mMCP-4) also has protective effects on murine allergic airway inflammation [47]. The change of the number of MC_{tc} indicates that MC_{tc} may play an important role in AR.

Although the total numbers of MCs and nerve fibre density were unchanged in patients with AR in comparison to the controls, the contacts between MCs, and particularly MC_{tc}, with nerve fibres were significantly increased in AR patients. These findings suggest that the MC-nerve interaction may contribute to the pathogenesis of AR.

In addition to the spatial association, MC-nerve interaction may occur by paracrine signalling via neuropeptides (CGRP, tachykinins [SP, NKA]) releasing from the nerve fibres and mediators, such as NGF, proteases, and histamine, from MCs. Our further attempts to characterize this neuroimmune crosstalk have shown that MCs in the nasal mucosa express sensory neuropeptide receptors and that the number of MCs containing NK1R, NK2R, and CGRPR increases in patients with AR. These results indicate that the function of MCs in the human nasal mucosa could be regulated by sensory nerves innervating the upper airway, and otherwise MC mediators may be able to activate sensory nerve fibers to induce neurogenic upper airway inflammation. The neuropeptide receptors NK1R and CGRPR were found in both MC subpopulations MC_t and MC_{tc}; however, these receptors were mainly expressed on MC_{tc}, and NK2R was only found in MC_{tc}. The preferred ligand for NK2R is NKA; however, SP can also act via NK2R. Thus, tachykinins may be an essential contributor in the regulation of MC_{tc}.

With respect to the receptors for tachykinins, the newly discovered MrgX family MrgX2 has been reported to be expressed on MCs [23]. In our study, this receptor was found on gland cells, but not in MCs, in the human nasal mucosa. Previous studies have shown that the skin MCs express MrgX2, but the lung-derived MCs and the human MC line HMC-1 do not display this receptor [48, 49]. Thus, it could be that only certain subpopulations of MCs express the MrgX2 receptor. In addition, these findings indicate that tachykinins may regulate nasal mucosa MCs via NK1R and NK2R rather than via MrgX2. Furthermore, the increase of mRNA expression of this receptor

in AR patients suggests that MrgX2 may contribute substantially to the mucus hypersecretion in AR.

Conversely, airway innervation can be activated and regulated by the inflammatory mediators of immune cells. The expression of PAR2- and TrkA-receptors on nerve fibres innervating the human nasal mucosa indicates that airway nerves can be controlled by proteases as well as MC-derived NGF. Supporting these present findings, the expression of histamine receptors and PAR2 have been reported on nerve fibres of human skin as well as on murine trigeminal ganglion neurons during inflammation of the upper airways [27, 28].

Conclusions

This study demonstrates for the first time an increase of MC-nerve contacts and neuropeptide receptor expression on MCs in patients with AR. Nasal nerve fibres were also found to express PAR2- and TrkA-receptors for MC mediators. Tachykinins may regulate the nasal mucosa MCs via NK1R and NK2R rather than via MrgX2. These findings suggest that targeting the key receptors of the MC-nerve interaction may prevent allergic airway inflammation such as AR.

Acknowledgements

This project was supported by the German Research Foundation (DFG, DI 1429/2-1) and the Stiftung Bergmannshilfswerks Luisenthal foundation to Prof. Q.T. Dinh.

Disclosure Statement

The authors declare no conflicts of interest.

References

1 Naclerio RM: Pathophysiology of perennial allergic rhinitis. Allergy 1997;52:7–13.
2 Sarin S, Undem B, Sanico A, Togias A: The role of the nervous system in rhinitis. J Allergy Clin Immunol 2006;118:999–1016.
3 Undem BJ, McAlexander M, Hunter DD: Neurobiology of the upper and lower airways. Allergy 1999;54(suppl 57):81–93.
4 Baraniuk JN: Pathogenesis of allergic rhinitis. J Allergy Clin Immunol 1997;99:S763–S772.
5 Pfaar O, Raap U, Holz M, Hormann K, Klimek L: Pathophysiology of itching and sneezing in allergic rhinitis. Swiss Med Wkly 2009;139:35–40.
6 Undem BJ, Carr MJ: Pharmacology of airway afferent nerve activity. Respir Res 2001;2:234–244.
7 Mosimann BL, White MV, Hohman RJ, Goldrich MS, Kaulbach HC, Kaliner MA: Substance P, calcitonin gene-related peptide, and vasoactive intestinal peptide increase in nasal secretions after allergen challenge in atopic patients. J Allergy Clin Immunol 1993;92:95–104.
8 Heppt W, Dinh QT, Cryer A, Zweng M, Noga O, Peiser C, Melvan M, Witt C, Fischer A, Groneberg DA: Phenotypic alteration of neuropeptide-containing nerve fibres in seasonal intermittent allergic rhinitis. Clin Exp Allergy 2004;34:1105–1110.
9 Lundblad L, Saria A, Lundberg JM, Anggard A: Increased vascular permeability in rat nasal mucosa induced by substance P and stimulation of capsaicin-sensitive trigeminal neurons. Acta Otolaryngol 1983;96:479–484.
10 Heaney LG, Cross LJ, Stanford CF, Ennis M: Substance P induces histamine release from human pulmonary mast cells. Clin Exp Allergy 1995;25:179–186.
11 Nilsson G, Rak S, Ahlstedt S: The influence of substance P on the proliferation of peripheral blood lymphocytes from normal individuals and birch pollen-allergic patients. Allergy 1987;42:516–523.
12 Williams CM, Galli SJ: The diverse potential effector and immunoregulatory roles of mast cells in allergic disease. J Allergy Clin Immunol 2000;105:847–859.
13 Galli SJ, Nakae S, Tsai M: Mast cells in the development of adaptive immune responses. Nat Immunol 2005;6:135–142.
14 Leon A, Buriani A, Dal TR, Fabris M, Romanello S, Aloe L, Levi-Montalcini R: Mast cells synthesize, store, and release nerve growth factor. Proc Natl Acad Sci USA 1994;91:3739–3743.
15 Wernersson S, Pejler G: Mast cell secretory granules: armed for battle. Nat Rev Immunol 2014;14:478–494.
16 Xiang Z, Nilsson G: IgE receptor-mediated release of nerve growth factor by mast cells. Clin Exp Allergy 2000;30:1379–1386.
17 Beaven MA: Our perception of the mast cell from Paul Ehrlich to now. Eur J Immunol 2009;39:11–25.
18 Pawankar R, Ra C: Heterogeneity of mast cells and T cells in the nasal mucosa. J Allergy Clin Immunol 1996;98:S248–S262.
19 Bradding P, Arthur G: Mast cells in asthma – state of the art. Clin Exp Allergy 2016;46:194–263.
20 Kulka M, Sheen CH, Tancowny BP, Grammer LC, Schleimer RP: Neuropeptides activate human mast cell degranulation and chemokine production. Immunology 2008;123:398–410.
21 Bauer O, Razin E: Mast cell-nerve interactions. News Physiol Sci 2000;15:213–218.
22 Kleij HP, Bienenstock J: Significance of conversation between mast cells and nerves. Allergy Asthma Clin Immunol 2005;1:65–80.
23 Lee MG, Dong X, Liu Q, Patel KN, Choi OH, Vonakis B, Undem BJ: Agonists of the MAS-related gene (Mrgs) orphan receptors as novel mediators of mast cell-sensory nerve interactions. J Immunol 2008;180:2251–2255.
24 Bresciani M, Laliberte F, Laliberte MF, Gramiccioni C, Bonini S: Nerve growth factor localization in the nasal mucosa of patients with persistent allergic rhinitis. Allergy 2009;64:112–117.
25 Tani E, Senba E, Kokumai S, Masuyama K, Ishikawa T, Tohyama M: Histamine application to the nasal mucosa induces release of calcitonin gene-related peptide and substance P from peripheral terminals of trigeminal ganglion: a morphological study in the guinea pig. Neurosci Lett 1990;112:1–6.
26 Mingomataj E, Dinh QT, Groneberg D, Feleszko W, Schmeck B, Joachim R, Noga O, Nagel S, Klapp BF, Fischer A: Trigeminal nasal-specific neurons respond to nerve growth factor with substance-P biosynthesis. Clin Exp Allergy 2008;38:1203–1211.

27 Dinh QT, Cryer A, Dinh S, Trevisani M, Georgiewa P, Chung F, Geppetti P, Heppt W, Klapp BF, Fischer A: Protease-activated receptor 2 expression in trigeminal neurons innervating the rat nasal mucosa. Neuropeptides 2005;39:461–466.
28 Nakasaki T, Masuyama K, Fukui H, Ogino S, Eura M, Samejima Y, Ishikawa T, Yumoto E: Effects of PAF on histamine H1 receptor mRNA expression in rat trigeminal ganglia. Prostaglandins Other Lipid Mediat 1999;58: 29–41.
29 Dudeck A, Suender CA, Kostka SL, von Stebut E, Maurer M: Mast cells promote Th1 and Th17 responses by modulating dendritic cell maturation and function. Eur J Immunol 2011;41:1883–1893.
30 Alving K, Sundstrom C, Matran R, Panula P, Hokfelt T, Lundberg JM: Association between histamine-containing mast cells and sensory nerves in the skin and airways of control and capsaicin-treated pigs. Cell Tissue Res 1991; 264:529–538.
31 Stead RH, Tomioka M, Quinonez G, Simon GT, Felten SY, Bienenstock J: Intestinal mucosal mast cells in normal and nematode-infected rat intestines are in intimate contact with peptidergic nerves. Proc Natl Acad Sci USA 1987;84:2975–2979.
32 Williams RM, Berthoud HR, Stead RH: Vagal afferent nerve fibres contact mast cells in rat small intestinal mucosa. Neuroimmunomodulation 1997;4:266–270.
33 Toyoda M, Morohashi M: Morphological assessment of the effects of cyclosporin A on mast cell – nerve relationship in atopic dermatitis. Acta Derm Venerol 1998;78:321–325.
34 Le DD, Rochlitzer S, Fischer A, Heck S, Tschernig T, Sester M, Bals R, Welte T, Braun A, Dinh QT: Allergic airway inflammation induces the migration of dendritic cells into airway sensory ganglia. Respir Res 2014;15:73.
35 KleinJan A, McEuen AR, Dijkstra MD, Buckley MG, Walls AF, Fokkens WJ: Basophil and eosinophil accumulation and mast cell degranulation in the nasal mucosa of patients with hay fever after local allergen provocation. J Allergy Clin Immunol 2000;106:677–686.
36 Fokkens WJ, Godthelp T, Holm AF, Blom H, Mulder PG, Vroom TM, Rijntjes E: Dynamics of mast cells in the nasal mucosa of patients with allergic rhinitis and non-allergic controls: a biopsy study. Clin Exp Allergy 1992; 22:701–710.
37 Caughey GH: Mast cell tryptases and chymases in inflammation and host defense. Immunol Rev 2007;217:141–154.
38 Miyazaki M, Takai S, Jin D, Muramatsu M: Pathological roles of angiotensin II produced by mast cell chymase and the effects of chymase inhibition in animal models. Pharmacol Ther 2006;112:668–676.
39 Wang T, Han SX, Zhang SF, Ning YY, Chen L, Chen YJ, He GM, Xu D, An J, Yang T, Zhang XH, Wen FQ: Role of chymase in cigarette smoke-induced pulmonary artery remodeling and pulmonary hypertension in hamsters. Respir Res 2010;11:36.
40 Kony S, Zureik M, Neukirch C, Leynaert B, Vervloet D, Neukirch F: Rhinitis is associated with increased systolic blood pressure in men: a population-based study. Am J Respir Crit Care Med 2003;167:538–543.
41 Kristan SS, Malovrh MM, Silar M, Kern I, Flezar M, Kosnik M, Suskovic S, Korosec P: Airway angiogenesis in patients with rhinitis and controlled asthma. Clin Exp Allergy 2009;39: 354–360.
42 He S, Walls AF: The induction of a prolonged increase in microvascular permeability by human mast cell chymase. Eur J Pharmacol 1998;352:91–98.
43 Groschwitz KR, Wu D, Osterfeld H, Ahrens R, Hogan SP: Chymase-mediated intestinal epithelial permeability is regulated by a protease-activating receptor/matrix metalloproteinase-2-dependent mechanism. Am J Physiol Gastrointest Liver Physiol 2013; 304:G479–G489.
44 Terakawa M, Tomimori Y, Goto M, Fukuda Y: Mast cell chymase induces expression of chemokines for neutrophils in eosinophilic EoL-1 cells and mouse peritonitis eosinophils. Eur J Pharmacol 2006;538:175–181.
45 Sommerhoff CP, Caughey GH, Finkbeiner WE, Lazarus SC, Basbaum CB, Nadel JA: Mast cell chymase. A potent secretagogue for airway gland serous cells. J Immunol 1989; 142:2450–2456.
46 Balzar S, Chu HW, Strand M, Wenzel S: Relationship of small airway chymase-positive mast cells and lung function in severe asthma. Am J Respir Crit Care Med 2005;171:431–439.
47 Waern I, Jonasson S, Hjoberg J, Bucht A, Abrink M, Pejler G, Wernersson S: Mouse mast cell protease 4 is the major chymase in murine airways and has a protective role in allergic airway inflammation. J Immunol 2009;183:6369–6376.
48 Fujisawa D, Kashiwakura J, Kita H, Kikukawa Y, Fujitani Y, Sasaki-Sakamoto T, Kuroda K, Nunomura S, Hayama K, Terui T, Ra C, Okayama Y: Expression of Mas-related gene X2 on mast cells is upregulated in the skin of patients with severe chronic urticaria. J Allergy Clin Immunol 2014;134:622–633.
49 Subramanian H, Kashem SW, Collington SJ, Qu H, Lambris JD, Ali H: PMX-53 as a dual CD88 antagonist and an agonist for Mas-related gene 2 (MrgX2) in human mast cells. Mol Pharmacol 2011;79:1005–1013.

Supplementary Material

Table S1: Antibodies used in this study

Antibody	Source	Dilution
Primary purified antibodies		
Rabbit polyclonal antibody PGP 9.5	Abcam, UK	1:200
Mouse Antibody Anti-Mast Cell Tryptase [AA1]	Abcam, UK	1:400
Goat Antibody Anti-Mast Cell Chymase	Abcam, UK	1:100
Rat Antibody Anti-Substance P [M09205]	Abcam, UK	1:200
Rabbit Polyclonal Antibody to TACR2 / NK2R	Acris, Germany	1:200
Rabbit Antibody to RAMP1 (FL-148) (For CGRP receptor)	Santa Cruz, USA	1:100
Rabbit Antibody Anti-TrkA (EP1058Y)	Abcam, UK	1:100
Mouse Antibody Anti-Human PAR2 (SAM11)	eBioscience, USA	1:100
Rabbit polyclonal MrgX2 antibody	Novus Biologicals	1:100
Rabbit anti Human Neurokinin 1 Receptors (NK1R)	Thermo scientific, USA	1:500
Isotype control purified antibodies		
Rabbit IgG	Dianova, Germany	1:5000
Rat IgG 2b kappa	BioLegend, USA	1:200
Goat IgG	R&D systems	1:300
Mouse Ig	BioLegend, USA	1:200
Secondary antibodies		
Alexa Fluor® 488 AffiniPure Fab Fragment Donkey Anti-Rabbit IgG (H+L)	Jackson ImmunoResearch, USA	1:400
Cy3 conjugated Donkey Anti-Rat IgG antibody	Jackson ImmunoResearch, USA	1:500
Cy3 conjugated Donkey Anti-Rabbit IgG antibody	Jackson ImmunoResearch, USA	1:500
DyLight 488 conjugated Donkey Anti-Goat IgG antibody	Jackson ImmunoResearch,USA	1:400
DyLight 649 conjugated Donkey Anti-Rabbit IgG antibody	Jackson ImmunoResearch, USA	1:400
Cy3 Conjugated Donkey Anti-Goat IgG antibody	Jackson ImmunoResearch, USA	1:500
Cy5 conjugated Donkey Anti-Rat IgG antibody	Jackson ImmunoResearch, USA	1:400

Table S2: List of primers used in this study

Gene	Primer sequences	Product (bp)	GenBank Accession
hNK1R (sense)	GCTGCCCTTCCACATCTTCT	157	NM_001058.3
hNK1R (anti-sense)	ACGGAACCTGTCATTGAGGC		
hNK2R (sense)[1]	GAGGCCGATGACGCTGTAG	94	NM_001057
hNK2R (anti-sense)[1]	CAAGACGCTCCTCCTGTACCA		
hCGRPR (sense)[2]	CTGCCAGGAGGC TAACTACG	298	NM_001308353.1
hCGRPR (anti-sense)[2]	GACCACGATGAAGGGGTAGA		
hMrgX2 (sense)	AAGAAGGGTGTTAAGGGGCAC	133	NM_054030.3
hMrgX2 (anti-sense)	CAGGGTCTCCTTGCCACAAA		
hβ-Actin (sense)	TTCCTTCCTGGGCATGGAGT	136	NM_001101.3
hβ-Actin (anti-sense)	AATGCCAGGGTACATGGT GG		

1. **Grassin-Delyle S, Naline E, Buenestado A, Risse PA, Sage E, Advenier C, Devillier P.** Expression and function of human hemokinin-1 in human and guinea pig airways. Respir Res 2010; 11:139.
2. **Gupta S, Mehrotra S, Villalon CM, Garrelds IM, de VR, van Kats JP, Sharma HS, Saxena PR, Maassenvandenbrink A.** Characterisation of CGRP receptors in human and porcine isolated coronary arteries: evidence for CGRP receptor heterogeneity. Eur J Pharmacol 2006; **530**:107-16.

5.2 Paper II

Title: Allergic airway inflammation induces the migration of dendritic cells into airway sensory ganglia

Authors: Duc Dung Le, Sabine Rochlitzer, Axel Fischer, Sebastian Heck, Thomas Tschernig, Martina Sester, Robert Bals, Tobias Welte, Armin Braun and Quoc Thai Dinh

Respiratory Research **2014 Jun 30;15:73.**

Le et al. Respiratory Research 2014, 15:73
http://respiratory-research.com/content/15/1/73

RESPIRATORY RESEARCH

RESEARCH Open Access

Allergic airway inflammation induces the migration of dendritic cells into airway sensory ganglia

Duc Dung Le[1], Sabine Rochlitzer[2], Axel Fischer[3], Sebastian Heck[1], Thomas Tschernig[4], Martina Sester[5], Robert Bals[6], Tobias Welte[7], Armin Braun[2] and Quoc Thai Dinh[1,6*]

Abstract

Background: A neuroimmune crosstalk between dendritic cells (DCs) and airway nerves in the lung has recently been reported. However, the presence of DCs in airway sensory ganglia under normal and allergic conditions has not been explored so far. Therefore, this study aims to investigate the localisation, distribution and proliferation of DCs in airway sensory ganglia under allergic airway inflammation.

Methods: Using the house dust mite (HDM) model for allergic airway inflammation BALB/c mice were exposed to HDM extract intranasally (25 μg/50 μl) for 5 consecutive days a week over 7 weeks. With the help of the immunohistochemistry, vagal jugular-nodose ganglia complex (JNC) sections were analysed regarding their expression of DC-markers (MHC II, CD11c, CD103), the neuronal marker PGP 9.5 and the neuropeptide calcitonin gene-related peptide (CGRP) and glutamine synthetase (GS) as a marker for satellite glia cells (SGCs). To address the original source of DCs in sensory ganglia, a proliferation experiment was also carried in this study.

Results: Immune cells with characteristic DC-phenotype were found to be closely located to SGCs and vagal sensory neurons under physiological conditions. The percentage of DCs in relation to neurons was significantly increased by allergic airway inflammation in comparison to the controls (HDM 51.38 ± 2.38% vs. control 28.16 ± 2.86%, $p < 0.001$). The present study also demonstrated that DCs were shown to proliferate in jugular-nodose ganglia, however, the proliferation rate of DCs is not significantly changed in the two treated animal groups (proliferating DCs/ total DCs: HDM 0.89 ± 0.38%, vs. control 1.19 ± 0.54%, $p = 0.68$). Also, increased number of CGRP-positive neurons was found in JNC after allergic sensitisation and challenge (HDM 31.16 ± 5.41% vs. control 7.16 ± 1.53%, $p < 0.001$).

Conclusion: The present findings suggest that DCs may migrate from outside into the ganglia to interact with sensory neurons enhancing or protecting the allergic airway inflammation. The increase of DCs as well as CGRP-positive neurons in airway ganglia by allergic airway inflammation indicate that intraganglionic DCs and neurons expressing CGRP may contribute to the pathogenesis of bronchial asthma. To understand this neuroimmune interaction in allergic airway inflammation further functional experiments should be carried out in future studies.

Keywords: House dust mite mouse model, Dendritic cells, Allergic airway inflammation, Sensory airway nerves, Neuroimmune interaction, CGRP

* Correspondence: Thai.Dinh@uks.eu
[1]Department of Experimental Pneumology and Allergology, Saarland University Faculty of Medicine, Kirrberger Strasse, Geb. 61.4, Homburg, Germany
[6]Department of Internal Medicine V, Pneumology, Allergology and Respiratory Critical Care Medicine, Saarland University Faculty of Medicine, Kirrberger Strasse, Geb. 61.4, Homburg, Germany
Full list of author information is available at the end of the article

Introduction

Allergic bronchial asthma is a chronic inflammatory respiratory disease characterised by airway obstruction, bronchial hyperreactivity and airway inflammation with the recruitment of a variety of immune cells including dendritic cells (DCs) [1-3]. DCs are phagocytic cells that are localised in many organs like in the skin, in the mucosa of the intestines, the upper airways, the lungs and the brain [2-5].

In the allergic sensitisation phase, DCs play a key role as professional antigen presenting cells in the allergic airway inflammation [3,4,6]. They capture the antigen, process and subsequently present it on the MHC class II molecules (MHC II) to naïve T lymphocytes in local lymph nodes leading to cascades of the Th2-immune allergic inflammatory processes [4,7,8]. Recently, the maturation and differentiation of DCs have been described to be modulated by many cytokines of immune cells as well as neuropeptides such as calcitonin gene-related peptide (CGRP) [9-11].

CGRP consists of 37 amino acids [12] and is biosynthesised and released from sensory neurons innervating the airways in response to different stimuli including allergic airway inflammation [12-14]. CGRP released from airway nerve fibres has the capacity to act as chemoattractant factor for different immune cells such as $CD4^+$ T-lymphocytes, $CD8^+$ T-lymphocytes, eosinophils and DCs and to induce the proliferation of airway epithelial cells [9,15-19]. On the other hand, DCs have the capacity to release neurotrophins, which can activate neurons leading to the production of neuropeptides causing neurogenic airway inflammation [20,21]. Previously, DCs were found to be frequently associated anatomically with CGRP-containing sensory nerve fibres of the airways and skin [22,23].

Peripheral airway sensory nerve fibres are known to be derived from the neuronal cell bodies which are located in jugular-nodose ganglia complex (JNC) and able to produce, store and release neuropeptides such as tachykinins and CGRP to cause neurogenic inflammation [24-26]. However, DCs in airway sensory ganglia have not been explored under normal and allergic airway conditions so far.

The present study, therefore, aimed to investigate the localisation, distribution and proliferation of DCs and CGRP immunoreactive (IR)-neurons in vagal sensory jugular-nodose ganglia under allergic airway inflammation by using a chronic house dust mite (HDM) mouse model.

Materials and methods

Animals

Female wild-type BALB/c-mice (6–8 weeks old) were purchased from Charles River. The animals were held in regular 12 h dark/light cycles at a temperature of 22°C and received laboratory food and tap water *ad libitum*. The animals were acclimatised for at least 2 weeks prior to the study. All animal experiments were performed in strict concordance with the German animal protection law and approved by the appropriate governmental authority (No. 10/0207 and 14/2013).

HDM-mouse model

To induce chronic allergic airway inflammation, BALB/c mice (n = 10) were exposed consecutively 5 days a week within a total period of 7 weeks by intranasal instillation of HDM extract (Greer Inc., Cat. No XPB82D3A2.5) with a dose of 25 μg protein in 50 μl saline. A second group of animals (n = 10) served as control and was treated intranasal with 50 μl saline. Analyses were performed 24 h after the last allergen challenge (Figure 1) [27,28].

In vivo proliferation study with EdU (5-ethynyl-2′-deoxyuridine)

EdU is a nucleoside analog of thymidine and is incorporated into DNA during active DNA synthesis. Controls and HDM-treated animals received an i.p. injection of 1 mg EdU (Invitrogen) in a volume of 200 μl 24 h before sacrifice.

Bronchoalveolar lavage fluid (BALF)

Bronchoalveolar lavage was performed by instillation of 0.8 ml ice-cold PBS twice as described before [10]. The BALF was centrifuged (320 × g, 10 min, 4°C) and the supernatants were removed. The cell pellets were resuspended in 0.5 ml PBS and counted automatically with a Casy® cell counter. Cytospots were prepared, stained according to Pappenheim and differential cell counts were evaluated.

Lung histology

For the assessment of the airway inflammation, acetone fixed lung cryosections of allergen sensitised/challenged and control animals were stained with hematoxylin and eosin. Cryosections (10 μm) were prepared using a cryostat (CM1950; Leica Cryostat, Nussloch, Germany) and stained with hematoxylin and eosin (H&E) and Periodic Acid Schiff (PAS) according to standard protocols.

Ganglion preparation

JNC ganglia were fixed in Zamboni solution (Morphisto – Evolutionsforschung und Anwendung GmbH, Frankfurt am Main, Germany) for six hours, rinsed overnight in 0.1 M phosphate buffered saline (PBS) and cryoprotected 24 h with 30% sucrose in 0.1 M PBS. All steps above were performed at 4°C. The ganglia were embedded in OCT and frozen in liquid nitrogen. Serial 8 μm sections of the ganglia were prepared using a cryostat

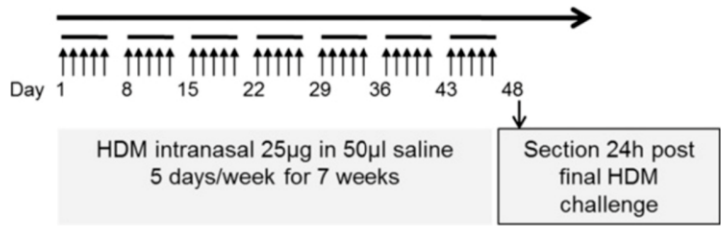


Figure 1 Allergic sensitisation and challenge protocol of chronic HDM mouse model. The animals were systemically sensitised by intranasal application of house dust mite 5 days a week for 7 weeks. All analyses were carried out 24 hours after the final challenge.

(Leica CM 1900, Bensheim, Germany), placed on APES (3-aminopropyltriethoxysilane) coated glass slides, dried at room temperature for 30 min and then stored in the freezer at −80°C.

Immunofluorescence staining

Every fourth section from serial sections of the JNC was dried at room temperature for 15 min and then rehydrated for 5 min in PBS. To reduce nonspecific antibody binding, the sections were incubated for 15 min at room temperature in 5% normal serum of the host species of the secondary antibody diluted in PBS. The sections were incubated with primary antibodies against PGP 9.5, MHC II, GS, CD11c, CD103, CD11b, F4/80, Iba1, GFAP, CGRP, or the appropriate isotype control antibodies (listed in Table 1) for 1 h at room temperature and then overnight at 4°C. After rinsing with 0.1 M PBS twice, the sections were incubated with secondary fluorescein conjugated antibodies (listed in Table 1) for 2 h at room temperature, for counterstaining the section then were incubated with 100 µl DAPI (0.5 µg/ml, Carl Roth, Germany) for 15 min at room temperature. Finally the sections were washed twice with 0.1 M PBS, once with double distilled water, mounted with fluorescent mounting medium Prolong Gold (Invitrogen) and covered with cover slips.

Quantitative analysis of MHC II IR-cells, CGRP ir-neurons and PGP 9.5 ir-neurons was visualised with epi-fluorescence microscopes (Axioskop 2 plus, Carl Zeiss and Olympus BX5), and cell counting was performed using the software ImageJ (National Institutes of Health). The evaluations of MHC II IR-cells and CGRP IR-neurons were normalised by the total neuron number and expressed as percentages.

Confocal images were acquired by LSM 510 META (Carl Zeiss, Jena, Germany). The Images were processed using Imaris 4.5.2 (Bitplane, Zurich, Switzerland).

EdU staining and quantification of EdU-labelled cells in JNC

EdU staining was conducted using Click-iT™ Cell Reaction Buffer Kit and Alexa Fluor 594 azide (Invitrogen) according to the manufacture's protocol. The JNC sections were rehydrated for 5 min in PBS and the blocked with 5% normal serum. The sections were incubated with 200 µl prepared Click-iT reaction cocktail for 30 min and then incubated with primary and secondary antibodies as described above. Primary antibodies included rat anti-mouse MHC class II (Biolegend) and rabbit anti-mouse PGP 9.5 (Abcam). Secondary detection was performed with donkey anti-rat Cy2 and donkey anti-rabbit DyLight 647 (Jackson ImmunoResearch) (Table 1). DAPI was used for counterstaining.

For the quantification, 16 sections of each mouse were analysed. Only the EdU-labelled and MHC II- and DAPI-positive cells were observed for quantification of proliferating cells. Evaluations of proliferating, MHC II positive cells with an EdU-labelled nucleus were normalised with the total MHC II positive cells and expressed as percentages.

Statistical analysis

Data is given as mean ± SEM. Statistical significance between groups was analysed with unpaired t-test using GraphPad Prism 4.03. Results of P values < 0.05 were considered significant.

Results

HDM induces allergic airway inflammation

HDM induced chronic allergic airway inflammation is indicated by the recruitment of eosinophils, neutrophils, lymphocytes and macrophages to the lung (Figure 2A). The histological analysis with H&E and PAS staining of the lung tissues showed massive infiltration of mononuclear cells (Figure 2B) and a high mucus secretion (Figure 2C) in HDM-sensitised and -challenged mice. In contrast, the lung sections of control mice did not contain inflammatory cell aggregates (Figure 2D) and no mucus secretion was detected (Figure 2E).

DCs are located in JNC and increased during allergic airway inflammation

JNC sections were analysed by immunofluorescent staining for the expression of pan-neuronal marker PGP 9.5 and MHC II to identify sensory neurons and DCs and assess the

Table 1 List of antibodies used in this study

Antibody	Source	Dilution
Primary purified antibodies		
Rabbit polyclonal antibody against mouse, human PGP 9.5	Abcam, Cambridge, UK	1:200
Rat monoclonal antibody against mouse I-A/I-E	BioLegend, San Diego, USA	1:200
Goat polyclonal antibody against mouse CGRP	Acris, Herford, Germany	1:400
Armenian hamster monoclonal antibody against mouse CD11c	Abcam, Cambridge, UK	1:100
Armenian hamster monoclonal antibody against mouse CD103	eBioscience, San Diego, USA	1:100
Rat monoclonal antibody against mouse CD11b	eBioscience, San Diego, USA	1:100
Rat monoclonal antibody against mouse F4/80	eBioscience, San Diego, USA	1:100
Rabbit polyclonal antibody against mouse glutamine synthetase (GS)	Abcam, Cambridge, UK	1:500
Rabbit Rabbit polyclonal antibody against Iba1	Wako Chemicals, Japan	1:300
Goat polyclonal antibody against GFAP	Abcam, Cambridge, UK	1:200
Isotype control purified antibodies		
Rabbit IgG	Dianova, Hamburg, Germany	1:5000
Rat IgG 2b kappa	BioLegend, San Diego, USA	1:200
Goat IgG	R&D systems,	1:300
Armenian Hamster IgG	BioLegend, San Diego, USA	1:200
Secondary antibodies		
DyLight 488 conjugated Donkey Anti-Rabbit IgG antibody	Jackson ImmunoResearch, INC., Baltimore, USA	1:400
Cy3 conjugated Donkey Anti-Rat IgG antibody	Jackson ImmunoResearch, INC., Baltimore, USA	1:500
Cy3 conjugated Donkey Anti-Rabbit IgG antibody	Jackson ImmunoResearch, INC., Baltimore, USA	1:400
DyLight 488 conjugated Donkey Anti-Goat IgG antibody	Jackson ImmunoResearch, INC., Baltimore, USA	1:400
Dylight 649 conjugated Goat Anti-Armenian Hamster IgG antibody	Jackson ImmunoResearch, INC., Baltimore, USA	1:400
DyLight 649 conjugated Donkey Anti-Rabbit IgG antibody	Jackson ImmunoResearch, INC., Baltimore, USA	1:400
Cy3 Conjugated Donkey Anti-Goat IgG	Jackson ImmunoResearch, INC., Baltimore, USA	1:500
Cy5 conjugated Donkey Anti-Rat IgG antibody	Jackson ImmunoResearch, INC., Baltimore, USA	1:400

anatomical localisation. Within the ganglia, neurons and nerve fibres were strongly immunoreactive for PGP 9.5 indicating that these cells were neurons and nerve fibres. Nerve fibres formed bundles crossing the ganglia. Under physiological and allergic conditions, DCs were observed to be widely distributed over the whole JNC and were located between the neurons and nerve fibres which were immunoreactive for PGP 9.5 (Figure 3).

To determine how the number of DCs in JNC changed during allergic airway inflammation, DCs and neurons in JNC of HDM-treated and control mice were quantified by using MHC II- and the pan-neuronal marker PGP 9.5- immunoreactivity. The percentage of DCs in relation to the overall neurons was significantly increased in HDM-treated mice (Figure 3C) if compared to the controls (Figure 3D) (DCs/neurons: HDM 51.38 ± 2.38% (n = 9) vs. control 28.16 ± 2.86 (n = 10), $p < 0.001$, Figure 3E).

To confirm whether the increase of DCs in JNC is caused by the targeted infiltration or global infiltration, the presence of MHC II-IR cells in trigeminal ganglia, which partly innervate the upper airways, has also been examined by immunohistochemistry. MHC II-IR cells have been found in trigeminal ganglia under physiological condition, and their numbers were not significantly changed by allergic airway inflammation (Figure 3F, G, H).

Phenotypic characterisation of DCs in JNC

To identify and characterise DCs phenotypically, various surface markers of DCs have been used for this study. In this respect, JNC sections were stained with antibodies against different markers of DCs such as MHC II, CD11c, CD11b, CD103, and Iba1. To discriminate DCs from macrophages, that also express these markers, the ganglia sections were additionally stained with the antibody against F4/80, which is the best known and extensively referenced marker for mouse macrophages. The staining results demonstrated a substantial number of cells in JNC that were strongly immunoreactive for MHC II, CD103 and Iba1. MHC II IR-cells have been found to be totally colocalised with CD103 and Iba1. The staining for CD11b and F4/80 marker did not show any immunoreactivity in MHC II IR-cells. Most

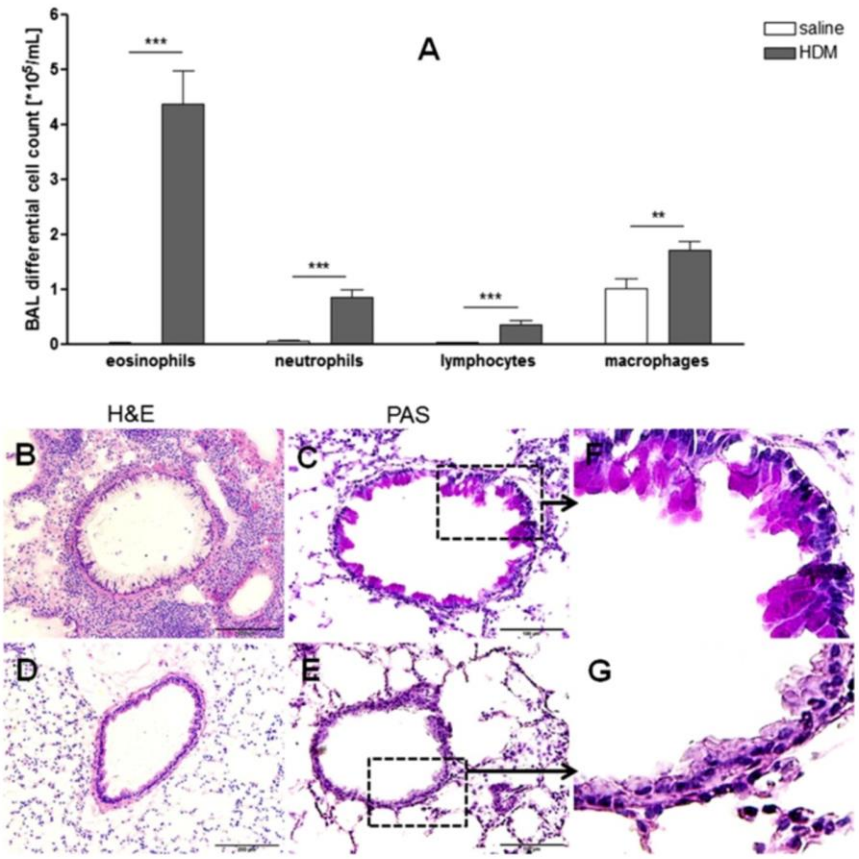


Figure 2 The inflammatory response in the lungs observed after continuous expose to HDM. Bronchoalveolar lavage fluid (BALF) cells differentiation **(A)**. Eosinophils, neutrophils, lymphocytes and macrophages in BALF demonstrated inflammation in the lungs of HDM-exposed mice, in comparison with control mice. Results are expressed as mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$ (unpaired two-tailed t-test). Representative photomicrographs of H&E (B,D,G) and PAS (C,E,F) stained lung sections. The lung sections from HDM-sensitised and challenged mice showed an infiltration of mononuclear cells **(B)** and a highly mucus secretion **(C)** in the airways, whereas the lungs of the control mice do not contain aggregated inflammatory cells **(D)** and mucus secretion **(E). F** and **G**: larger magnifications of **C** and **E**. Scale bars of **B** and **D**: 200 μm, scale bars of **C** and **E**: 100 μm.

of the MHC II-IR cells were immunoreactive for CD11c (CD11c-IR cells/MHC II-IR cells: HDM group: 98.84 ± 0.70% (n = 4), control group: 97.36 ± 0.60% (n = 5)). These findings demonstrated a subpopulation of DCs in JNC with these markers MHC II^+ $CD11c^+$ $CD103^+$ $Iba1^+$ $F4/80^-$ $CD11b^-$ (Figure 4).

Discrimination between DCs and glia cells in JNC

The expression of MHC II molecules has been described not only in conventional antigen-presenting cells (APC) but also in human trigeminal satellite glia cells (SGCs) [29]. Therefore, the discriminations of DCs from SGCs and other glia cells in mouse airway ganglia were determined. JNC sections were stained with the SGC marker glutamine synthetase (GS), astrocyte marker glial fibrillary acidic protein (GFAP) and the APC marker MHC II. The results showed that the GS positive cells were located around neurons and formed an envelope around the sensory neurons. SGCs in JNC were found to be small-sized cells with a thin cytoplasm and a small cell nucleus without APC phenotype. These cells were negative for MHC II-immunoreactivity indicating that they were not identical with DCs, characterised with MHC II. Furthermore, GS positive cells display a different anatomical structure than the MHC II positive cells identified as DCs in JNC. Further analysis demonstrated that MHC II-IR cells in JNC are negative for GFAP and therefore they do not belong to astrocyte subpopulation of glia cells (Figure 5).

Increase of CGRP-immunoreactive-neurons in JNC in allergic airway inflammation

Similarly to the finding for DCs, the percentage of CGRP ir-neurons in relation to the JNC-overall neurons was also found to be significantly elevated in HDM-treated

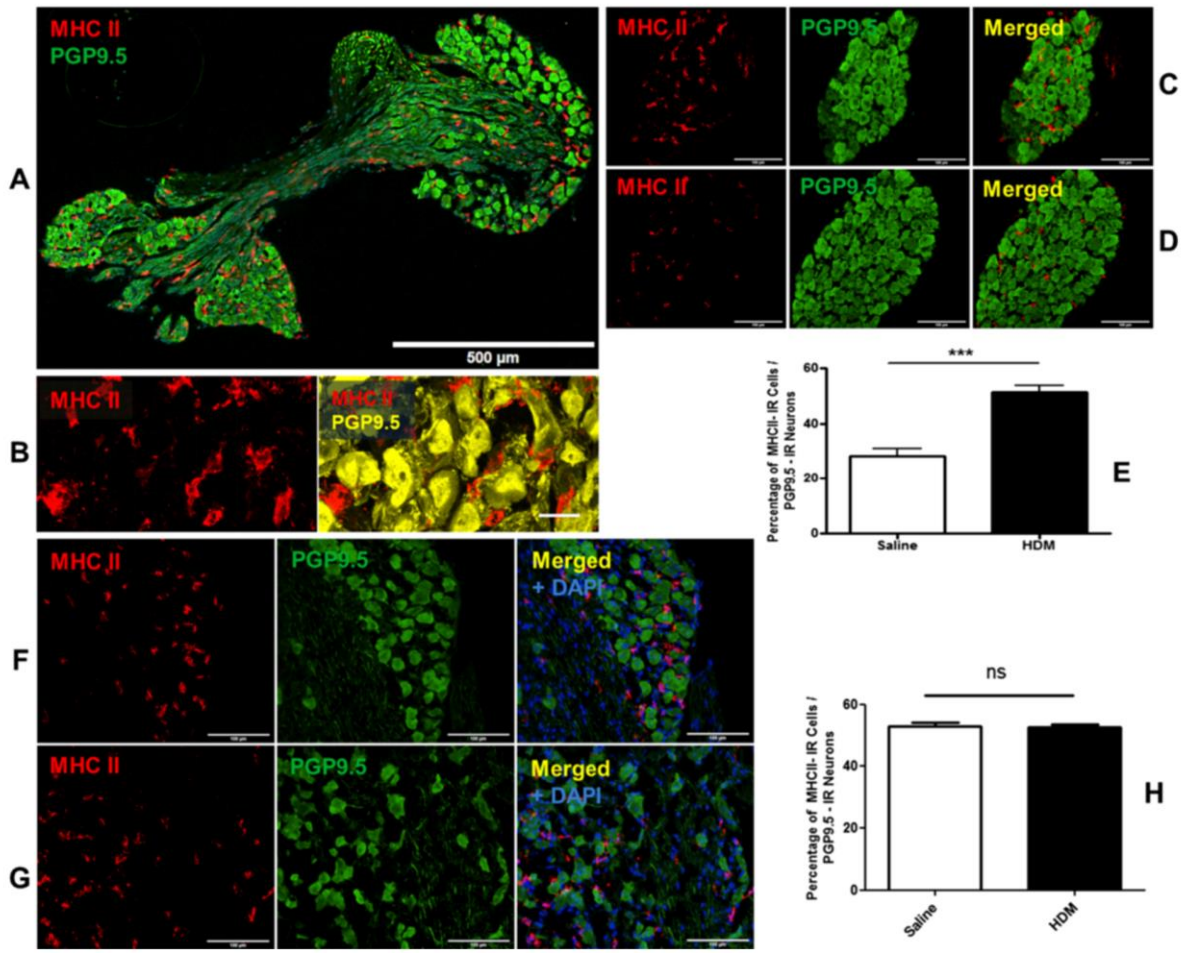


Figure 3 Quantification of dendritic cells in JNC and trigeminal ganglia. Zamboni fixed sections of JNC were stained with MHC II for DCs and PGP 9.5 for neurons. **A**: Overview of a JNC section stained for MHC II and PGP 9.5. **B**: An enlargement of DCs and neurons by using confocal microscope LSM 510 META (Carl Zeiss, Jena, Germany) with a 40x objective. Immunofluorescence analysis revealed amount of DCs, which are located next to the neuron in JNC of HDM treated mice **(C)** as well as control mice **(D)** and their number increased significantly during allergic airway inflammation. **E**: The quantification of DCs in JNC of control and HDM-treated mice. The results were expressed as percentages of MHC II positive cells in relation to PGP 9.5 positive neurons, H HDM 51.38 ± 2.38% (n = 9) vs. control 28.16 ± 2.86 (n = 10), $p < 0.001$. MHC II-IR cells have been found in trigeminal ganglia of control **(F)** and HDM treated mice **(G)**. **H**: The quantification of MHC II-IR cells in trigeminal ganglia of control and HDM groups. The results were expressed as percentages of MHC II-IR cells in relation to PGP 9.5 positive neurons, HDM 52.69 ± 0.86% (n = 4) vs control 52.90 ± 1.36% (n = 4). Images **A**, **C**, **D**, **F**, **G** were acquired with Olympus BX51 (Tokyo, Japan), Scale bar in **A**: 500 µm, in **B**: 20 µm, in **C**, **D**, **F**, **G**: 100 µm.

mice (Figure 6A) when compared to the control mice (Figure 6B) (CGRP-positive neurons/total neurons: HDM 31.16 ± 5.41% (n = 9) vs. control 7.16 ± 1.53% (n = 10), $p < 0.001$, Figure 6C). Additionally, MHC II-positive cells have been found to have an overall distribution on the JNC slices without any preferentially location to CGRP-positive neurons (Figure 6D).

Proliferating DCs in JNC during allergic airway inflammation

To examine whether DCs proliferated in JNC or migrated from outside into the ganglia during allergic airway inflammation, the animals were injected with EdU, which is incorporated with DNA of dividing cells. Systemic administration of EdU 24 h before analysis assured the detection of proliferating cells. The proliferating DCs in two groups were evaluated and expressed as percentage of MHC II positive cells with EdU-labelled nucleus/ total MHC II positive cells. The results showed no significant change of proliferating DCs ratio between two groups (proliferating DCs/ total DCs: HDM 0.89 ± 0.38%, (n = 4) vs. control 1.19 ± 0.54%, (n = 5), $p = 0.68$, Figure 7G). Additionally, proliferation of neurons as well as SGCs was not found in the experiments. Further EdU experiments for

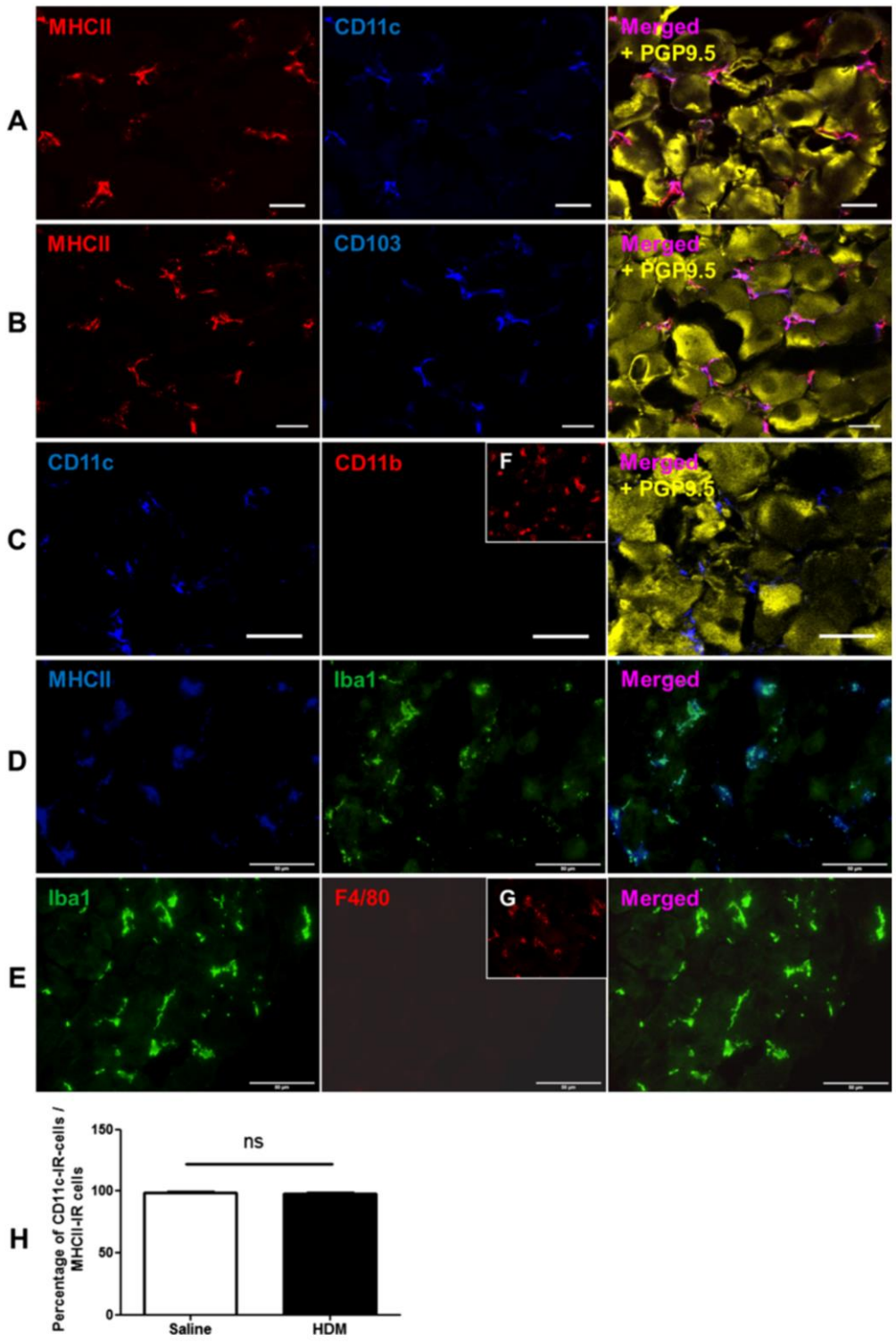


Figure 4 (See legend on next page.)

(See figure on previous page.)
Figure 4 Phenotypic characterisation of DCs in JNC. JNC sections were stained for different cell markers and images were acquired with confocal microscope LSM 510 META (Carl Zeiss, Jena, Germany) using a 40x objective and Olympus BX51 (Tokyo, Japan) using a 20x, 40x objectives. The Immunofluorescence analysis showed the immune cells in JNC positive for MHC II and CD11c **(A)**, positive for MHC II and CD103 **(B)**, positive for CD11c and negative for CD11b **(C)**, positive for MHC II and Iba1 **(D)**, positive for Iba1 and negative for F4/80 **(E)**. **F**: Positive control of CD11b antibody in the lung tissue. **G**: Positive control of CD11c antibody in trigeminal ganglion. Yellow cells in **A**, **B**, **C** are neurons, which are stained with antibody against PGP 9.5. **H**: The quantification of CD11c-IR cells in JNC, the results were expressed as percentages of CD11c-IR cells in relation to MHC II–IR cells. Scale bars represent in **A**, **B**, **C**: 20 μm, in **D**, **E**: 50 μm.

the positive controls have been carried out in lung tissue of HDM treated mice. The finding showed a large population of MHC II-IR cells of the lung also strongly positive for EdU, showing the DCs proliferation in the lung (Figure 7H).

Discussion

Dendritic cells play a pivotal role as antigen sampling and presenting cells in the initiation and development of allergic response such as allergic airway inflammation [3,4,8]. Previous studies have revealed that DCs were found to be localised in the neighbourhood of peripheral CGRP-positive nerve fibres in the airways [22]. The role of this DC-nerve interaction in allergic airway inflammation, however, has not been clear yet.

CGRP has been discussed to have the capacity to modulate DCs during the allergic airway inflammation. CGRP is known to be produced by the cell bodies located in JNC, anterogradely transported and released by the peripheral nerve fibres innervating the airways [12-14]. In this respect, the present study aimed to investigate the localisation, distribution and proliferation of DCs in JNC by using HDM mouse model for allergic airway inflammation.

After continuous intranasal administration with HDM extract, a chronic airway inflammation was induced as previously reported [27]. In accordance to other studies, inhalation of HDM extract led to a significant increase of the recruitment of eosinophils as well as a substantial number of neutrophils, macrophages and lymphocytes [27,28]. With the characteristic of mixed eosinophilic and neutrophilic inflammation, the HDM mouse model becomes an interesting animal model that may closely reflect the situation of severe bronchial asthma in human [1].

Interestingly, for the first time, cells expressing MHC II molecules were identified in JNC of mice under physiological conditions. The immunofluorescence analysis revealed that these cells belong to the immune cell population rather than to the nervous system as they were non-reactive for the pan-neuronal marker PGP 9.5. Additionally, these immune cells exhibited DC phenotypes as they displayed high immunoreactivity for MHC II and CD103 while negative for CD11b- and F4/80-immunoreactivity. Most (about 98%) of MHC II-IR cells were immunoreactive for CD11c indicating these cells are DCs. MHC II-IR cells have also been found to be totally colocalised with Iba1, which has been reported to be expressed in

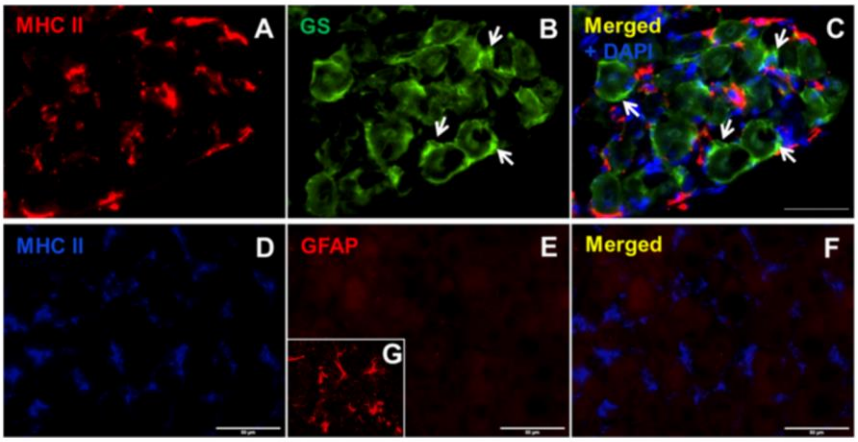


Figure 5 Distinction between DCs and glia cells in JNC. A: DCs are immunoreactive with MHC II. **B**: Satellite glia cells (SGCs, arrows) are immunoreactive for glutamine synthetase (GS). **C**: MHC II-IR cells have no overlaps with SGCs immunoreactive for GS, the separate cell nucleus of SGCs and DCs are stained with DAPI. The dendritic cells with immunoreactivity for MHC II **(D)** were also stained with astrocyte marker GFAP, these dendritic cells expressed no GFAP **(E)**. **F**: merged representation of MHC II and GFAP. **G**: positive control of GFAP antibody in mouse brain section. Scale bars: 50 μm.

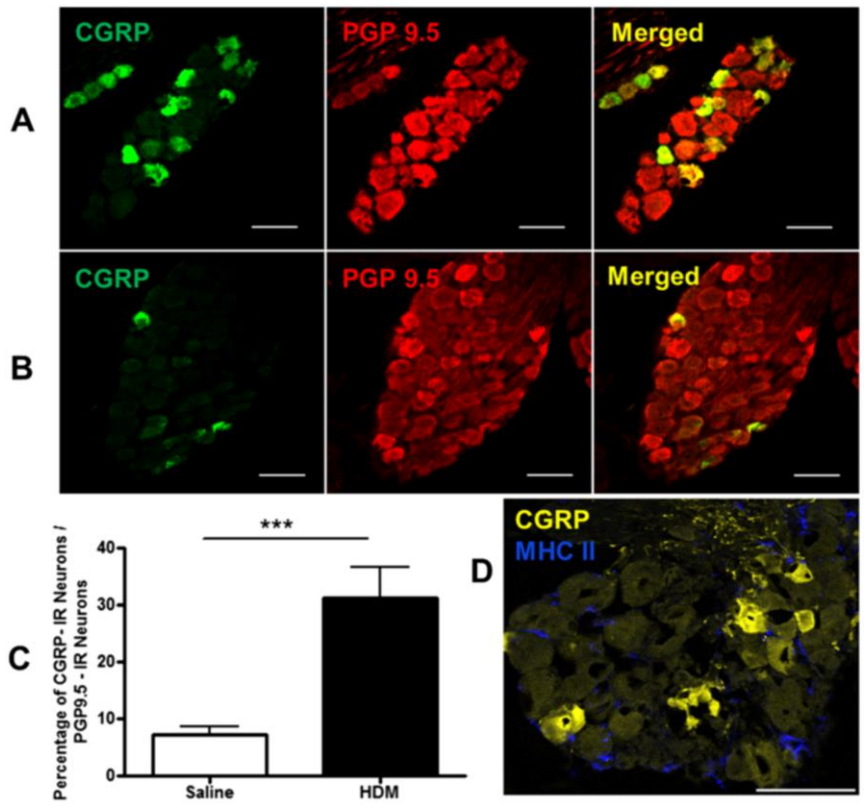


Figure 6 Increased numbers of CGRP-positive neurons under allergic condition. The JNC sections of HDM treated animals and control animals were stained with antibodies against neuropeptides CGRP and PGP 9.5. The amount of CGRP positive neurons increased significantly during allergic airway inflammation **(A)** in comparison to that of the controls **(B)**. Scale bars: 50 μm. Images were acquired with Axioskop 2 plus (Zeiss) using a 40x objective. **C**: The quantification of CGRP ir-neurons in JNC of control and HDM-treated mice. The results were expressed as percentage of CGRP-IR-neurons in relation to all PGP 9.5 positive neurons, HDM 31.16 ± 5.41% (n = 9) vs. control 7.16 ± 1.53% (n = 10), $p < 0.001$. **D**: MHC II-positive cells distribute overall on the JNC slices without any preferentially location to CGRP-positive neurons. Image was acquired with confocal microscope LSM 510 META (Carl Zeiss, Jena, Germany), scale bar: 50 μm.

DCs in the brain and other organs such as intestine and skin [5,30,31]. A very small population with MHC II-IR did not express CD11c (<2%). These cell types are still unknown and remain to be investigated in future study.

The localisation of DCs within the sensory ganglia was found to be widely distributed on the whole ganglia and between SGCs and/or neurons. Recently, other neuronal cells, such as SGCs in human trigeminal ganglion, have also been shown to express MHC II [29]. However, the present findings revealed that SGCs in mouse JNC to be negative for MHC II. The contrary findings of the SGC population may be caused by the ganglionic- and species-specific differences between mouse and human [29,32]. The exact role of SGCs in JNC under physiological and allergic situations remains to be investigated in future experiments.

For the discrimination of DC migration into the ganglia as a targeted infiltration and systemic infiltration, further studies have been carried out in trigeminal ganglia. In contrast to the JNC, where the numbers of DCs were significantly increased during allergic airway inflammation, the numbers of MHC II-IR cells in trigeminal ganglia, however, were not changed. These contrary findings may be caused by the ganglionic- or organ-specific differences between JNC and trigeminal ganglia. Anatomically, trigeminal ganglia are located inside the cranium, whereas JNC are found to be under the skullbase. Additionally, the two ganglia have different embryonic development.

The findings of the increase of DCs in the JNC lead to further questions of their origin. The results of the proliferation study suggest that DCs may move from the systemic blood circulation into JNC by passing through the ganglion-blood barrier. In contrast to the brain–blood barrier, the ganglion-blood barrier is known to be only discontinuously formed [33]. Alternatively, DCs may reach the JNC along the vagus from the peripheral airways by retrograde migration when their numbers in the airways and the blood system were to be enhanced during allergic

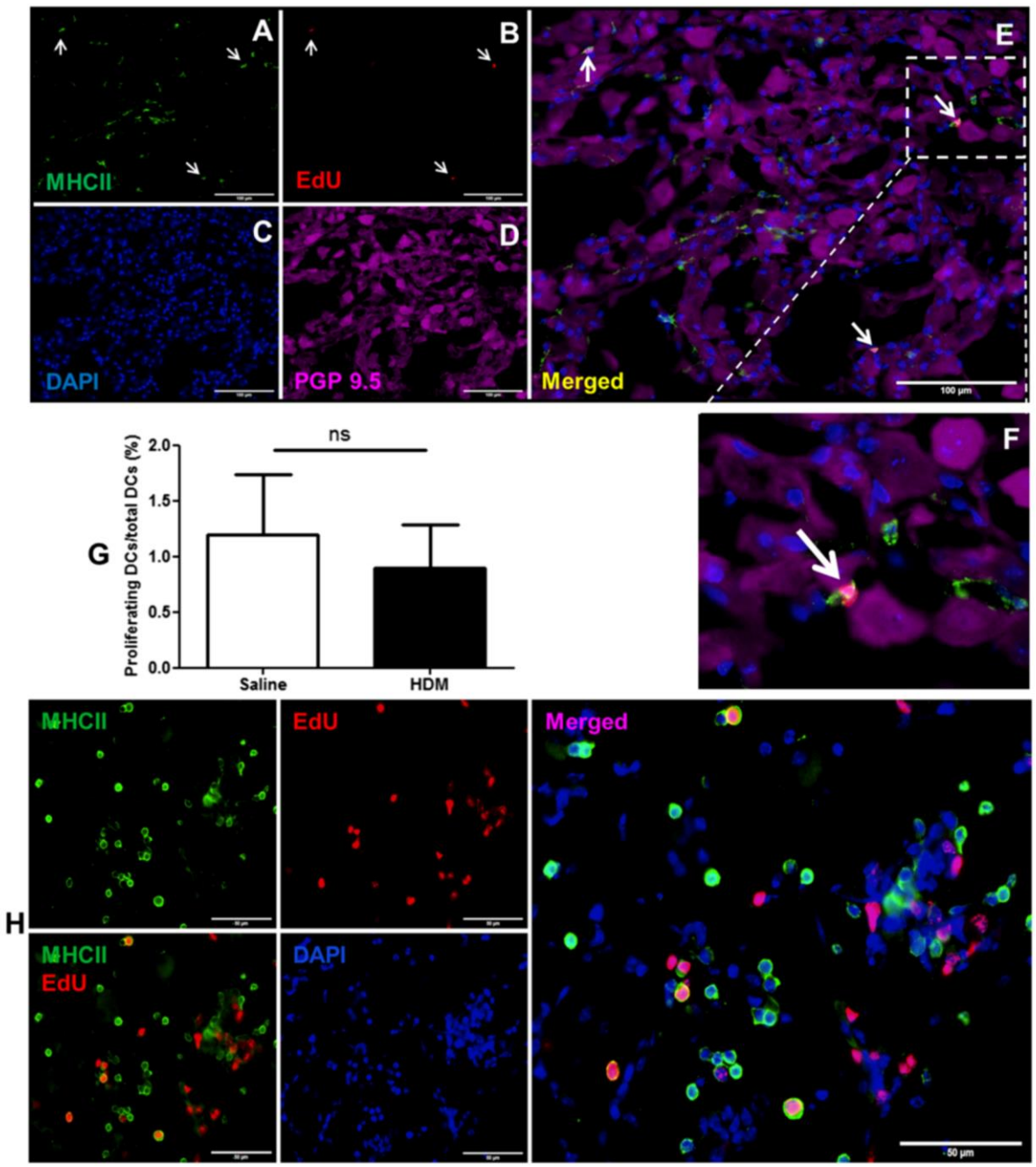


Figure 7 Quantitative analysis of proliferating DCs in JNC. A: MHC II staining of DCs. **B**: EdU detection of proliferating DCs (arrows). **C**: Nucleus staining with DAPI. **D**: Neurons positive for PGP 9.5. **E**: Merged representation of the images shown in **A-D**. **F**: An enlargement of EdU positive cell. **G**: Quantification of proliferating DCs in JNC of HDM treated and control mice. Ganglion sections were stained for EdU, MHC II, PGP 9.5 and nucleus. The proliferating DCs, which are positive with MHC II and EdU labelled nucleus, and total number of DCs were evaluated. The results were expressed as percentages of proliferating DCs/total DCs. The proliferation rate of DCs in HDM treated mice 0.89 ± 0.38% (n = 4) and control mice 1.19 ± 0.54% (n = 5), p = 0.68. **H**: The positive control of EdU in the lung. The figures showed amount of proliferating MHC II-IR cells and other cell types in the lung. Scale bars: 100 μm in **A**, **B**, **C**, **D**, 50 μm in **H**.

airway inflammation [34,35]. The presence of DCs in JNC also may play a substantial role in the immune response to protect the neurons against viral and bacterial infection, virus replication and bacterial spread without any neuronal destruction [36-39]. In this respect, other immune cells like T cells and macrophages were recently found inside the HSV-1 latently infected trigeminal ganglia [36]. However, the origin and function of the DCs in JNC under normal and allergic airway conditions remains to be answered in future studies.

With respect to the neuroimmune interaction, maturation, migration and function of DCs during allergic airway inflammation have been reported to be modulated by neuropeptides like the calcitonin gene-related peptide (CGRP), tachykinins and vasoactive intestinal peptide (VIP) released from airway nerves fibers [9,10,13,14]. In view of modulatory effects of CGRP on DCs, sensory neurons in JNC were investigated for CGRP-expression. The significant increase of CGRP-IR-neurons and the elevated numbers of DCs in allergic airway response suggest that there may be a functional relationship between DCs and CGRP-IR-neurons.

The increased of CGRP-IR-neurons in HDM-treated mice may have an influence on the migration, proliferation or function of DCs in JNC. With respect to the effect of CGRP in allergic airway inflammation, a proinflammatory or antiinflammatory role of CGRP in allergic airway inflammation is therefore still controversially discussed [9,10,12,40]. The intragangionic release of CGRP within JNC has not been demonstrated so far in vivo. Previous study on rat has reported about the release of CGRP from isolated JNC after stimulation with different substances such as capsaicin, nitric oxide donor sodium nitroprusside [41]. Our findings showed an increase of CGRP-positive neurons in JNC, but a release of CGRP from the neuron into the ganglia could not be demonstrated in the present study. Based on this observation, we suggest that CGRP may be paracrinely released from the neurons or anterogradely transported to the peripheral nerve endings and liberated into the airways. The exact role of CGRP in sensory neurons concerning the modulation of DC-function during allergic airway response remains to be elucidated in future studies.

Conclusion

The present study revealed for the first time the existence of immune cells with DC phenotype within JNC. The increase of DCs and CGRP-positive neurons in airway ganglia caused by allergic airway inflammation indicates that ganglia DCs and neurons expressing CGRP may contribute to the pathogenesis of bronchial asthma. These findings demonstrate that DCs may migrate from outside into the ganglia to interact with sensory neurons enhancing or protecting the allergic airway inflammation. Further studies should be carried out to explore the interaction of DCs and neurons in airway sensory ganglia during allergic airway inflammation.

Abbreviations

APC: Antigen-presenting cell; APES: 3-Aminopropyltriethoxysilane; BALF: Bronchoalveolar lavage fluid; CGRP: Calcitonin gene-related peptide; DC: Dendritic cells; DAPI: 4', 6-diamidino-2-phenylindol dihydrochloride; EdU: 5-ethynyl-2'-deoxyuridine; GFAP: Glial fibrillary acidic protein; GS: Glutamine synthetase; HDM: House dust mite; H&E: Hematoxylin and eosin; Iba1: Ionized calcium binding adapter molecule 1; IR: Immunoreactive; JNC: Jugular-nodose ganglia complex; LPS: Lipopolysaccharide; MHC II: Major histocompatibility class II; PAS: Periodic Acid Schiff; PBS: Phosphate buffered saline; PGP 9.5: Protein gene product 9.5; SGC: Satellite glia cell; VIP: Vasoactive intestinal peptide.

Competing interests

None of the authors have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Authors' contributions

The authors AF, TT, RB, TW, AB, MS QTD designed the study. DDL, SR, SH performed animal experiments and analysed the data. DDL, QTD wrote the manuscript. All authors read and approved the manuscript.

Acknowledgments

This project was supported by GRK 1441 "Regulation of the Allergic Inflammatory Response in Lung and Skin" to Prof. T. Welte and Prof. Q.T. Dinh, by the German Research Foundation (DFG, DI 1429/2-1) and by the foundation "Stiftung Bergmannshilfswerks Luisenthal" to Prof. Q.T. Dinh, by SFB587 B4 and by IMI UBIOPRED to Prof. A. Braun.

Author details

[1]Department of Experimental Pneumology and Allergology, Saarland University Faculty of Medicine, Kirrberger Strasse, Geb. 61.4, Homburg, Germany. [2]Department of Airway Immunology, Fraunhofer Institute for Toxicology and Experimental Medicine, German Centre for Lung Research (DZL), Hannover, Germany. [3]Institute of Occupational Medicine, Charité - Universitätsmedizin Berlin, Free University and Humboldt University, Berlin, Germany. [4]Institute of Anatomy and Cell Biology, Saarland University Faculty of Medicine, Homburg, Germany. [5]Department of Transplant and Infection Immunology, Saarland University, Homburg, Germany. [6]Department of Internal Medicine V, Pneumology, Allergology and Respiratory Critical Care Medicine, Saarland University Faculty of Medicine, Kirrberger Strasse, Geb. 61.4, Homburg, Germany. [7]Department of Pneumology, Hannover Medical School, German Centre for Lung Research (DZL), Hannover, Germany.

Received: 21 January 2014 Accepted: 23 June 2014
Published: 30 June 2014

References

1. Barnes PJ: **Pathophysiology of allergic inflammation.** *Immunol Rev* 2011, **242:**31–50.
2. Gill MA: **The role of dendritic cells in asthma.** *J Allergy Clin Immunol* 2012, **129:**889–901.
3. Lambrecht BN: **Dendritic cells and the regulation of the allergic immune response.** *Allergy* 2005, **60:**271–282.
4. Novak N, Haberstok J, Geiger E, Bieber T: **Dendritic cells in allergy.** *Allergy* 1999, **54:**792–803.
5. Bulloch K, Miller MM, Gal-Toth J, Milner TA, Gottfried-Blackmore A, Waters EM, Kaunzner UW, Liu K, Lindquist R, Nussenzweig MC, Steinman RM, McEwen BS: **CD11c/EYFP transgene illuminates a discrete network of dendritic cells within the embryonic, neonatal, adult, and injured mouse brain.** *J Comp Neurol* 2008, **508:**687–710.
6. Steinman RM, Banchereau J: **Taking dendritic cells into medicine.** *Nature* 2007, **449:**419–426.
7. Vermaelen KY, Carro-Muino I, Lambrecht BN, Pauwels RA: **Specific migratory dendritic cells rapidly transport antigen from the airways to the thoracic lymph nodes.** *J Exp Med* 2001, **193:**51–60.

8. Lambrecht BN, Hammad H: **The role of dendritic and epithelial cells as master regulators of allergic airway inflammation.** *Lancet* 2010, **376:**835–843.
9. Dunzendorfer S, Kaser A, Meierhofer C, Tilg H, Wiedermann CJ: **Cutting edge: peripheral neuropeptides attract immature and arrest mature blood-derived dendritic cells.** *J Immunol* 2001, **166:**2167–2172.
10. Rochlitzer S, Veres TZ, Kuhne K, Prenzler F, Pilzner C, Knothe S, Winkler C, Lauenstein HD, Willart M, Hammad H, Müller M, Krug N, Lambrecht BN, Braun A: **The neuropeptide calcitonin gene-related peptide affects allergic airway inflammation by modulating dendritic cell function.** *Clin Exp Allergy* 2011, **41:**1609–1621.
11. Joos GF, Germonpre PR, Pauwels RA: **Role of tachykinins in asthma.** *Allergy* 2000, **55:**321–337.
12. Groneberg DA, Quarcoo D, Frossard N, Fischer A: **Neurogenic mechanisms in bronchial inflammatory diseases.** *Allergy* 2004, **59:**1139–1152.
13. Dinh QT, Mingomataj E, Quarcoo D, Groneberg DA, Witt C, Klapp BF, Braun A, Fischer A: **Allergic airway inflammation induces tachykinin peptides expression in vagal sensory neurons innervating mouse airways.** *Clin Exp Allergy* 2005, **35:**820–825.
14. Fischer A, McGregor GP, Saria A, Philippin B, Kummer W: **Induction of tachykinin gene and peptide expression in guinea pig nodose primary afferent neurons by allergic airway inflammation.** *J Clin Invest* 1996, **98:**2284–2291.
15. Barnes PJ: **Neurogenic inflammation in the airways.** *Respir Physiol* 2001, **125:**145–154.
16. Davies D, Medeiros MS, Keen J, Turner AJ, Haynes LW: **Eosinophil chemotactic peptide sequences in rat alpha-CGRP. Activation of a novel trophic action by neutral endopeptidase 24.11.** *Ann N Y Acad Sci* 1992, **657:**405–411.
17. Foster CA, Mandak B, Kromer E, Rot A: **Calcitonin gene-related peptide is chemotactic for human T lymphocytes.** *Ann N Y Acad Sci* 1992, **657:**397–404.
18. Lambrecht BN: **Immunologists getting nervous: neuropeptides, dendritic cells and T cell activation.** *Respir Res* 2001, **2:**133–138.
19. Kawanami Y, Morimoto Y, Kim H, Nakamura T, Machida K, Kido T, Asonuma E, Yatera K, Yoshii C, Kido M: **Calcitonin gene-related peptide stimulates proliferation of alveolar epithelial cells.** *Respir Res* 2009, **10:**8.
20. Braun A, Lommatzsch M, Mannsfeldt A, Neuhaus-Steinmetz U, Fischer A, Schnoy N, Lewin GR, Renz H: **Cellular sources of enhanced brain-derived neurotrophic factor production in a mouse model of allergic inflammation.** *Am J Respir Cell Mol Biol* 1999, **21:**537–546.
21. Noga O, Peiser M, Altenahr M, Schmeck B, Wanner R, Dinh QT, Hanf G, Suttorp N: **Selective induction of nerve growth factor and brain-derived neurotrophic factor by LPS and allergen in dendritic cells.** *Clin Exp Allergy* 2008, **38:**473–479.
22. Veres TZ, Rochlitzer S, Shevchenko M, Fuchs B, Prenzler F, Nassenstein C, Fischer A, Welker L, Holz O, Muller M, Krug N, Braun A: **Spatial interactions between dendritic cells and sensory nerves in allergic airway inflammation.** *Am J Respir Cell Mol Biol* 2007, **37:**553–561.
23. Hosoi J, Murphy GF, Egan CL, Lerner EA, Grabbe S, Asahina A, Granstein RD: **Regulation of Langerhans cell function by nerves containing calcitonin gene-related peptide.** *Nature* 1993, **363:**159–163.
24. Groth M, Helbig T, Grau V, Kummer W, Haberberger RV: **Spinal afferent neurons projecting to the rat lung and pleura express acid sensitive channels.** *Respir Res* 2006, **7:**96.
25. Dinh QT, Groneberg DA, Peiser C, Joachim RA, Frossard N, Arck PC, Klapp BF, Fischer A: **Expression of substance P and nitric oxide synthase in vagal sensory neurons innervating the mouse airways.** *Regul Pept* 2005, **126:**189–194.
26. Dinh QT, Groneberg DA, Peiser C, Springer J, Joachim RA, Arck PC, Klapp BF, Fischer A: **Nerve growth factor-induced substance P in capsaicin-insensitive vagal neurons innervating the lower mouse airway.** *Clin Exp Allergy* 2004, **34:**1474–1479.
27. Johnson JR, Wiley RE, Fattouh R, Swirski FK, Gajewska BU, Coyle AJ, Gutierrez-Ramos JC, Ellis R, Inman MD, Jordana M: **Continuous exposure to house dust mite elicits chronic airway inflammation and structural remodeling.** *Am J Respir Crit Care Med* 2004, **169:**378–385.
28. Tomlinson KL, Davies GC, Sutton DJ, Palframan RT: **Neutralisation of interleukin-13 in mice prevents airway pathology caused by chronic exposure to house dust mite.** *PLoS One* 2010, **5**(10):e13136. doi:10.1371/journal.pone.0013136.
29. van Velzen M, Laman JD, Kleinjan A, Poot A, Osterhaus AD, Verjans GM: **Neuron-interacting satellite glial cells in human trigeminal ganglia have an APC phenotype.** *J Immunol* 2009, **183:**2456–2461.
30. Chen ZW, Ahren B, Ostenson CG, Cintra A, Bergman T, Moller C, Fuxe K, Mutt V, Jornvall H, Efendic S: **Identification, isolation, and characterization of daintain (allograft inflammatory factor 1), a macrophage polypeptide with effects on insulin secretion and abundantly present in the pancreas of prediabetic BB rats.** *Proc Natl Acad Sci U S A* 1997, **94:**13879–13884.
31. Orsmark C, Skoog T, Jeskanen L, Kere J, Saarialho-Kere U: **Expression of allograft inflammatory factor-1 in inflammatory skin disorders.** *Acta Derm Venereol* 2007, **87:**223–227.
32. Hanani M: **Satellite glial cells: more than just 'rings around the neuron'.** *Neuron Glia Biol* 2010, **6:**1–2.
33. Ten Tusscher MP, Klooster J, Vrensen GF: **Satellite cells as blood-ganglion cell barrier in autonomic ganglia.** *Brain Res* 1989, **490:**95–102.
34. Veres TZ, Voedisch S, Spies E, Valtonen J, Prenzler F, Braun A: **Aeroallergen challenge promotes dendritic cell proliferation in the airways.** *J Immunol* 2013, **190:**897–903.
35. van Rijt LS, Prins JB, Leenen PJ, Thielemans K, de Vries VC, Hoogsteden HC, Lambrecht BN: **Allergen-induced accumulation of airway dendritic cells is supported by an increase in CD31(hi)Ly-6C(neg) bone marrow precursors in a mouse model of asthma.** *Blood* 2002, **100:**3663–3671.
36. Theil D, Derfuss T, Paripovic I, Herberger S, Meinl E, Schueler O, Strupp M, Arbusow V, Brandt T: **Latent herpesvirus infection in human trigeminal ganglia causes chronic immune response.** *Am J Pathol* 2003, **163:**2179–2184.
37. Tsiang H, Ceccaldi PE, Lycke E: **Rabies virus infection and transport in human sensory dorsal root ganglia neurons.** *J Gen Virol* 1991, **72**(Pt 5):1191–1194.
38. Li XQ, Fu ZF, Alvarez R, Henderson C, Tripp RA: **Respiratory syncytial virus (RSV) infects neuronal cells and processes that innervate the lung by a process involving RSV G protein.** *J Virol* 2006, **80:**537–540.
39. Antinone SE, Smith GA: **Retrograde axon transport of herpes simplex virus and pseudorabies virus: a live-cell comparative analysis.** *J Virol* 2010, **84:**1504–1512.
40. Voedisch S, Rochlitzer S, Veres TZ, Spies E, Braun A: **Neuropeptides control the dynamic behavior of airway mucosal dendritic cells.** *PLoS One* 2012, **7:**e45951.
41. Strecker T, Koulchitsky S, Dieterle A, Neuhuber WL, Weyand M, Messlinger K: **Release of calcitonin gene-related peptide from the jugular-nodose ganglion complex in rats–a new model to examine the role of cardiac peptidergic and nitrergic innervation.** *Neuropeptides* 2008, **42:**543–550.

doi:10.1186/1465-9921-15-73
Cite this article as: Le *et al.*: Allergic airway inflammation induces the migration of dendritic cells into airway sensory ganglia. *Respiratory Research* 2014 **15**:73.

5.3 Paper III

Title: Steroid treatment reduces the allergic airway inflammation and does not alter the increased numbers of dendritic cells and CGRP-expressing neurons in airway sensory ganglia

Authors: Duc Dung Le*****, Ulrike Funck*****, Sabine Wronski, Sebastian Heck, Thomas Tschernig, Markus Bischoff, Martina Sester, Christian Herr, Robert Bals, Tobias Welte, Armin Braun and Quoc Thai Dinh

***Neuroimmunomodulation*. 2016;23(1):18-26**

***** These authors contributed equally to the study

Original Paper

NeuroImmunoModulation

Neuroimmunomodulation 2016;23:18–26
DOI: 10.1159/000440622

Received: June 1, 2015
Accepted after revision: August 22, 2015
Published online: October 8, 2015

Steroid Treatment Reduces Allergic Airway Inflammation and Does Not Alter the Increased Numbers of Dendritic Cells and Calcitonin Gene-Related Peptide-Expressing Neurons in Airway Sensory Ganglia

Duc Dung Le[a] Ulrike Funck[a] Sabine Wronski[f, h] Sebastian Heck[a] Thomas Tschernig[d] Markus Bischoff[e] Martina Sester[b] Christian Herr[c] Robert Bals[c] Tobias Welte[g, h] Armin Braun[f, h] Quoc Thai Dinh[a, c]

Departments of [a]Internal Medicine V – Experimental Pneumology and Allergology, [b]Transplant and Infection Immunology and [c]Internal Medicine V – Pneumology, Allergology and Respiratory Critical Care Medicine, and Institutes of [d]Anatomy and Cell Biology and [e]Medical Microbiology and Hygiene, Saarland University Faculty of Medicine, Homburg, [f]Department of Airway Immunology, Fraunhofer Institute for Toxicology and Experimental Medicine, [g]Department of Pneumology, Hannover Medical School, and [h]German Centre for Lung Research (DZL), Hannover, Germany

Key Words
Dendritic cells · Calcitonin gene-related peptide · Jugular-nodose ganglion complex · Neuroimmune interaction · Steroid treatment

Abstract
Objectives: Our previous data demonstrated that allergic airway inflammation induces migration of dendritic cells (DC) into airway sensory jugular and nodose ganglia (jugular-nodose ganglion complex; JNC). Here we investigated the effects of steroid treatment regarding the expression and migration of DC and calcitonin gene-related peptide (CGRP)-immunoreactive neurons of vagal sensory ganglia during allergic airway inflammation. ***Methods:*** A house dust mite (HDM) model for allergic airway inflammation was used. The mice received 0.3 mg fluticasone propionate per kilogram of body weight in the last 9 days. JNC slices were analyzed on MHC II, the neuronal marker PGP9.5, and the neuropeptide CGRP. ***Results:*** Allergic airway inflammation increased the numbers of DC and CGRP-expressing neurons in the JNC significantly in comparison to the controls (DC/neurons: HDM 44.58 ± 1.6% vs. saline 33.29 ± 1.6%, $p < 0.05$; CGRP-positive neurons/total neurons: HDM 30.65 ± 1.9% vs. saline 19.49 ± 2.3%, $p < 0.05$). Steroid treatment did not have any effect on the numbers of DC and CGRP-expressing neurons in the JNC compared to HDM-treated mice. ***Conclusions:*** The present findings indicate an important role of DC and CGRP-containing neurons in the pathogenesis of allergic airway inflammation. However, steroid treatment did not have an effect on the population of DC and neurons displaying CGRP in the JNC, whereas steroid treatment was found to suppress allergic airway inflammation.

Introduction

Allergic bronchial asthma is characterized by reversible airway obstruction, airway hyperresponsiveness (AHR), mucus hypersecretion, and chronic airway in-

D.D.L. and U.F. contributed equally to this work.

KARGER

© 2015 S. Karger AG, Basel
1021–7401/15/0231–0018$39.50/0

E-Mail karger@karger.com
www.karger.com/nim

Prof. Quoc Thai Dinh, MD
Department of Experimental Pneumology and Allergology, Saarland University Hospital and Saarland University Faculty of Medicine, Kirrberger Strasse, Geb. 61.4
DE–66421 Homburg/Saar (Germany)
E-Mail Thai.Dinh@uniklinikum-saarland.de

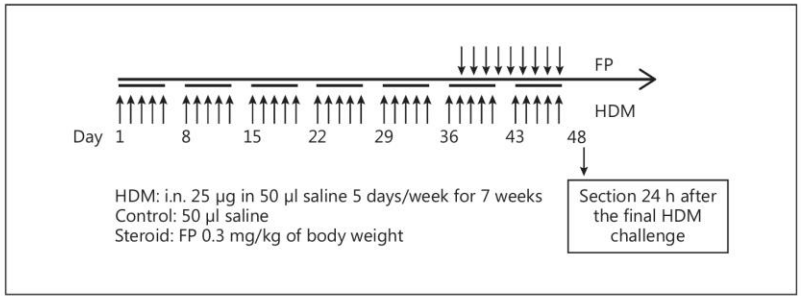


Fig. 1. Treatment protocol of the chronic HDM mouse model. The animals were systemically sensitized by intranasal application of HDM extract 5 days a week for 7 weeks. FP treatment was administered i.n. at 0.3 mg/kg of body weight in a volume of 50 µl on the final 9 days. All analyses were carried out 24 h after the final challenge.

flammation with recruitment of various immune cells, including dendritic cells (DC) [1–3]. As antigen-presenting and phagocytic cells, DC are known to play a key role in the allergic immune response and to be localized in many organs like the skin, the mucosa of the intestines, and the lungs [2–5].

DC have been shown to have the capacity to activate T lymphocytes, induce immune tolerance, and maintain immune memory [3, 5–7]. Recently, the maturation and differentiation of DC have been described to be modulated by many cytokines of immune cells as well as neuropeptides such as the calcitonin gene-related peptide (CGRP) released by airway sensory nerve fibers [8–13].

CGRP consists of 37 amino acids [14] and is biosynthesized and released from sensory neurons innervating the airways in response to different stimuli including allergic airway inflammation [12, 14, 15]. CGRP released from airway nerve fibers has the capacity to act as a chemoattractant factor for different immune cells such as CD4+ T lymphocytes, CD8+ T lymphocytes [16, 17], eosinophils [18, 19], and DC [20] and to induce the proliferation of airway epithelial cells [21]. Besides, as a chemoattractant for inflammatory cells, CGRP plays an important role, with multiple effects in the pathogenesis of allergic airway inflammation [22, 23]. CGRP has been proposed to induce bronchoconstriction and vasodilatation in airway diseases [24, 25].

Fluticasone propionate (FP) has been commonly used for therapy of bronchial asthma and chronic obstructive pulmonary disease (COPD). Treatment with FP has been demonstrated to reduce the expression of sensory neuropeptides such as substance P and CGRP [26, 27] and to inhibit the migration of DC [28].

In our previous study, DC were found to be localized in the jugular-nodose ganglion complex (JNC) under physiologic conditions, and their number was significantly increased during allergic airway inflammation by migration from outside into the ganglia [4]. However, the impact of FP on neuropeptide expression and on the migration of DC in airway sensory ganglia has been not explored so far.

The present study, therefore, aimed to investigate the anti-inflammatory effects of steroid treatment on the presence and the distribution of DC and CGRP-immunoreactive neurons in vagal sensory jugular-nodose ganglia under allergic airway inflammation.

Materials and Methods

Animals

Female wild-type BALB/c mice (6–8 weeks old) were purchased from Charles River. The animals were held in regular 12-hour dark/light cycles at a room temperature of 22°C and received laboratory food and tap water ad libitum. The animals were acclimatized for at least 2 weeks prior to the study. All animal experiments were performed in strict concordance with the German animal protection law and were approved by the appropriate governmental authority (No. 10/0207).

House Dust Mite Mouse Model

The application of house dust mites (HDM) to induce chronic allergic airway inflammation has been reported previously [4]. Shortly, BALB/c mice (n = 6) were exposed consecutively 5 days a week for a total of 7 weeks to intranasal (i.n.) instillation of HDM extract (catalog No. XPB82D3A2.5; Greer Inc.) at a dose of 25 µg protein in 50 µl saline. The control groups (n = 6) were treated i.n. with 50 µl saline. Analyses were performed 24 h after the last allergen challenge (fig. 1).

Steroid Treatment

To assess steroid efficacy in HDM-treated animals, one group (n = 6) was treated i.n. with FP at 0.3 mg/kg of body weight in a volume of 50 µl 1 h before HDM application daily during the final 9 days before necropsy. The animals in the nontreated groups received 50 µl of vehicle i.n. instead. The body weights of the steroid-treated animals were monitored daily.

Table 1. Antibodies for immunofluorescence staining

Antibody	Source	Dilution
Primary purified antibodies		
Rabbit polyclonal antibody against mouse, human PGP9.5	Abcam, Cambridge, UK	1:200
Rat monoclonal antibody against mouse I-A/I-E	BioLegend, San Diego, Calif., USA	1:200
Goat polyclonal antibody against mouse CGRP	Acris, Herford, Germany	1:400
Isotype control purified antibodies		
Rabbit IgG	Dianova, Hamburg, Germany	1:5,000
Rat IgG 2b kappa	BioLegend	1:200
Goat IgG	R&D Systems	1:300
Secondary antibodies		
DyLight 488-conjugated donkey anti-rabbit IgG antibody	Jackson ImmunoResearch Inc., Baltimore, Md., USA	1:400
Cy3-conjugated donkey anti-rat IgG antibody	Jackson ImmunoResearch	1:500
Cy3-conjugated donkey anti-rabbit IgG antibody	Jackson ImmunoResearch	1:400
DyLight 488-conjugated donkey anti-goat IgG antibody	Jackson ImmunoResearch	1:400
DyLight 649-conjugated donkey anti-rabbit IgG antibody	Jackson ImmunoResearch	1:400

Ganglion Preparation

JNC ganglia were fixed immediately in Zamboni solution (Morphisto Evolutionsforschung und Anwendung GmbH, Frankfurt am Main, Germany) for 6 h, rinsed overnight in 0.1 M phosphate-buffered saline (PBS), and cryoprotected for 24 h with 30% sucrose in 0.1 M PBS. All of the above described steps were performed at 4°C. The ganglia were embedded in optimal cutting temperature medium and frozen in liquid nitrogen. Serial 8-µm sections of the ganglia were prepared using a cryostat (Leica CM 1900; Bensheim, Germany), placed on APES (3-aminopropyltrieth-oxysilane)-coated glass slides, dried at room temperature for 30 min, and then stored in the freezer at –80°C.

Immunofluorescence Staining

The immunofluorescence staining has been described previously [4]. Briefly, every fourth section from serial sections of JNC was dried at room temperature for 15 min and then rehydrated for 5 min in PBS. To reduce nonspecific antibody binding, the sections were incubated for 15 min at room temperature in 5% normal serum of the host species of the secondary antibody diluted in PBS. The sections were incubated with primary antibodies against PGP9.5, MHC II, CGRP, or the appropriate isotype control antibodies (table 1) for 1 h at room temperature and then overnight at 4°C. After rinsing with 0.1 M PBS twice, the sections were incubated with secondary fluorescein-conjugated antibodies (table 1) for 2 h at room temperature; for counterstaining, the sections were then incubated with 100 µl DAPI (0.5 µg/ml; Carl Roth, Germany) for 15 min at room temperature. Finally the sections were washed twice with 0.1 M PBS and once with double distilled water, mounted with the fluorescent mounting medium Prolong Gold (Invitrogen), and covered with coverslips.

For quantitative analysis of MHC II-immunoreactive cells, CGRP-immunoreactive neurons, and PGP9.5-immunoreactive neurons, 16 serial ganglion sections per mouse were stained and visualized with an epifluorescence microscope (Axioskop 2 Plus; Carl Zeiss, Jena, Germany), and cell counting was performed using the software ImageJ (National Institutes of Health). The evaluations of MHC II-immunoreactive cells and CGRP-immunoreactive neurons were normalized by the total neuron number and expressed as percentages.

Confocal images were acquired by LSM 510 META (Carl Zeiss). The Images were processed using Imaris 4.5.2 (Bitplane, Zurich, Switzerland).

Lung Histology

Cryosections of optimal cutting temperature medium-embedded lungs (10 µm) were prepared using a cryostat (Leica CM 1900) and then fixed in cold acetone for 10 min. Lung cryosections were taken for assessment of airway inflammation by using hematoxylin and eosin (H&E) staining and periodic acid Schiff (PAS) staining.

Statistical Analysis

Data are given as means ± SEM. The data were analyzed using GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, Calif., USA). Comparisons between groups were analyzed by ANOVA with Bonferroni adjustment. p values <0.05 were considered statistically significant.

Results

Allergic Airway Inflammation after HDM and Steroid Treatment

Histological analysis with H&E and PAS staining of the lung tissues showed massive infiltration of mononuclear cells (fig. 2, H&E) and a high level of mucus secretion (fig. 2, PAS) in HDM- and HDM+FP-treated mice. In contrast, the lung sections of control mice did not con-

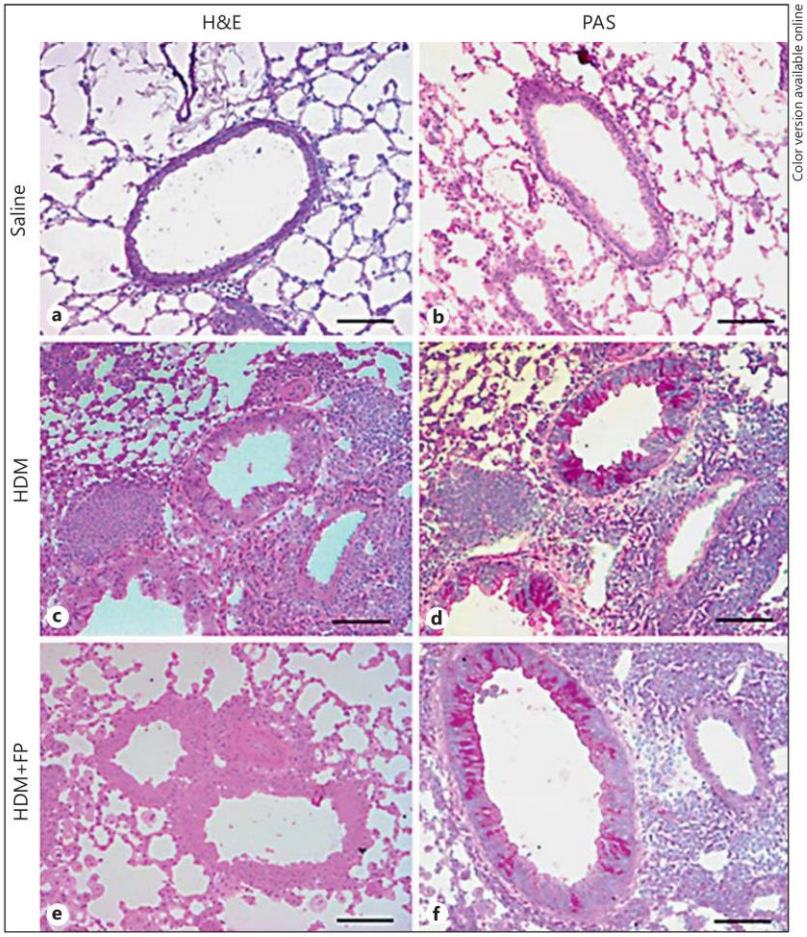


Fig. 2. Effects of chronic HDM and steroid treatments in the lung. Photomicrographs of H&E- (left) and PAS-stained (right) lung sections. The lung sections of control mice (**a**, **b**) did not contain aggregated inflammatory cells or mucus secretion, whereas lung sections from HDM (**c**, **d**) and HDM+FP (**e**, **f**) mice showed infiltration of mononuclear immune cells and a highly mucus secretion in the airways. Scale bars = 100 μm.

tain inflammatory cell aggregates (fig. 2a) and no mucus secretion was detected (fig. 2b). Chronic allergic airway inflammation induced by HDM treatment was indicated by the recruitment of eosinophils, neutrophils, lymphocytes, and macrophages to the lung and the increased lung resistance in response to methacholine. Steroid treatment reduced the number of eosinophils in BAL and the AHR as previously reported [29].

Steroid Treatment Did Not Alter the DC in the JNC

To identify sensory neurons and DC in the JNC, the ganglia sections were analyzed with immunofluorescence for expression of the pan-neuronal marker PGP9.5 and the DC marker MHC II. Under physiological and allergic airway conditions, DC have been found to be widely distributed over the whole JNC, and they were located between the neurons and nerve fibers (fig. 3).

We wished to determine how the number of DC in the JNC changed during allergic airway inflammation and steroid treatment. The analysis of immunofluorescence staining showed that the number of DC in the JNC was significantly increased in HDM-treated animals (DC/neurons: HDM 44.58 ± 1.6%, n = 6 vs. saline 33.29 ± 1.6%, n = 6, $p < 0.05$; fig. 3j).

Steroid treatment had no effect on the migration of DC into the JNC in either group of HDM-treated mice (DC/neurons: HDM 44.58 ± 1.6%, n = 6 vs. HDM+FP 43.69 ± 3.0%, n = 6, $p > 0.05$; fig. 3j).

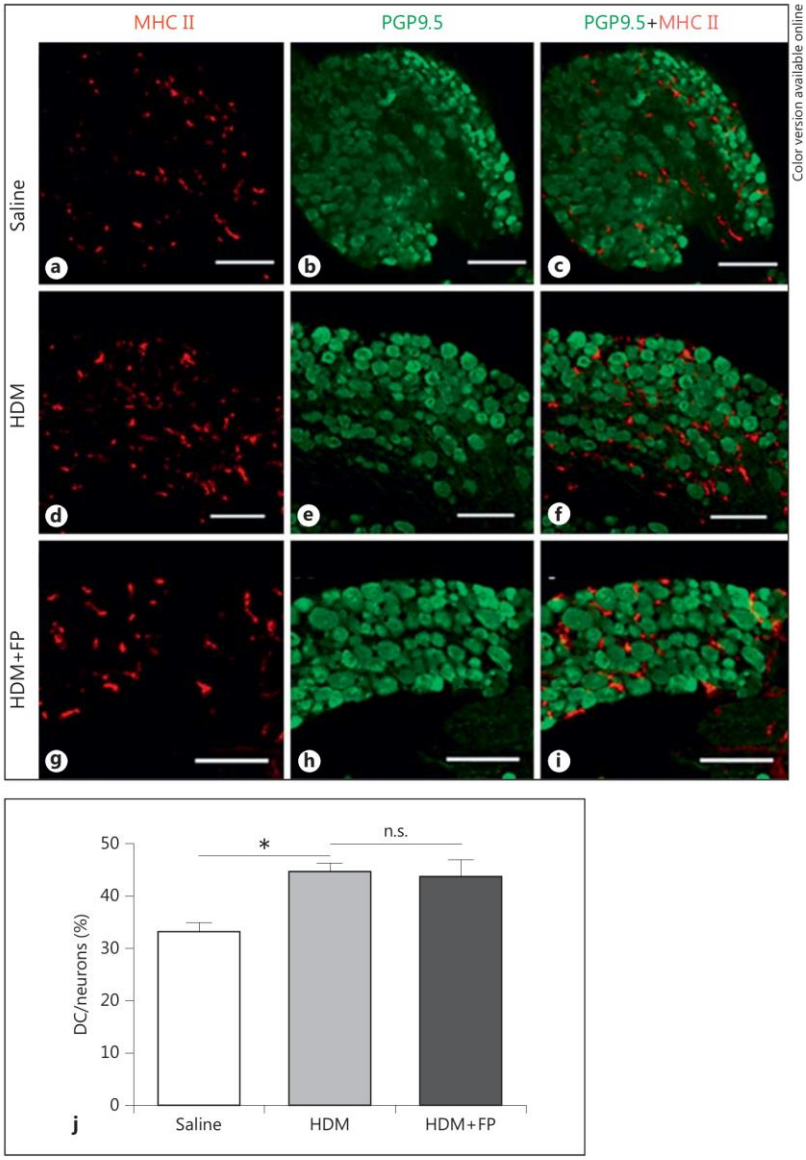


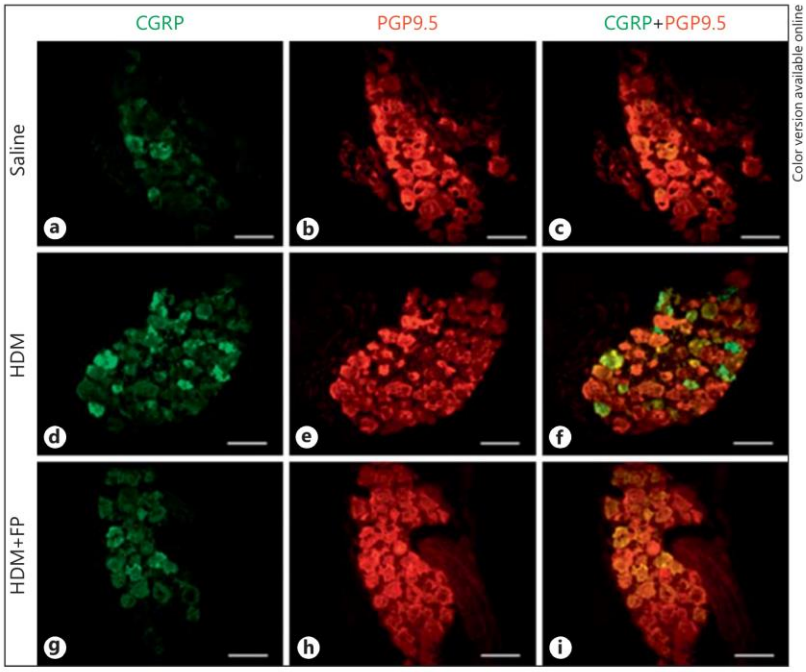
Fig. 3. Quantification of DC in the JNC. Sixteen ganglion sections per mouse (n = 6) were stained with MHC II antibody for DC and PGP9.5 for neurons, and the images were acquired using an epifluorescence microscope (Axioskop 2 Plus). The DC (MHC II), neurons (PGP9.5), and merged picture (PGP9.5+MHC II) of the JNC of the control (**a–c**), HDM- (**d–f**), HDM+FP-treated (**g–i**) groups are shown. **j** Quantification of DC in the JNC of all groups. Results are expressed as percentages of MHC II-positive cells in relation to PGP9.5-positive neurons. Data are given as means ± SEM. n.s. = Not significant. * $p \leq 0.05$. Scale bars = 100 μm.

CGRP-Expressing Neurons Were Not Affected by Steroid Treatment

Similar to our previous findings, the percentage of CGRP ir-neurons in relation to JNC-overall neurons was also found to be significantly increased by HDM-induced allergic airway inflammation (CGRP-positive neurons/total neurons: HDM 30.65 ± 1.9, n = 6% vs. saline 19.49 ± 2.3%, n = 6, $p < 0.05$; fig. 4j).

Steroid treatment did not have any effect on the number of CGRP-expressing neurons in the JNC (CGRP-positive neurons/total neurons: HDM 30.65 ± 1.9, n = 6% vs. HDM+FP 27.35 ± 2.5%, n = 6, $p > 0.05$; fig. 4j).

The distribution of DC in correlation with CGRP-expressing neurons in the JNC during allergic airway inflammation and after steroid treatment was observed. DC were found to be distributed overall on the JNC



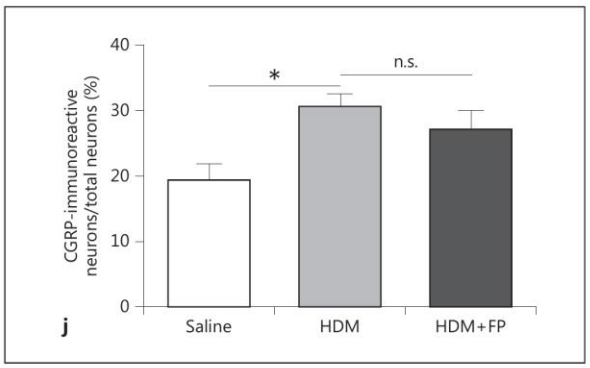


Fig. 4. Quantitative analysis of CGRP-expressing neurons in the JNC. Sixteen sections per mouse (n = 6) were stained with antibodies against the neuropeptides CGRP and PGP9.5, and images were acquired with an Axioskop 2 Plus epifluorescence microscope using a ×40 objective. CGRP-expressing neurons in the JNC are shown for the control group (**a–c**), the HDM-treated group (**d–f**), and HDM+FP-treated mice (**g–i**). **j** Quantification of CGRP-expressing neurons in the JNC of all groups. Results are expressed as the percentage of CGRP-immunoreactive neurons in relation to all PGP9.5-positive neurons. Data are given as means ± SEM. n.s. = Not significant. * $p \leq 0.05$. Scale bars = 50 μm.

slices of all animals groups without any preferential localization to CGRP-positive neurons in all groups (fig. 5).

Discussion

DC represent a phenotypically heterogeneous malleable population with an important biological function in many immune responses such as allergic airway inflammation. There is increasing evidence showing the interactions of DC with airway nerves under allergic conditions. Previous studies have shown that tachykinins and CGRP may have the capacity to regulate the migration of immune cells such as DC [16, 19, 20]. Recently, an increase in the number of DC and CGRP-expressing neurons in the JNC caused by HDM-induced allergic airway inflammation was found. These findings suggest that CGRP may contribute to the migration of immune cells such as DC into airway sensory ganglia and to enhance or suppress airway inflammation [4, 30]. In correspondence with our previous results, DC and CGRP-expressing neurons in the JNC were enhanced after treatment with HDM. DC were found to be distributed overall on the

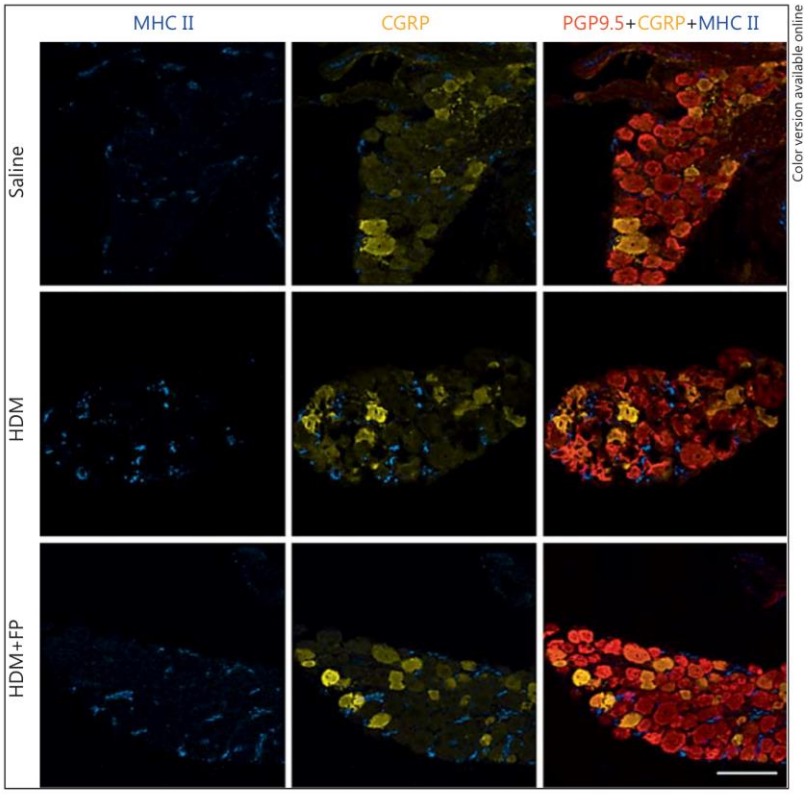


Fig. 5. Distribution of DC in correlation with CGRP-expressing neurons in the JNC. MHC II-positive cells distribute overall on the JNC slices of all animal groups without any preferred localization to CGRP-positive neurons. Images were acquired using a confocal microscope (LSM 510 META). Scale bar = 50 μm.

JNC slices of all animals groups, without any preferential localization to CGRP-positive neurons. Supporting the present findings, neuropeptides such as CGRP have been found to be released from airway nerves to the lung during allergic and infectious airway inflammation [31, 32]. However, the functional role of intraganglionic DC and CGRP in allergic airway inflammation remains poorly understood.

Vagal sensory ganglia have been found not only to convey afferent information from the lung to the central nervous system but also to induce neurogenic inflammation and allergic airway inflammation by releasing numerous neuropeptides from peripheral nerve fibers [9, 11]. Neuropeptides such as CGRP and tachykinins released from airway nerves have the capacity to stimulate the proliferation and recruitment of immune cells to the airways [18–21]. On the other hand, immune cells have been shown to activate airway nerves via cytokines and neurotrophic factors such as neurotrophins (nerve growth factor and brain-derived neurotrophic factor) [30, 33]. CGRP may act as a chemoattractant factor for the migration of DC into airway ganglia, leading to an increase in the intraganglionic DC population. This neuroimmune cross talk could somehow enhance allergic airway inflammation.

Intraganglionic DC in the JNC may also play a key role in the protection of neurons against viral and bacterial infection [34–37]. With respect to immune cells in ganglions, T cells and macrophages have been found in HSV-1-infected trigeminal ganglia [34].

To investigate the effect of steroids on intraganglionic DC and CGRP in sensory ganglia, FP was intranasally delivered on the final 9 days. FP has been commonly used for therapy of bronchial asthma and obstructive pulmonary disease. Treatment with FP has been demonstrated to improve the clinical response in allergic diseases via a decrease in tachykinins [38, 39]. As expected, in the present study, the treatment of FP was found to reduce allergic airway inflammation via a decrease in inflammatory cells like eosinophils in BALF and lung histology and to

reduce AHR [29]. However, FP did not inhibit the migration of DC or alter the number of CGRP-expressing neurons in airway sensory neurons. Although the previous study reported that FP treatment reduces the migration of plasmacytoid DC in the lungs, this effect does not occur with intraganglionic DC, which are also characterized by CD11c+CD11b– [4, 28]. The result indicates that the increase in intraganglionic DC occurs independently of processes inhibited by steroids. This finding is unexpected und surprising as systemic steroids have been established for the treatment of inflammation in neurological diseases. The blood-brain barrier seems to be no obstacle for systemic steroids. In contrast to the central nervous system, vagal sensory ganglia are enclosed by a capsule, so this may be one of the reasons for the survival and persistence of viruses like the herpesvirus in trigeminal ganglia. Otherwise, steroid treatment may not have an effect on airway nerves in terms of neuropeptide biosynthesis and release.

With respect to the steroid effect, the migration of intraganglionic DC could be effected by long-term treatment with FP and not short-term treatment within 9 days. The present findings demonstrate that the effects of FP on functions of intraganglionic DC remain unknown.

Unilateral vagotomy of allergic sensitized and challenged mice may help to investigate whether the increase in intraganglionic DC requires intact axons of the vagus nerve and if this is caused by a neurotrophic effect or a systemic response. However, vagotomy can lead to neuronal degeneration with alteration of neuropeptide biosynthesis and release and therefore may not reflect the real-life situation in mice. In this regard, further experiments need to be carried out in the future.

In conclusion, the present results demonstrated that the allergic airway inflammation induced by HDM led to an increase in the numbers of DC and CGRP-expressing neurons in sensory ganglia and that these numbers were affected by steroid treatment. These findings indicate that short-term steroid treatment did not have effects on migration of DC into the JNC, whereas steroid treatment was found to suppress allergic airway inflammation.

Acknowledgments

This project was supported by the GRK 1441 Research Training Group (Prof. T. Welte and Prof. Q.T. Dinh), by the German Research Foundation (DFG, DI 1429/2-1) and the Stiftung Bergmannshilfswerks Luisenthal foundation (Prof. Q.T. Dinh), and by SFB587 B4 and IMI UBIOPRED (Prof. A. Braun).

References

1 Barnes PJ: Pathophysiology of allergic inflammation. Immunol Rev 2011;242:31–50.
2 Gill MA: The role of dendritic cells in asthma. J Allergy Clin Immunol 2012;129:889–901.
3 Lambrecht BN: Dendritic cells and the regulation of the allergic immune response. Allergy 2005;60:271–282.
4 Le DD, Rochlitzer S, Fischer A, Heck S, Tschernig T, Sester M, Bals R, Welte T, Braun A, Dinh QT: Allergic airway inflammation induces the migration of dendritic cells into airway sensory ganglia. Respir Res 2014;15:73.
5 Novak N, Haberstok J, Geiger E, Bieber T: Dendritic cells in allergy. Allergy 1999;54:792–803.
6 Lipscomb MF, Masten BJ: Dendritic cells: immune regulators in health and disease. Physiol Rev 2002;82:97–130.
7 Manicassamy S, Pulendran B: Dendritic cell control of tolerogenic responses. Immunol Rev 2011;241:206–227.
8 Dinh QT, Cryer A, Dinh S, Trevisani M, Georgiewa P, Chung F, Geppetti P, Heppt W, Klapp BF, Fischer A: Protease-activated receptor 2 expression in trigeminal neurons innervating the rat nasal mucosa. Neuropeptides 2005;39:461–466.
9 Rochlitzer S, Veres TZ, Kuhne K, Prenzler F, Pilzner C, Knothe S, Winkler C, Lauenstein HD, Willart M, Hammad H, Muller M, Krug N, Lambrecht BN, Braun A: The neuropeptide calcitonin gene-related peptide affects allergic airway inflammation by modulating dendritic cell function. Clin Exp Allergy 2011;41:1609–1621.
10 Piedimonte G: Contribution of neuroimmune mechanisms to airway inflammation and remodeling during and after respiratory syncytial virus infection. Pediatr Infect Dis J 2003;22:S66–S74.
11 Barnes PJ: Neurogenic inflammation in the airways. Respir Physiol 2001;125:145–154.
12 Dinh QT, Mingomataj E, Quarcoo D, Groneberg DA, Witt C, Klapp BF, Braun A, Fischer A: Allergic airway inflammation induces tachykinin peptides expression in vagal sensory neurons innervating mouse airways. Clin Exp Allergy 2005;35:820–825.
13 Martling CR, Matran R, Alving K, Hokfelt T, Lundberg JM: Innervation of lower airways and neuropeptide effects on bronchial and vascular tone in the pig. Cell Tissue Res 1990;260:223–233.
14 Groneberg DA, Quarcoo D, Frossard N, Fischer A: Neurogenic mechanisms in bronchial inflammatory diseases. Allergy 2004;59:1139–1152.
15 Fischer A, McGregor GP, Saria A, Philippin B, Kummer W: Induction of tachykinin gene and peptide expression in guinea pig nodose primary afferent neurons by allergic airway inflammation. J Clin Invest 1996;98:2284–2291.
16 Foster CA, Mandak B, Kromer E, Rot A: Calcitonin gene-related peptide is chemotactic for human T lymphocytes. Ann NY Acad Sci 1992;657:397–404.
17 Lambrecht BN: Immunologists getting nervous: neuropeptides, dendritic cells and T cell activation. Respir Res 2001;2:133–138.
18 Manley HC, Haynes LW: Eosinophil chemotactic response to rat CGRP-1 is increased after exposure to trypsin or guinea pig lung particulate fraction. Neuropeptides 1989;13:29–34.
19 Davies D, Medeiros MS, Keen J, Turner AJ, Haynes LW: Eosinophil chemotactic peptide sequences in rat alpha-CGRP: activation of a novel trophic action by neutral endopeptidase 24.11. Ann NY Acad Sci 1992;657:405–411.

20 Dunzendorfer S, Kaser A, Meierhofer C, Tilg H, Wiedermann CJ: Cutting edge: peripheral neuropeptides attract immature and arrest mature blood-derived dendritic cells. J Immunol 2001;166:2167–2172.
21 Kawanami Y, Morimoto Y, Kim H, Nakamura T, Machida K, Kido T, Asonuma E, Yatera K, Yoshii C, Kido M: Calcitonin gene-related peptide stimulates proliferation of alveolar epithelial cells. Respir Res 2009;10:8.
22 Davies D, Spicer BA, Smith H, Haynes LW: Effect of neonatal capsaicin on peptide-containing primary afferent fibres, eosinophil distribution and hyperresponsiveness in rat lung tissue following experimentally induced eosinophilia. Neuroimmunomodulation 1994;1:308–314.
23 Kawamura N, Tamura H, Obana S, Wenner M, Ishikawa T, Nakata A, Yamamoto H: Differential effects of neuropeptides on cytokine production by mouse helper T cell subsets. Neuroimmunomodulation 1998;5:9–15.
24 Palmer JB, Cuss FM, Mulderry PK, Ghatei MA, Springall DR, Cadieux A, Bloom SR, Polak JM, Barnes PJ: Calcitonin gene-related peptide is localised to human airway nerves and potently constricts human airway smooth muscle. Br J Pharmacol 1987;91:95–101.
25 DeWitt BJ, Cheng DY, Caminiti GN, Nossaman BD, Coy DH, Murphy WA, Kadowitz PJ: Comparison of responses to adrenomedullin and calcitonin gene-related peptide in the pulmonary vascular bed of the cat. Eur J Pharmacol 1994;257:303–306.
26 Schaper C, Gustavus B, Koch B, Ewert R, Felix SB, Kunkel G, Noga O, Glaser S: Effect of fluticasone on neuropeptides in nasal lavage in persistent allergic rhinitis. J Investig Allergol Clin Immunol 2010;20:214–221.
27 Han SH, An HJ, Song JY, Shin DE, Kwon YD, Shim JS, Lee SC: Effects of corticosteroid on the expressions of neuropeptide and cytokine mRNA and on tenocyte viability in lateral epicondylitis. J Inflamm (Lond) 2012;9:40.
28 Lommatzsch M, Kraeft U, Troebs L, Garbe K, Bier A, Stoll P, Klammt S, Kuepper M, Bratke K, Virchow JC: Fluticasone impact on airway dendritic cells in smokers: a randomized controlled trial. Respir Res 2013;14:114.
29 Rochlitzer S, Hoymann HG, Muller M, Braun A: No exacerbation but impaired anti-viral mechanisms in a rhinovirus-chronic allergic asthma mouse model. Clin Sci (Lond) 2014;126:55–65.
30 Veres TZ, Rochlitzer S, Shevchenko M, Fuchs B, Prenzler F, Nassenstein C, Fischer A, Welker L, Holz O, Muller M, Krug N, Braun A: Spatial interactions between dendritic cells and sensory nerves in allergic airway inflammation. Am J Respir Cell Mol Biol 2007;37:553–561.
31 Dakhama A, Kanehiro A, Makela MJ, Loader JE, Larsen GL, Gelfand EW: Regulation of airway hyperresponsiveness by calcitonin gene-related peptide in allergen sensitized and challenged mice. Am J Respir Crit Care Med 2002;165:1137–1144.
32 Tan Y, Yang T, Liu S, Liu H, Xiang Y, Qu F, Li H, Qin X: Infection with respiratory syncytial virus alters peptidergic innervation in the lower airways of guinea pigs. Exp Physiol 2008;93:1284–1291.
33 Braun A, Lommatzsch M, Mannsfeldt A, Neuhaus-Steinmetz U, Fischer A, Schnoy N, Lewin GR, Renz H: Cellular sources of enhanced brain-derived neurotrophic factor production in a mouse model of allergic inflammation. Am J Respir Cell Mol Biol 1999;21:537–546.
34 Theil D, Derfuss T, Paripovic I, Herberger S, Meinl E, Schueler O, Strupp M, Arbusow V, Brandt T: Latent herpesvirus infection in human trigeminal ganglia causes chronic immune response. Am J Pathol 2003;163:2179–2184.
35 Tsiang H, Ceccaldi PE, Lycke E: Rabies virus infection and transport in human sensory dorsal root ganglia neurons. J Gen Virol 1991;72:1191–1194.
36 Li XQ, Fu ZF, Alvarez R, Henderson C, Tripp RA: Respiratory syncytial virus (RSV) infects neuronal cells and processes that innervate the lung by a process involving RSV G protein. J Virol 2006;80:537–540.
37 Antinone SE, Smith GA: Retrograde axon transport of herpes simplex virus and pseudorabies virus: a live-cell comparative analysis. J Virol 2010;84:1504–1512.
38 Schaper C, Gustavus B, Koch B, Ewert R, Felix SB, Kunkel G, Noga O, Glaser S: Effect of fluticasone on neuropeptides in nasal lavage in persistent allergic rhinitis. J Investig Allergol Clin Immunol 2010;20:214–221.
39 Callebaut I, Vandewalle E, Hox V, Bobic S, Jorissen M, Stalmans I, De Vries A, Scadding G, Hellings PW: Nasal corticosteroid treatment reduces substance P levels in tear fluid in allergic rhinoconjunctivitis. Ann Allergy Asthma Immunol 2012;109:141–146.

5.4 Paper IV

Title: Allergic airway inflammation induces migration of mast cell populations into the mouse airway

Authors: David Schmit*****, Duc Dung Le*****, Sebastian Heck, Markus Bischoff, Thomas Tschernig, Christian Herr, Christoph Beisswenger, Peter Kobelt, Phillipp Moritz Lepper, Kian Fan Chung, Robert Bals and Quoc Thai Dinh

Cell Tissue Res (2017). doi:10.1007/s00441-017-2597-9

* These authors contributed equally to the study

Cell Tissue Res
DOI 10.1007/s00441-017-2597-9

REGULAR ARTICLE

Allergic airway inflammation induces migration of mast cell populations into the mouse airway

David Schmit[1] · Duc Dung Le[1] · Sebastian Heck[1] · Markus Bischoff[2] · Thomas Tschernig[3] · Christian Herr[4] · Christoph Beisswenger[4] · Peter Kobelt[5] · Phillipp Moritz Lepper[4] · Kian Fan Chung[6] · Robert Bals[4] · Quoc Thai Dinh[1,4]

Received: 6 June 2016 / Accepted: 23 February 2017

Abstract Mast cells (MCs) and airway nerves play an important role in allergic asthma. However, little is known about the MCs and their interaction with airway nerves during allergic airway inflammation. This study aims to investigate the distribution and proliferation of MC populations in different lung compartments, along with the association of mast cells with nerve endings, using a house dust mite (HDM) model for allergic airway inflammation. BALB/c mice were exposed to HDM extract intranasally (25 μg/50 μl) for 5 consecutive days a week over 7 weeks. Immunofluorescence and Edu stains were used to examine the colocalisation of MCs and nerves and the proliferation of MCs, respectively. HDM treatment caused an increased migration of MCs into bronchi, alveolar parenchyma and airway vessels. The proportions of tryptase-chymase expressing MC (MC_{TC}) increased significantly in the bronchi and the alveolar parenchyma but not in the vascular tissues, by allergic airway inflammation. The association of MCs with nerves was found only in the bronchi and there were no changes in comparison of controls to HDM-treated animals. The present study shows a strong migration of tryptase expressing MC (MC_T) and MC_{TC} into the bronchi and the alveolar parenchyma, as well as of MC_T in the vascular compartment under HDM treatment. This supports the hypothesis that these mast cell populations may contribute to allergic airway inflammation.

Keywords Mast cells · Allergic airway inflammation · Tryptase · Chymase · Neuroimmune InteractionDavid Schmit and Duc Dung Le contributed equally to this work.

Introduction

Throughout recent decades, the prevalence of bronchiale asthma has increased dramatically and especially in industrialized countries (Braman 2006). About 300 million people worldwide are suffering from bronchial asthma. Asthma is characterized by symptoms such as increased airway hyperresponsiveness, bronchial obstruction, secretion of mucus, chronic airway inflammation and airway remodeling. It is now considered as a chronic complex disease consisting in different phenotypes (Wenzel 2006, 2012). As the immunology of the mouse has been well characterized and genetically modified or altered mice are available, the mouse is frequently used as model for allergic airway inflammation and may help to reveal this heterogenity of the disease.

Mast cells play a key role in allergic dieseases by releasing amounts of biologically active molecules from their secretory granules. These include histamine, prostaglandins (PGs), leukotrienes (LTs), nerve growth factor (NGF) and various MC-specific proteases, such as tryptase and chymase (Galli

David Schmit and Duc Dung Le contributed equally to this work.

✉ Quoc Thai Dinh
Thai.Dinh@uniklinikum-saarland.de

[1] Department of Experimental Pneumology and Allergology, Saarland University Hospital and Saarland University Faculty of Medicine, Kirrberger Strasse, Geb. 61.4, 66421 Homburg/Saar, Germany

[2] Institute of Medical Microbiology and Hygiene, Saarland University Hospital, Homburg, Germany

[3] Institute of Anatomy and Cell Biology, Saarland University Hospital, Homburg, Germany

[4] Department of Internal Medicine V, Saarland University Hospital, Homburg, Germany

[5] Center for Internal Medicine and Dermatology, Division of General Internal and Psychosomatic Medicine, Charité – Universitätsmedizin, Berlin, Germany

[6] Thoracic Medicine, National Heart & Lung Institute, Imperial College, London, UK

Published online: 25 March 2017

et al. 2005; Leon et al. 1994; Brown et al. 2008; Wernersson and Pejler 2014; Xiang and Nilsson 2000). In humans, only one chymase has been identified and the mast cells are classified into two subpopulations: MC_T containing only tryptase and MC_{TC} that contains chymase in addition to tryptase (Pawankar and Ra 1996; Bradding and Arthur 2016). In contrast, rodents have been shown to express a number of chymases such as mast cell protease (mMCP)-1, mMCP-2, mMCP-4 and mMCP-5 with distinct proteolytic properties. With respect to their biological effects, mMCP-4 expressed by mouse MCs have been suggested to be likely the functional homologue of human chymase (Tchougounova et al. 2003). In the airway, MC populations are not homogenous and each lung compartment contains its own specific sub-group (Andersson et al. 2009). Thus, these cells differ remarkably in their morphology, mediator content and histochemical characteristics as well as their response to external stimuli (Kitamura 1989; Oskeritzian et al. 2005). Although the important role of MCs in allergic asthma is undoubted, their precise function in asthma remains controversial. The reports about MCs in the airway in recent years have renewed the interest in the role of MCs in allergic asthma (Robinson 2004; Cyphert et al. 2011). MCs have been found to be increased within the airway smooth muscle in asthmatic patients (Brightling et al. 2002).

Activation of MCs leads to the release of different mediators, which can affect various cell types, such as immune cells, epithelium, endothelium and airway smooth muscle in different compartments of the airway (Hatanaka et al. 1986; Carter and Bradding 2011; Robinson 2004). Mast cell mediators can also influence neuronal activity as well as the release of neuropeptides (Weigand et al. 2009; Forsythe and Bienenstock 2012). Reciprocally, MCs can be activated by various stimuli, including neuropeptides (Persson et al. 1998; Busse and Lemanske 2001). MCs were found to be associated with nerve fibers in animal and human tissue under physiological and pathophysiological conditions (Jarvikallio et al. 2003; Alving et al. 1991; Stead et al. 1987). Human airways are innervated by numerous cholinergic, adrenergic and non-adrenergic non-cholinergic neurons that may contribute to inflammatory processes (Barnes 1986, 1992; Dinh et al. 2004a, b). The potential role of MCs is well documented and includes their interaction with neurokinins, neurotrophins, and tachykinins (van Houwelingen et al. 2002; Groneberg et al. 2007; Dinh et al. 2005). Additionally, MCs have been suggested to play a particular role in interaction with the nervous system in chemical- and irritant-induced asthma (Hox et al. 2013; Devos et al. 2016).

This study aims to investigate the distribution, proliferation and proportion of MC subpopulations in different lung compartments (bronchi, alveolar parenchyma and vessels) by using a HDM mouse model of allergic airway inflammation. Futhermore, the mast cell–nerve contacts in the airways are studied in different lung compartments.

Methods

Animals

Female wild-type BALB/c-mice (6–8 weeks old; Janvier Labs, France) were held in regular 12-h dark/light cycles at a temperature of 22 °C and received laboratory food and tap water ad libitum. The animals were adapted to the new environment for at least 2 weeks prior to the study. All animal experiments were performed in strict concordance with the German animal protection law and approved by the authorities of Saarland (No. 14/2013).

The HDM-mouse model for allergic airway inflammation

Chronic allergic airway inflammation was induced by exposing 8 mice on 5 consecutive days a week over a total period of 7 weeks to HDM extract intranasally (Greer). A dose of 25 μg protein in 50 μl saline was used. A second group of animals ($n = 8$) served as control and was treated via the same route with 50 μl saline. Analyses were performed 24 h after the last allergen challenge (Fig. 1).

In vivo proliferation study with EdU (5-ethynyl-2′-deoxyuridine)

EdU is a nucleoside analog of thymidine and is incorporated into DNA during active DNA synthesis. The animals received an i.p. injection of 1 mg EdU (Invitrogen) in a volume of 200 μl 24 h before sacrifice.

Bronchoalveolar lavage fluid (BALF)

To perform bronchoalveolar lavage, 0.8 ml ice-cold PBS was installed twice. The total cell count in BALF was determined. The BALF was centrifuged (320*g*, 10 min, 4 °C) and the supernatants were removed. Cytospots were prepared and stained according to Diff Quick (Medion Diagnostics) and differential cell counts were evaluated.

Histological stains

Zambonie fixed lung cryosections (8 μm) were made using a cryostat (CM1950; Leica Cryostat, Germany). Airway inflammation was assessed by staining cryosections with hematoxylin and eosin (H&E) and Periodic Acid Schiff (PAS) according to standard protocols. The measurement of airway epithelium thickness (area measurement) and the length of basement membrane was performed by using software Axio Vision

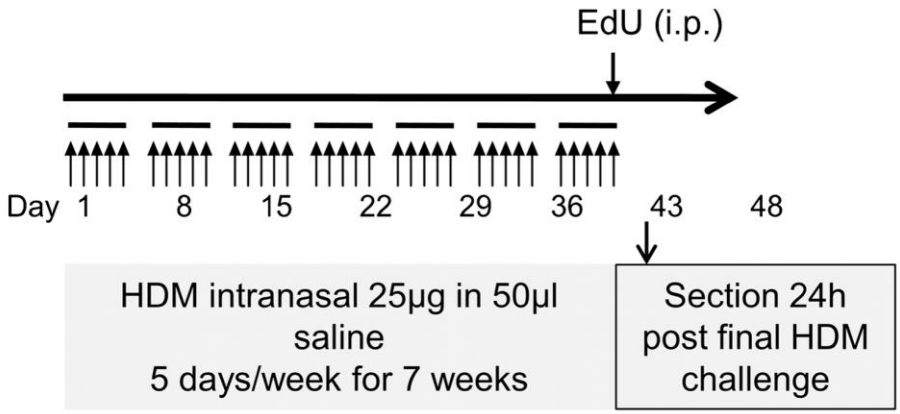


Fig. 1 HDM treatment protocol. The animals were systemically sensitized by intranasal application of house dust mite 5 days a week for 7 weeks. EdU was injected (i.p.) 1 day before analysis

(Carl Zeiss). The thickness of airway epithelium was expressed as mean of area (μm^2) per 1 µm basement membrane. The assessment of airway epithelial goblet cells was expressed as mean of number of goblet cells per 1 mm basement membrane.

Immunofluorescence staining

Immunofluorescence staining has been described previously (Le et al. 2014). Briefly, cryosections of the lungs were dried at room temperature for 15 min and then rehydrated in PBS for 5 min. To reduce nonspecific antibody binding, the sections were incubated for 15 min at room temperature in 5% normal serum of the host species of the secondary antibody diluted in PBS. The sections were incubated with primary antibodies (chicken anti-mouse PGP 9.5, 1:400; Acris Antibodies; rabbit anti-mouse tryptase, 1:500; Abcam; goat anti-mouse mMCP-4, 1:400; Acris Antibodies) or the appropriate isotope control antibodies for 1 h at room temperature and then overnight at 4 °C. After rinsing twice with 0.1 M PBS, the sections were incubated with secondary fluorescein-conjugated antibodies (Jackson ImmunoResearch) (donkey anti-rabbit Cy3, 1:400; donkey anti-chicken Cy2, 1:400; donkey anti-goat, 1:400) for 2 h at room temperature. Finally, the sections were washed twice with 0.1 M PBS, once with double-distilled water, mounted with fluorescent mounting medium Fluoroshield (Sigma Aldrich) and covered with cover slips.

Quantitative analysis of mast cells was visualized with epifluorescence microscopes (Olympus BX5 and Axio Imager M2; Carl Zeiss) and cell counting was performed manually. Sixteen lung slices per animals were analyzed and all quantifications were carried out on blinded sections and controlled by a second investigator. The evaluations of solely tryptase ir-cells (MC_T) and of tryptase ir- and mMCP-4 ir-cells (MC_{TC}) were analyzed in bronchi, alveolar parenchyma and vessels and expressed as densities (cells\mm^2) and as percentages of the total MC numbers ($MC_{TOT} = MC_T + MC_{TC}$). The numbers of MC_T and MC_{TC} with direct contact to PGP 9.5 ir-neurons was expressed as percentages of the specific MC subtype (total MC_T or total MC_{TC}).

EdU staining for proliferating analysis

As previously described, EdU staining was conducted using Click-iT™ Cell Reaction Buffer Kit and Alexa Fluor 594 azide according to the manufacturer's protocol (Invitrogen) (Le et al. 2014). The lung sections were rehydrated for 5 min in PBS and then blocked with 5% normal serum. The sections were incubated with 200 µl prepared Click-iT reaction cocktail for 30 min and then incubated with antibodyrabbit anti-mouse tryptase (1:500; Abcam) and secondary antibody donkey anti-rabbit Cy2 (1:400; Jackson ImmunoResearch) as described above. DAPI was used for counterstaining.

Measurement of serum total IgE and cytokines in BALF

The concentration of total IgE in serum and IL4 and IL13 in the BALF supernatants was assessed by using Mouse IgE Ready-SET-Go, Mouse IL-4 ELISA Ready-SET-Go and Mouse IL-13 ELISA Ready-Set-Go (eBiosciene, San Diego, CA, USA), as per the manufacturer's instructions. The limits of detection were 4 ng/ml for IgE and 4 pg/ml for IL-4 and IL-13.

Statistical analysis

Data are given as mean ± SEM. The Kolmogorov–Smirnov test analyzed the Gaussian distribution. Statistical significance between groups was analyzed with an unpaired *t* test using GraphPad Prism 4.03. P values < 0.05 were considered significant.

Results

HDM treatment induces allergic inflammation in the airways

HDM treatment-induced allergic airway inflammation is characterized by the recruitment of eosinophils, neutrophils, lymphocytes and macrophages into the airways (Fig. 2a, b). HDM-treated mice showed increased levels of total serum IgE (HDM 82,64 ± 13 ng/ml $n = 8$ vs. saline 674.3 ± 62.75 ng/ml $n = 8$, $P < 0.0001$) and of Th2 cytokines IL4 (HDM 22.19 ± 2.32 pg/ml $n = 8$ vs. saline 1.84 ± 1.11 pg/ml $n = 8$, $P < 0.0001$) and IL13 in the BALF (HDM 59.93 pg/ml ± 12.25 vs. saline undetectable, $P < 0.0001$) (Fig. 2c–e)

The histological analysis using H&E and PAS staining of different lung tissues showed massive infiltration of mononuclear cells and a distinct mucus secretion (Fig. 2f', f"') in HDM-treated mice. In contrast, the lung sections of control mice did not contain inflammatory cell aggregates and no mucus secretion was detected (Fig. 2f, f"). Semi-quantitative analysis also showed a significant increase of airway epithelium thickness (HDM 12.85 ± 1.23 $\mu m^2/mm$ $n = 5$ vs. saline 26.66 ± 3.06 $\mu m^2/\mu m$ $n = 5$, $P < 0{,}0031$) and the number of airway epithelial goblet cells (HDM 0.05 ± 0.03 cells/mm $n = 5$ vs. saline 61.37 ± 4.23 cells/mm $n = 5$, $P < 0.0001$) in HDM-treated mice in comparision to controls (Fig. 2g, h).

Mast cells increased significantly during allergic inflammation in most lung compartments

Mast cells were detected by staining antibody against mouse tryptase. The contribution of MC in lung compartments was quantified and expressed as cells per mm^2. Under inflammatory conditions, the highest density of total MCs was found in conducting airways with the lowest concentration in alveolar tissue. Allergic airway inflammation induced a significant increase of MCs in the bronchi (HDM 24.48 ± 2.49 $n = 8$ vs. saline 4.78 ± 0.98 $n = 8$, $P < 0.0001$), alveolar parenchyma (HDM 5.21 ± 0.70 $n = 8$ vs. saline 0.40 ± 0.09 $n = 8$,

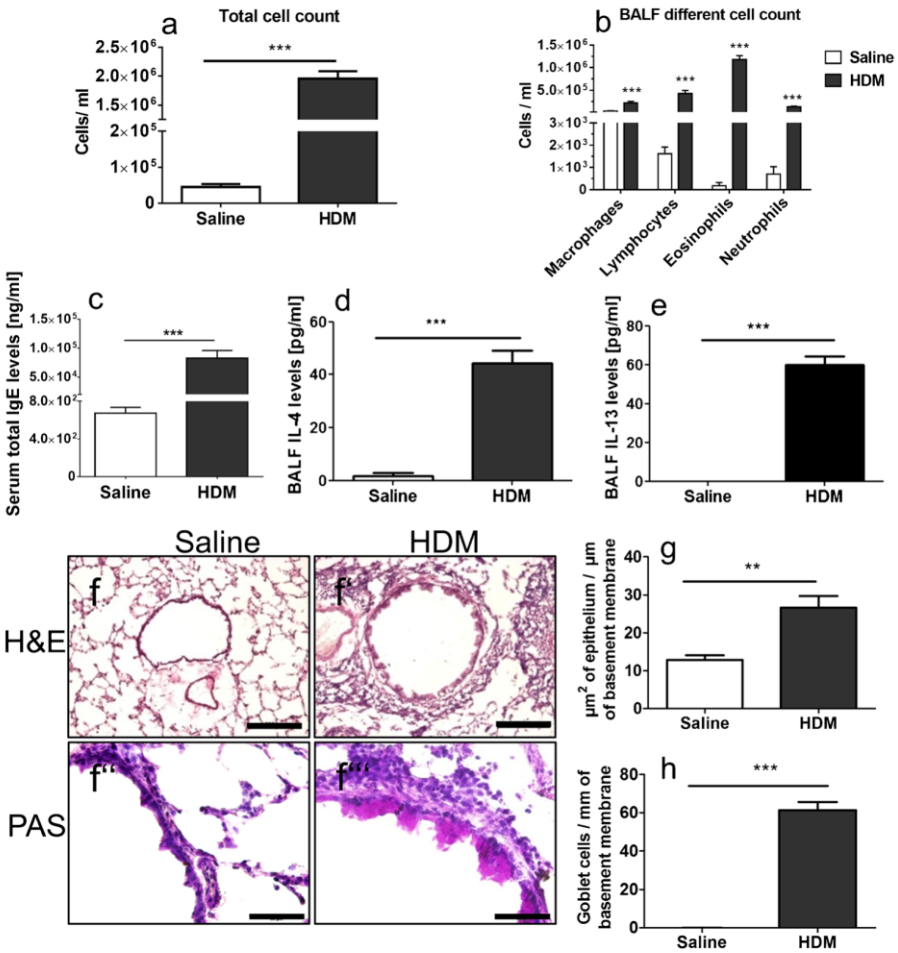


Fig. 2 HDM induces allergic airway inflammation. Bronchoalveolar lavage fluid (BALF) total cell count (**a**) and cell differentiation (**b**). Eosinophils, neutrophils, lymphocytes and macrophages in BALF demonstrated inflammation in the lungs of HDM-exposed mice, in comparison with control mice. Total resum IgE level (**c**) and the cytokine levels of interleukin IL-4 (**d**) and IL-13 (**e**) in the bronchoalveolar lavage (BAL) fluid. **f**–**f"'** Representative photomicrographs of H&E- and PAS-stained lung sections. The lung sections from HDM-sensitised and -challenged mice showed an infiltration of mononuclear cells (*H&E*) and a highly mucus secretion (*PAS*) in the airways, whereas the lungs of the control mice do not contain aggregated inflammatory cells and mucus secretion. Quantification of airway epithelial thickness (**g**) and airway epithelial goblet cells (**h**) as mean per 1 mm of basement membrane. Results are expressed as mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$ (unpaired two-tailed *t* test). *Scale bars* (H&E) 200 µm, (PAS) 50 µm

$P < 0.0001$) and airway vessels (HDM 18.98 ± 2.95 $n = 8$ vs. saline 1.47 ± 0.40 $n = 8$, $P < 0.0001$) (Fig. 3)

HDM treatment induced the migration of MC into the airways

To examine whether MCs proliferated in the lung or migrated from outside into the airways during allergic airway inflammation, the animals were injected with EdU, which was incorporated with the DNA of dividing cells. Systemic administration of EdU 24 h before analysis assured the detection of proliferating cells. The proliferating MCs in two groups were examined (double-positive with EdU and tryptase). The results showed that proliferating MCs were not detected in the lung of controls and HDM treated animals (Fig. 4a, b–b"'). Furthermore, experiments were caried out to examine the survival of MCs during allergic inflammation. Lung tissues were stained with survival marker phosphor Akt1 (Ser473). All mast cells in controls and HDM group were found negative with Akt1 (Fig. 4c, d–d"', e).

Proportional changes of MC populations in allergic airway inflammation

MCs were differentiated using staining against tryptase and mMCP-4 (mouse chymase) followed by immunofluorescence imaging. MC_T were positive only for tryptase, whereas MC_{TC} expressed both enzymes. Under baseline conditions, MC_T were the predominant subtype in all lung compartments with the highest proportion in vascular tissue, whereas MC_{TC} were found only in the bronchus and alveolar lung parenchyma but not in the vessels. The highest fraction of MC_{TC} appeared in the bronchi. The proportion of MC_{TC} in lung compartments was quantified and expressed as a percentage (MC_{TC}/MC_{TOT}). Allergic airway inflammation induced significant increases in the proportions of MC_{TC} in changes in the bronchi (HDM 7.37 ± 0.75%, $n = 8$ vs. saline 2.0 ± 1.30% $n = 8$, $P = 0.0032$) and alveolar parenchyma (HDM 4.00 ± 0.94% $n = 8$ vs. saline 1.00 ± 0.86% $n = 8$, $P = 0.0346$). In vascular tissue, these changes were not detectable (Fig. 5).

MC-nerve interaction in the lung

The pan-neuronal marker PGP 9.5 was used to detect nerves in the conducting airways. Under physiological conditions, a minority of MC_T could be found in the proximity of nerve fibers. MC_{TC} did not form any anatomic link. During lung inflammation, proportional changes of MC_T and MC_{TC} with contact to nerves were not detectable (% MC_T having contact with nerve: HDM 13.75 ± 1.34%, $n = 8$ vs. saline 23.13 ± 5.09% $n = 8$, $P = 0.0969$) (% MC_{TC} having contact with nerve: HDM 5.87 ± 4,24%, $n = 8$, saline were not detectable) (Fig. 6)

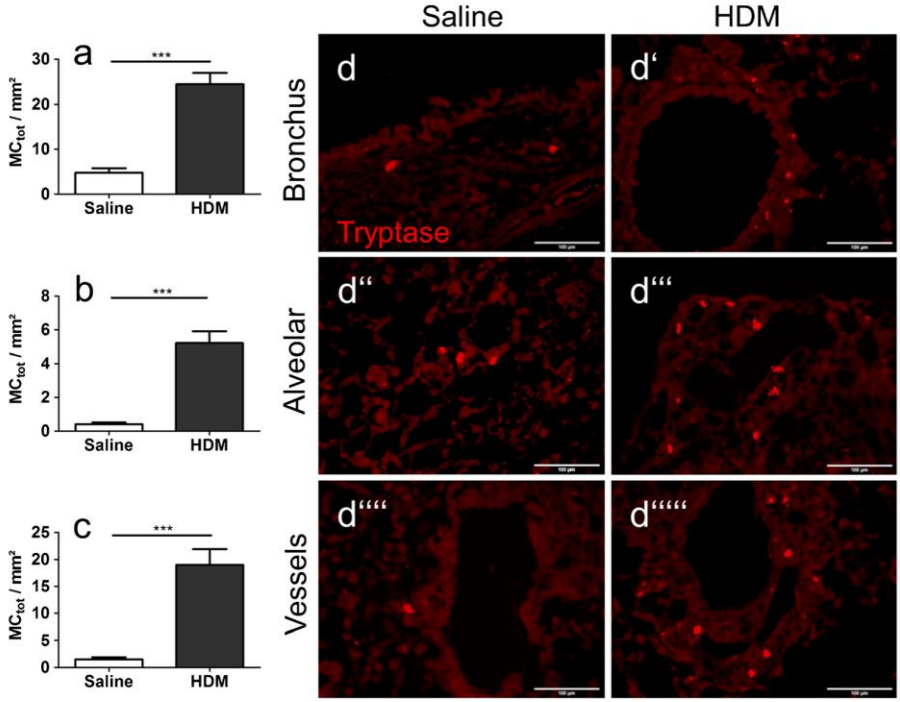


Fig. 3 Contribution of mast cells in the lung. Quantification of mast cells in bronchus (**a**), alveolar (**b**) and vessels. Results are expressed as cells per ml. Microphotograph of mouse lungs showing present of MCs (*red*, stained with antibody against tryptase) in bronchus, alveolar and vessels. Results are expressed as mean ± SEM, ***$P < 0.001$ (unpaired two-tailed *t* test). *Scale bars* 100 μl

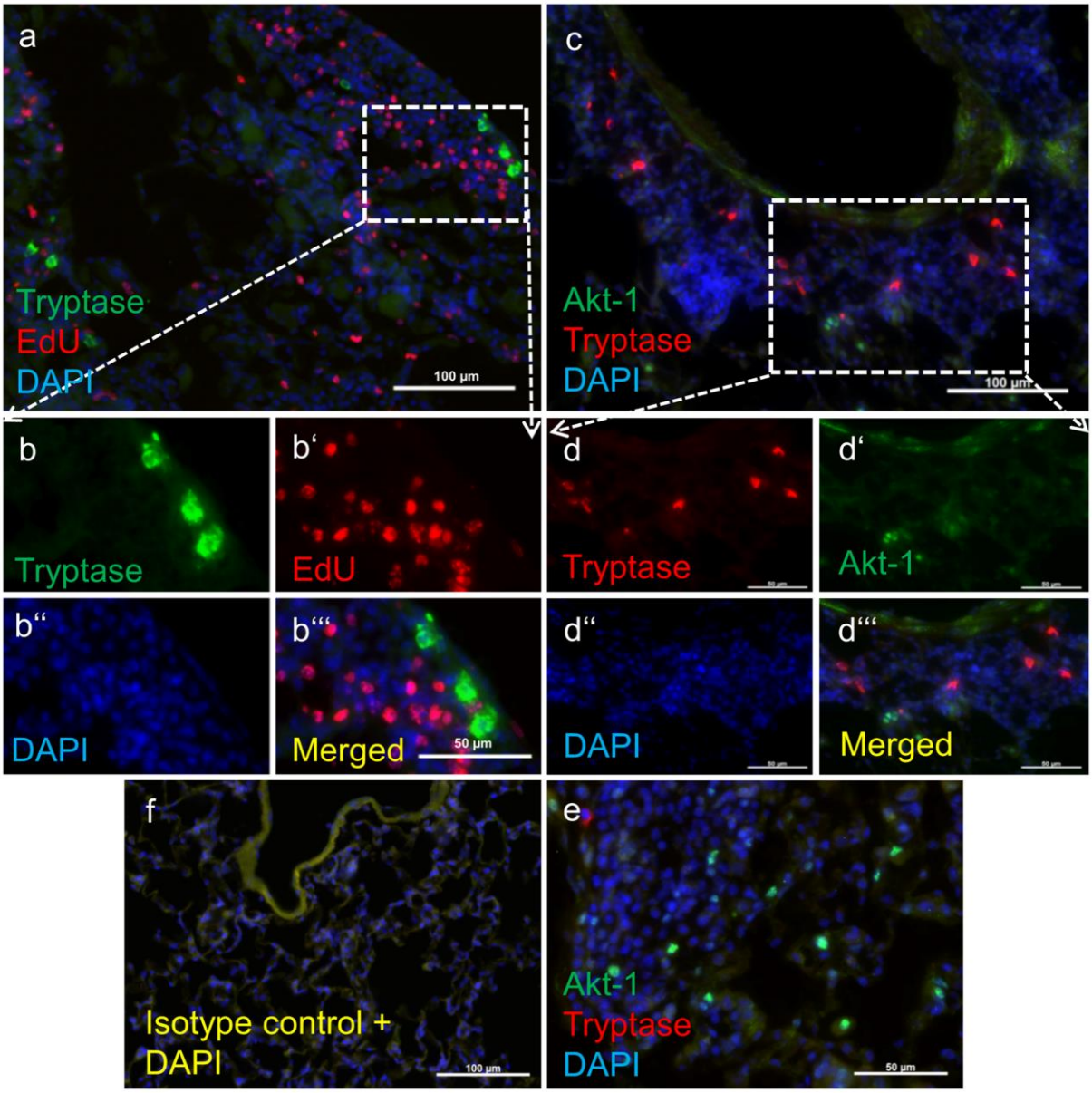


Fig. 4 Proliferating analysis of MCs in the lungs. **a** Fluorescence staining for tryptase (*green*), Edu (*red*) and DAPI (*blue*). No proliferating mast cells (*green*) were found in the lungs of controls and HDM-treated mice. **b**–**b**”’ Large magnifications of mast cells in (**a**). **c** Immunofluorescence staining of survival marker Akt1 (*green*), tryptase (*red*) and DAPI (*blue*) in the mouse lung tissues. All mast cells in controls and HDM-treated mice were negative for Akt1. **d**–**d**”’ Large magnifications of (**c**). **e** The Akt1-positive cells were found in the lung tissues. **f** Negative control for immunofluorescence staining. *Scale bars* (**a**, **c**, **f**) 100 µm, (**b**–**b**”’, **d**–**d**”’, **e**) 50 µm

Discussion

Our study revealed a significant increase of MCs in the bronchi, alveolar and vascular tissues during allergic airway inflammation. The number of MC_{TC} subpopulations rose significantly in bronchi and alveolar parenchyma but not in vascular tissues. The anatomic associations between MCs and nerves were found only in bronchi and there were no changes after HDM treatment. Mast cells form a very heterogeneous cell population displaying multiple functions (Erjefalt 2014). Therefore, investigating their contribution and the proportion in anatomical compartments is essential for the study of the role of mast cells in allergic airway inflammation. Their particular distribution and phenotypical appearance (MC_T and

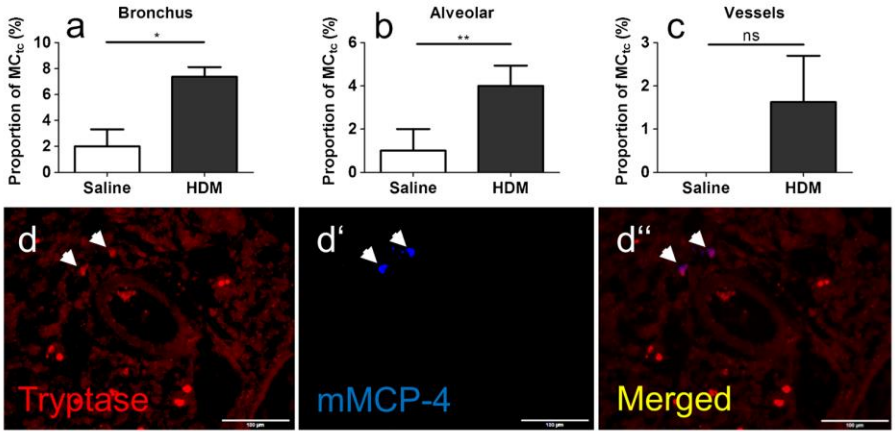


Fig. 5 Proportion of MC_{TC} subpopulations **a**–**c** The proportion of MC_{tc} bronchus, alveolar and vessels in the lung of controls and HDM-treated mice. The results are expressed as the percentage of MC_{TC} to the number of MCtot ($MC_T + MC_{TC}$). **d**–**d**" Fluorescence staining showing tryptase (*red*), mMCP4 (mouse chymase) (*magenta*, stained with antibody against mMCP4) and tryptase-mMCP-4 double-positive mast cells (*arrows*), indicating two subpopulations of MCs in the lung: tryptase-positive (MC_T) and tryptase-mMCP-4 (chymase)-positive (MC_{TC}). Results are expressed as mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$ (unpaired two-tailed *t* test). *Scale bars* 100 μm

MC_{TC}), as well as their interaction with the nervous system, are still of interest and remain unexplored in mouse models for allergic airway inflammation.

Analysis of mast cell numbers in human asthma show divergent results. While some authors report decreasing numbers, others reveal higher numbers of MCs and their subtypes in different parts of asthmatic airways (Andersson et al. 2009). These differences may be due to variations in asthma severity, the use of different methods and analysis criteria or effects of glucocorticoid treatments. The results of this study show some important features described in human asthma. Under physiological conditions, human MCs can be found in all lung

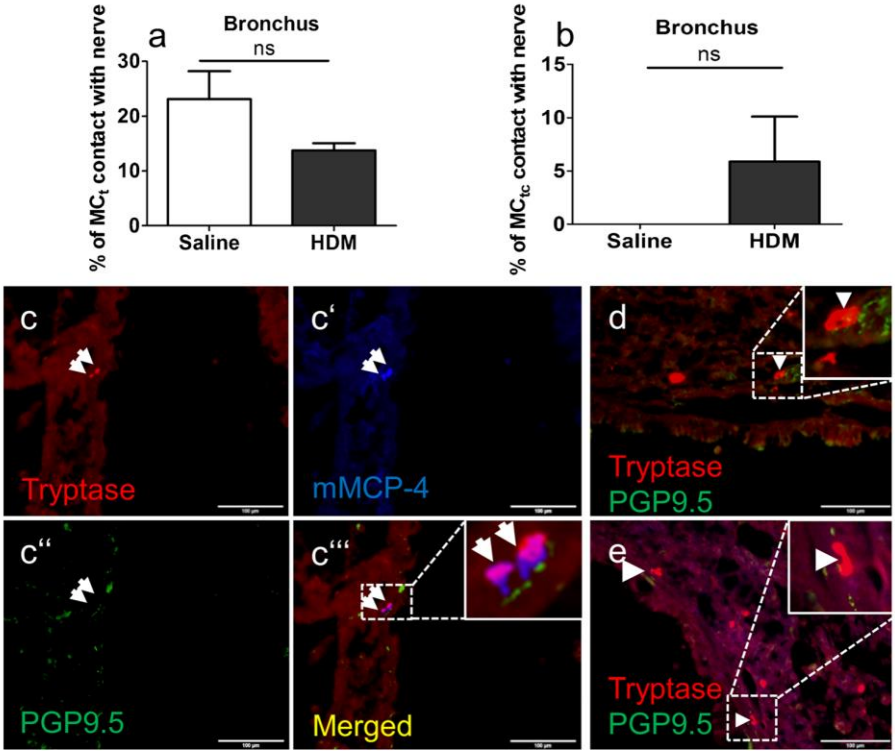


Fig. 6 Mast cell–nerve contacts in the lung. The anatomic associations between MCs and nerves were only found in bronchus. The quantitative analysis of MC_T (**a**) and MC_{TC} (**b**) contact with nerves. The results are expressed as the percentage of MC_T and MC_{TC} associating with nerves to the numbers of MC_T and MC_{TC}, respectively. **c**–**c**''' Microphotograph showing the association between MC_{TC} (*arrows*) and nerve fibers (*green*, stained with antibody against PGP 9.5) in bronchus. **d**, **e** The association between MC_T (*arrows*) and nerve fibers (*green*)

compartments, with MC_T being the predominant subtype in bronchi and alveolar parenchyma (Andersson et al. 2009). Our HDM mouse model shows a similar distribution, whereas direct comparison elicits fewer MC numbers and even smaller proportions of MC_{TC} in the murine airways under baseline conditions. These findings could point to different roles in promoting early allergic inflammation.

The proliferation study of MCs showed that MCs did not proliferate and survive after HDM treatment. Therefore, we believe that these MCs migrate from the systemic circulation into the airways and lung during allergic inflammation.

Under inflammatory conditions, MCs increased in human lung tissue especially towards the periphery and the alveolar parenchyma (Andersson et al. 2011). This increase seems to be accompanied by an elevated proportion of MC_{TC} in small airways and alveolar compartments (Balzar et al. 2005). This HDM model also shows a rise of MC_{TC} in bronchi and even alveolar parenchyma and therefore reproduces a key feature of human asthma. Furthermore, we demonstrate a proportional increase of MC_{TC} in conducting airways as well as in the alveolar compartment. This seems to represent the human situation in severe uncontrolled asthma, as has been described before in asthmatics (Andersson et al. 2011). As it is known that under some conditions the number of MC_{TC} in small airways and alveolar parenchyma correlates especially with better lung function (Balzar et al. 2005), their regulative role has to be analyzed in the HDM mouse model. Nevertheless, it has to be taken into consideration that the total MC numbers have been shown to be much higher in asthmatic lung and airways, especially in distal airways, than in HDM-treated mice.

The roles of MCs in allergic asthma are still not fully understood. Previous studies have shown that MCs may have an essential role in airway remodeling, especially on increased smooth muscle mass, subepithelial fibrosis, epithelial alterations and neovascularization, by releasing proteases and growth factors (Okayama et al. 2007). MCs have been found to infiltrate the airway smooth muscle in asthma and to have a relationship with airway smooth muscle hypertrophy (Brightling et al. 2002; Amin et al. 2005). Tryptase has been shown to have the capacity to induce the proliferation of airway epithelial cells (Cairns and Walls 1996). Asthmatics as well as HDM-treated mice show increased vascularity that correlates with disease severity (Rydell-Tormanen et al. 2008; Salvato 2001). Especially, human MC_{TC} secrete vascular endothelial growth factor (VEGF), which contributes significantly to the vasculature angionesis (Chetta et al. 2003; Abdel-Rahman et al. 2006). Our results show that there is an increase of MC_T but not MC_{TC} in the vascular compartment under inflammatory conditions and that MC_{TC} are even absent under baseline conditions. Comparisons to the human situation are difficult due to a lack of data. Nevertheless, the role of MC_{TC} in the vascular remodeling process has to be questioned in this model.

Moreover, mast cells proteases have been reported to increase the migration of immune cells, including neutrophils and eosinophils (Terakawa et al. 2006; He and Walls 1998). Mast cell chymase was revealed to be a potent secretagogue for airway serous gland cells (Sommerhoff et al. 1989).

With respect to MC–nerve interaction, histological studies show that these cells are closely related to the central and peripheral nervous systems (Bienenstock et al. 1988). This connection is not limited solely to anatomical but also shares numerous functional aspects (Bienenstock et al. 1991; Weigand et al. 2009). In the context of an "axon reflex", MCs activate nerve fibers, which in turn modulate the inflammatory processes via the release of tachykinins and neuropeptides (e.g., SP, NKA, CGRP) (Barnes 1986). Our analysis reveals an anatomical connection between MC_T and bronchial nerve fibers in the HDM mouse model. Under morphological aspects, this connection was not changed following HDM treatment. Whether this colocalization therefore comes out by chance is very unlikely in view of the previous investigations (Alving et al. 1991) but it implicates the need for functional analysis with the focus on the expression of specific tachykinin (SP, NKA) and CGRP and neuropeptide receptors on MCs and in electrophysiological experiments.

In conclusion, the present study reveals for the first time that there is a migration of MC_T and MC_{TC} into the bronchi and the alveolar parenchyma as well as an increase of MC_T in the vascular compartment following HDM treatment. This supports the idea that MCs contribute to allergic airway inflammation in the HDM mouse model and may also implicate the key features of severe human asthma. Furthermore, it shows that at least MC_T interacts with airway nerves but that there is the need for further functional investigations concerning this interaction in bronchial asthma.

Acknowledgements This project was supported and funded by the foundation 'Stiftung Bergmannshilfswerk Luisenthal' to Prof. Dr. Quoc Thai Dinh. We would like to thank Ms Janine Schlicker for her technical assistance.

Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

References

Abdel-Rahman AM, el-Sahrigy SA, Bakr SI (2006) A comparative study of two angiogenic factors: vascular endothelial growth factor and angiogenin in induced sputum from asthmatic children in acute attack. Chest 129:266–271

Alving K, Sundstrom C, Matran R, Panula P, Hokfelt T, Lundberg JM (1991) Association between histamine-containing mast cells and sensory nerves in the skin and airways of control and capsaicin-treated pigs. Cell Tissue Res 264:529–538

Amin K, Janson C, Boman G, Venge P (2005) The extracellular deposition of mast cell products is increased in hypertrophic airways smooth muscles in allergic asthma but not in nonallergic asthma. Allergy 60:1241–1247

Andersson CK, Mori M, Bjermer L, Lofdahl CG, Erjefalt JS (2009) Novel site-specific mast cell subpopulations in the human lung. Thorax 64:297–305

Andersson CK, Bergqvist A, Mori M, Mauad T, Bjermer L, Erjefalt JS (2011) Mast cell-associated alveolar inflammation in patients with atopic uncontrolled asthma. J Allergy Clin Immunol 127:905–912

Balzar S, Chu HW, Strand M, Wenzel S (2005) Relationship of small airway chymase-positive mast cells and lung function in severe asthma. Am J Respir Crit Care Med 171:431–439

Barnes PJ (1986) Asthma as an axon reflex. Lancet 1:242–245

Barnes PJ (1992) Modulation of neurotransmission in airways. Physiol Rev 72:699–729

Bienenstock J, Perdue M, Blennerhassett M, Stead R, Kakuta N, Sestini P, Vancheri C, Marshall J (1988) Inflammatory cells and the epithelium. Mast cell/nerve interactions in the lung in vitro and in vivo. Am Rev Respir Dis 138:S31–S34

Bienenstock J, MacQueen G, Sestini P, Marshall JS, Stead RH, Perdue MH (1991) Mast cell/nerve interactions in vitro and in vivo. Am Rev Respir Dis 143:S55–S58

Bradding P, Arthur G (2016) Mast cells in asthma—state of the art. Clin Exp Allergy 46:194–263

Braman SS (2006) The global burden of asthma. Chest 130:4S–12S

Brightling CE, Bradding P, Symon FA, Holgate ST, Wardlaw AJ, Pavord ID (2002) Mast-cell infiltration of airway smooth muscle in asthma. N Engl J Med 346:1699–1705

Brown JM, Wilson TM, Metcalfe DD (2008) The mast cell and allergic diseases: role in pathogenesis and implications for therapy. Clin Exp Allergy 38:4–18

Busse WW, Lemanske RF Jr (2001) Asthma. N Engl J Med 344:350–362

Cairns JA, Walls AF (1996) Mast cell tryptase is a mitogen for epithelial cells. Stimulation of IL-8 production and intercellular adhesion molecule-1 expression. J Immunol 156:275–283

Carter RJ, Bradding P (2011) The role of mast cells in the structural alterations of the airways as a potential mechanism in the pathogenesis of severe asthma. Curr Pharm Des 17:685–698

Chetta A, Zanini A, Foresi A, Del DM, Castagnaro A, D'Ippolito R, Baraldo S, Testi R, Saetta M, Olivieri D (2003) Vascular component of airway remodeling in asthma is reduced by high dose of fluticasone. Am J Respir Crit Care Med 167:751–757

Cyphert JM, Kovarova M, Koller BH (2011) Unique populations of lung mast cells are required for antigen-mediated bronchoconstriction. Clin Exp Allergy 41:260–269

Devos FC, Boonen B, Alpizar YA, Maes T, Hox V, Seys S, Pollaris L, Liston A, Nemery B, Talavera K, Hoet PH, Vanoirbeek JA (2016) Neuro-immune interactions in chemical-induced airway hyperreactivity. Eur Respir J 48:380–392

Dinh QT, Groneberg DA, Peiser C, Springer J, Joachim RA, Arck PC, Klapp BF, Fischer A (2004a) Nerve growth factor-induced substance P in capsaicin-insensitive vagal neurons innervating the lower mouse airway. Clin Exp Allergy 34:1474–1479

Dinh QT, Groneberg DA, Witt C, Peiser C, Cifuentes LB, Frossard N, Klapp BF, Fischer A (2004b) Expression of tyrosine hydroxylase and neuropeptide tyrosine in mouse sympathetic airway-specific neurons under normal situation and allergic airway inflammation. Clin Exp Allergy 34:1934–1941

Dinh QT, Mingomataj E, Quarcoo D, Groneberg DA, Witt C, Klapp BF, Braun A, Fischer A (2005) Allergic airway inflammation induces tachykinin peptides expression in vagal sensory neurons innervating mouse airways. Clin Exp Allergy 35:820–825

Erjefalt JS (2014) Mast cells in human airways: the culprit? Eur Respir Rev 23:299–307

Forsythe P, Bienenstock J (2012) The mast cell-nerve functional unit: a key component of physiologic and pathophysiologic responses. Chem Immunol Allergy 98:196–221

Galli SJ, Nakae S, Tsai M (2005) Mast cells in the development of adaptive immune responses. Nat Immunol 6:135–142

Groneberg DA, Fischer TC, Peckenschneider N, Noga O, Dinh QT, Welte T, Welker P (2007) Cell type-specific regulation of brain-derived neurotrophic factor in states of allergic inflammation. Clin Exp Allergy 37:1386–1391

Hatanaka K, Imakita M, Go S, Yamamoto A (1986) Effects of degranulation of mast cells on proliferation of mesenchymal cells in the mesentery of mice. Cell Tissue Res 246:53–56

He S, Walls AF (1998) Human mast cell chymase induces the accumulation of neutrophils, eosinophils and other inflammatory cells in vivo. Br J Pharmacol 125:1491–1500

Hox V, Vanoirbeek JA, Alpizar YA, Voedisch S, Callebaut I, Bobic S, Sharify A, De Vooght V, Van Gerven L, Devos F, Liston A, Voets T, Vennekens R, Bullens DM, De VA, Hoet P, Braun A, Ceuppens JL, Talavera K, Nemery B, Hellings PW (2013) Crucial role of transient receptor potential ankyrin 1 and mast cells in induction of nonallergic airway hyperreactivity in mice. Am J Respir Crit Care Med 187:486–493

Jarvikallio A, Harvima IT, Naukkarinen A (2003) Mast cells, nerves and neuropeptides in atopic dermatitis and nummular eczema. Arch Dermatol Res 295:2–7

Kitamura Y (1989) Heterogeneity of mast cells and phenotypic change between subpopulations. Annu Rev Immunol 7:59–76

Le DD, Rochlitzer S, Fischer A, Heck S, Tschernig T, Sester M, Bals R, Welte T, Braun A, Dinh QT (2014) Allergic airway inflammation induces the migration of dendritic cells into airway sensory ganglia. Respir Res 15:73

Leon A, Buriani A, Dal TR, Fabris M, Romanello S, Aloe L, Levi-Montalcini R (1994) Mast cells synthesize, store, and release nerve growth factor. Proc Natl Acad Sci U S A 91:3739–3743

Okayama Y, Ra C, Saito H (2007) Role of mast cells in airway remodeling. Curr Opin Immunol 19:687–693

Oskeritzian CA, Zhao W, Min HK, Xia HZ, Pozez A, Kiev J, Schwartz LB (2005) Surface CD88 functionally distinguishes the MCTC from the MCT type of human lung mast cell. J Allergy Clin Immunol 115:1162–1168

Pawankar R, Ra C (1996) Heterogeneity of mast cells and T cells in the nasal mucosa. J Allergy Clin Immunol 98:S248–S262

Persson CG, Erjefalt JS, Greiff L, Andersson M, Erjefalt I, Godfrey RW, Korsgren M, Linden M, Sundler F, Svensson C (1998) Plasma-derived proteins in airway defence, disease and repair of epithelial injury. Eur Respir J 11:958–970

Robinson DS (2004) The role of the mast cell in asthma: induction of airway hyperresponsiveness by interaction with smooth muscle? J Allergy Clin Immunol 114:58–65

Rydell-Tormanen K, Johnson JR, Fattouh R, Jordana M, Erjefalt JS (2008) Induction of vascular remodeling in the lung by chronic house dust mite exposure. Am J Respir Cell Mol Biol 39:61–67

Salvato G (2001) Quantitative and morphological analysis of the vascular bed in bronchial biopsy specimens from asthmatic and non-asthmatic subjects. Thorax 56:902–906

Sommerhoff CP, Caughey GH, Finkbeiner WE, Lazarus SC, Basbaum CB, Nadel JA (1989) Mast cell chymase. A potent secretagogue for airway gland serous cells. J Immunol 142:2450–2456

Stead RH, Tomioka M, Quinonez G, Simon GT, Felten SY, Bienenstock J (1987) Intestinal mucosal mast cells in normal and nematode-infected rat intestines are in intimate contact with peptidergic nerves. Proc Natl Acad Sci U S A 84:2975–2979

Tchougounova E, Pejler G, Abrink M (2003) The chymase, mouse mast cell protease 4, constitutes the major chymotrypsin-like activity in peritoneum and ear tissue. A role for mouse mast cell protease 4 in thrombin regulation and fibronectin turnover. J Exp Med 198:423–431

Terakawa M, Tomimori Y, Goto M, Fukuda Y (2006) Mast cell chymase induces expression of chemokines for neutrophils in eosinophilic EoL-1 cells and mouse peritonitis eosinophils. Eur J Pharmacol 538:175–181

van Houwelingen AH, Kool M, de Jager SC, Redegeld FA, van Heuven-Nolsen D, Kraneveld AD, Nijkamp FP (2002) Mast cell-derived TNF-alpha primes sensory nerve endings in a pulmonary hypersensitivity reaction. J Immunol 168:5297–5302

Weigand LA, Myers AC, Meeker S, Undem BJ (2009) Mast cell-cholinergic nerve interaction in mouse airways. J Physiol 587: 3355–3362

Wenzel SE (2006) Asthma: defining of the persistent adult phenotypes. Lancet 368:804–813

Wenzel SE (2012) Asthma phenotypes: the evolution from clinical to molecular approaches. Nat Med 18:716–725

Wernersson S, Pejler G (2014) Mast cell secretory granules: armed for battle. Nat Rev Immunol 14:478–494

Xiang Z, Nilsson G (2000) IgE receptor-mediated release of nerve growth factor by mast cells. Clin Exp Allergy 30:1379–1386

6 References

1. Meggs WJ. The Toxicant Induction of Irritant Asthma, Rhinitis, and Related Conditions. 19-61. 2013. SPRINGER. Ref Type: Edited Book

2. Sahin-Yilmaz A, Naclerio RM. Anatomy and physiology of the upper airway. Proc Am Thorac Soc 2011; 8:31-9.

3. van CP, Sys L, De BT, Watelet JB. Anatomy and physiology of the nose and the paranasal sinuses. Immunol Allergy Clin North Am 2004; 24:1-17.

4. Computational Fluid and Particle Dynamics in the Human Respiratory System. 19-44. 2013. Springer. Ref Type: Edited Book

5. Widdicombe J. Physiologic control. Anatomy and physiology of the airway circulation. Am Rev Respir Dis 1992; 146:S3-S7.

6. In-Depth Review of Allergic Rhinitis; http://www.worldallergy.org. http://www.worldallergy.org/professional/allergic_diseases_center/rhinitis/rhinitis_indepth.php . 2015. Ref Type: Internet Communication

7. Small P, Kim H. Allergic rhinitis. Allergy Asthma Clin Immunol 2011; 7 Suppl 1:S3.

8. Baraniuk JN. Pathogenesis of allergic rhinitis. J Allergy Clin Immunol 1997; 99:S763-S772.

9. Greiner AN, Hellings PW, Rotiroti G, Scadding GK. Allergic rhinitis. Lancet 2011; 378:2112-22.

10. Pawankar R, Mori S, Ozu C, Kimura S. Overview on the pathomechanisms of allergic rhinitis. Asia Pac Allergy 2011; 1:157-67.

11. Tran NP, Vickery J, Blaiss MS. Management of rhinitis: allergic and non-allergic. Allergy Asthma Immunol Res 2011; 3:148-56.

12. Kim D, Baraniuk JN. Neural aspects of allergic rhinitis. Curr Opin Otolaryngol Head Neck Surg 2007; 15:268-73.

13. Galli SJ, Tsai M. IgE and mast cells in allergic disease. Nat Med 2012; 18:693-704.

14. Galli SJ, Nakae S, Tsai M. Mast cells in the development of adaptive immune responses. Nat Immunol 2005; 6:135-42.

15. Grimbaldeston MA, Metz M, Yu M, Tsai M, Galli SJ. Effector and potential immunoregulatory roles of mast cells in IgE-associated acquired immune responses. Curr Opin Immunol 2006; 18:751-60.

16. Brown JM, Wilson TM, Metcalfe DD. The mast cell and allergic diseases: role in pathogenesis and implications for therapy. Clin Exp Allergy 2008; 38:4-18.

17. Bauer O, Razin E. Mast Cell-Nerve Interactions. News Physiol Sci 2000; 15:213-8.

18. Kleij HP, Bienenstock J. Significance of Conversation between Mast Cells and Nerves. Allergy Asthma Clin Immunol 2005; 1:65-80.

19. Pfaar O, Raap U, Holz M, Hormann K, Klimek L. Pathophysiology of itching and sneezing in allergic rhinitis. Swiss Med Wkly 2009; 139:35-40.

20. Sarin S, Undem B, Sanico A, Togias A. The role of the nervous system in rhinitis. J Allergy Clin Immunol 2006; 118:999-1016.

21. Pawankar R, Canonica GW, ST Holgate ST, Lockey RF, Blaiss M. The WAO White Book on Allergy (Update. 2013). 2013.

22. Fanta CH. Asthma. N Engl J Med 2009; 360:1002-14.

23. Holgate ST, Arshad HS, Roberts GC, Howarth PH, Thurner P, Davies DE. A new look at the pathogenesis of asthma. Clin Sci (Lond) 2010; 118:439-50.

24. Holgate ST. Pathogenesis of asthma. Clin Exp Allergy 2008; 38:872-97.

25. Nauta AJ, Engels F, Knippels LM, Garssen J, Nijkamp FP, Redegeld FA. Mechanisms of allergy and asthma. Eur J Pharmacol 2008; 585:354-60.

26. Barnes PJ. Pathophysiology of allergic inflammation. Immunol Rev 2011; 242:31-50.

27. Holgate ST. Pathogenesis of asthma. Clin Exp Allergy 2008; 38:872-97.

28. Barnes PJ. Pathophysiology of allergic inflammation. Immunol Rev 2011; 242:31-50.

29. Barnes PJ. Neurogenic inflammation in the airways. Respir Physiol 2001; 125:145-54.

30. Lambrecht BN, Hammad H. The airway epithelium in asthma. Nat Med 2012; 18:684-92.

31. Barnes PJ. Neuropeptides and asthma. Am Rev Respir Dis 1991; 143:S28-S32.

32. Groneberg DA, Quarcoo D, Frossard N, Fischer A. Neurogenic mechanisms in bronchial inflammatory diseases. Allergy 2004; 59:1139-52.

33. Joos GF, Germonpre PR, Pauwels RA. Neural mechanisms in asthma. Clin Exp Allergy 2000; 30 Suppl 1:60-5.

34. Canning BJ, Fischer A. Neural regulation of airway smooth muscle tone. Respir Physiol 2001; 125:113-27.

35. Dinh QT, Mingomataj E, Quarcoo D, Groneberg DA, Witt C, Klapp BF, Braun A, Fischer A. Allergic airway inflammation induces tachykinin peptides expression in vagal sensory neurons innervating mouse airways. Clin Exp Allergy 2005; 35:820-5.

36. Galli SJ, Tsai M, Piliponsky AM. The development of allergic inflammation. Nature 2008; 454:445-54.

37. Holgate ST. The epidemic of allergy and asthma. Nature 1999; 402:B2-B4.

38. EAACI Global Atlas of Allergy. European Academy of Allergy and Clinical Immunology, 2014.

39. Abdul Ghaffar. Immunology - Chapter seventeen - Hypersensitivity reactions. Microbiology and Immunology On-line . 2016. Ref Type: Online Source

40. Kenneth M.Murphy, Paul Travers, Mark Walport. Janeway's Immunobiology. 7th Edition Edn. Garland Science, 2008.

41. Warrington R, Watson W, Kim HL, Antonetti FR. An introduction to immunology and immunopathology. Allergy Asthma Clin Immunol 2011; 7 Suppl 1:S1.

42. Lambrecht BN. Dendritic cells and the regulation of the allergic immune response. Allergy 2005; 60:271-82.

43. Novak N, Haberstok J, Geiger E, Bieber T. Dendritic cells in allergy. Allergy 1999; 54:792-803.

44. Nassenstein C, Kutschker J, Tumes D, Braun A. Neuro-immune interaction in allergic asthma: role of neurotrophins. Biochem Soc Trans 2006; 34:591-3.

45. Nassenstein C, Kammertoens T, Veres TZ, Uckert W, Spies E, Fuchs B, Krug N, Braun A. Neuroimmune crosstalk in asthma: dual role of the neurotrophin receptor p75NTR. J Allergy Clin Immunol 2007; 120:1089-96.

46. Veres TZ, Rochlitzer S, Shevchenko M, Fuchs B, Prenzler F, Nassenstein C, Fischer A, Welker L, Holz O, Muller M, Krug N, Braun A. Spatial interactions between dendritic cells and sensory nerves in allergic airway inflammation. Am J Respir Cell Mol Biol 2007; 37:553-61.

47. Rochlitzer S, Veres TZ, Kuhne K, Prenzler F, Pilzner C, Knothe S, Winkler C, Lauenstein HD, Willart M, Hammad H, Muller M, Krug N, Lambrecht BN, Braun A. The neuropeptide calcitonin gene-related peptide affects allergic airway inflammation by modulating dendritic cell function. Clin Exp Allergy 2011; 41:1609-21.

48. Veres TZ, Shevchenko M, Krasteva G, Spies E, Prenzler F, Rochlitzer S, Tschernig T, Krug N, Kummer W, Braun A. Dendritic cell-nerve clusters are sites of T cell proliferation in allergic airway inflammation. Am J Pathol 2009; 174:808-17.

49. Lambrecht BN. Immunologists getting nervous: neuropeptides, dendritic cells and T cell activation. Respir Res 2001; 2:133-8.

50. Joos GF. The role of sensory neuropeptides in the pathogenesis of bronchial asthma. Clin Exp Allergy 1989; 19 Suppl 1:9-13.

51. Kakurai M, Monteforte R, Suto H, Tsai M, Nakae S, Galli SJ. Mast cell-derived tumor necrosis factor can promote nerve fiber elongation in the skin during contact hypersensitivity in mice. Am J Pathol 2006; 169:1713-21.

52. Leon A, Buriani A, Dal TR, Fabris M, Romanello S, Aloe L, Levi-Montalcini R. Mast cells synthesize, store, and release nerve growth factor. Proc Natl Acad Sci U S A 1994; 91:3739-43.

53. van Houwelingen AH, Kool M, de Jager SC, Redegeld FA, van Heuven-Nolsen D, Kraneveld AD, Nijkamp FP. Mast cell-derived TNF-alpha primes sensory nerve endings in a pulmonary hypersensitivity reaction. J Immunol 2002; 168:5297-302.

54. Dinh QT, Suhling H, Fischer A, Braun A, Welte T. [Innervation of the airways in asthma bronchiale and chronic obstructive pulmonary disease (COPD)]. Pneumologie 2011; 65:283-92.

55. Undem BJ, McAlexander M, Hunter DD. Neurobiology of the upper and lower airways. Allergy 1999; 54 Suppl 57:81-93.

56. Mingomataj E, Dinh QT, Groneberg D, Feleszko W, Schmeck B, Joachim R, Noga O, Nagel S, Klapp BF, Fischer A. Trigeminal nasal-specific neurons respond to nerve growth factor with substance-P biosynthesis. Clin Exp Allergy 2008; 38:1203-11.

57. Dinh QT, Groneberg DA, Mingomataj E, Peiser C, Heppt W, Dinh S, Arck PC, Klapp BF, Fischer A. Expression of substance P and vanilloid receptor (VR1) in trigeminal sensory neurons projecting to the mouse nasal mucosa. Neuropeptides 2003; 37:245-50.

58. Baraniuk JN, Lundgren JD, Okayama M, Goff J, Mullol J, Merida M, Shelhamer JH, Kaliner MA. Substance P and neurokinin A in human nasal mucosa. Am J Respir Cell Mol Biol 1991; 4:228-36.

59. Lane AP. Nasal anatomy and physiology. Facial Plast Surg Clin North Am 2004; 12:387-95, v.

60. Klaassen AB, van Megen YJ, Kuijpers W, van den Broek P. Autonomic innervation of the nasal mucosa. ORL J Otorhinolaryngol Relat Spec 1988; 50:32-41.

61. Baraniuk JN, Kaliner MA. Neuropeptides and nasal secretion. J Allergy Clin Immunol 1990; 86:620-7.

62. Driessen AK, Farrell MJ, Mazzone SB, McGovern AE. The Role of the Paratrigeminal Nucleus in Vagal Afferent Evoked Respiratory Reflexes: A Neuroanatomical and Functional Study in Guinea Pigs. Front Physiol 2015; 6:378.

63. Mazzone SB, Undem BJ. Vagal Afferent Innervation of the Airways in Health and Disease. Physiol Rev 2016; 96:975-1024.

64. Grace MS, Dubuis E, Birrell MA, Belvisi MG. Pre-clinical studies in cough research: role of Transient Receptor Potential (TRP) channels. Pulm Pharmacol Ther 2013; 26:498-507.

65. Chang RB, Strochlic DE, Williams EK, Umans BD, Liberles SD. Vagal Sensory Neuron Subtypes that Differentially Control Breathing. Cell 2015; 161:622-33.

66. Springall DR, Cadieux A, Oliveira H, Su H, Royston D, Polak JM. Retrograde tracing shows that CGRP-immunoreactive nerves of rat trachea and lung originate from vagal and dorsal root ganglia. J Auton Nerv Syst 1987; 20:155-66.

67. Dinh QT, Klapp BF, Fischer A. [Airway sensory nerve and tachykinins in asthma and COPD]. Pneumologie 2006; 60:80-5.

68. Dinh QT, Groneberg DA, Peiser C, Mingomataj E, Joachim RA, Witt C, Arck PC, Klapp BF, Fischer A. Substance P expression in TRPV1 and trkA-positive dorsal root ganglion neurons innervating the mouse lung. Respir Physiol Neurobiol 2004; 144:15-24.

69. Dunzendorfer S, Kaser A, Meierhofer C, Tilg H, Wiedermann CJ. Cutting edge: peripheral neuropeptides attract immature and arrest mature blood-derived dendritic cells. J Immunol 2001; 166:2167-72.

70. Dunzendorfer S, Wiedermann CJ. Neuropeptides and the immune system: focus on dendritic cells. Crit Rev Immunol 2001; 21:523-57.

71. Bellibas SE. The effect of human calcitonin gene-related peptide on eosinophil chemotaxis in the rat airway. Peptides 1996; 17:563-4.

72. Foster CA, Mandak B, Kromer E, Rot A. Calcitonin gene-related peptide is chemotactic for human T lymphocytes. Ann N Y Acad Sci 1992; 657:397-404.

73. van der Velden VH, Hulsmann AR. Autonomic innervation of human airways: structure, function, and pathophysiology in asthma. Neuroimmunomodulation 1999; 6:145-59.

74. Undem BJ, Carr MJ. The role of nerves in asthma. Curr Allergy Asthma Rep 2002; 2:159-65.

75. Barnes PJ. Neuropeptides in human airways: function and clinical implications. Am Rev Respir Dis 1987; 136:S77-S83.

76. Joos GF, Germonpre PR, Pauwels RA. Role of tachykinins in asthma. Allergy 2000; 55:321-37.

77. Groneberg DA, Quarcoo D, Frossard N, Fischer A. Neurogenic mechanisms in bronchial inflammatory diseases. Allergy 2004; 59:1139-52.

78. Heaney LG, Cross LJ, Stanford CF, Ennis M. Substance P induces histamine release from human pulmonary mast cells. Clin Exp Allergy 1995; 25:179-86.

79. Nilsson G, Rak S, Ahlstedt S. The influence of substance P on the proliferation of peripheral blood lymphocytes from normal individuals and birch pollen-allergic patients. Allergy 1987; 42:516-23.

80. Lambrecht BN. Immunologists getting nervous: neuropeptides, dendritic cells and T cell activation. Respir Res 2001; 2:133-8.

81. Tatemoto K, Nozaki Y, Tsuda R, Konno S, Tomura K, Furuno M, Ogasawara H, Edamura K, Takagi H, Iwamura H, Noguchi M, Naito T. Immunoglobulin E-independent activation of mast cell is mediated by Mrg receptors. Biochem Biophys Res Commun 2006; 349:1322-8.

82. McNeil BD, Pundir P, Meeker S, Han L, Undem BJ, Kulka M, Dong X. Identification of a mast-cell-specific receptor crucial for pseudo-allergic drug reactions. Nature 2015; 519:237-41.

83. Fujisawa D, Kashiwakura J, Kita H, Kikukawa Y, Fujitani Y, Sasaki-Sakamoto T, Kuroda K, Nunomura S, Hayama K, Terui T, Ra C, Okayama Y. Expression of Mas-related gene X2 on mast cells is upregulated in the skin of patients with severe chronic urticaria. J Allergy Clin Immunol 2014; 134:622-33.

84. Subramanian H, Kashem SW, Collington SJ, Qu H, Lambris JD, Ali H. PMX-53 as a dual CD88 antagonist and an agonist for Mas-related gene 2 (MrgX2) in human mast cells. Mol Pharmacol 2011; 79:1005-13.

85. Wimalawansa SJ. Calcitonin gene-related peptide and its receptors: molecular genetics, physiology, pathophysiology, and therapeutic potentials. Endocr Rev 1996; 17:533-85.

86. Springer J, Geppetti P, Fischer A, Groneberg DA. Calcitonin gene-related peptide as inflammatory mediator. Pulm Pharmacol Ther 2003; 16:121-30.

87. Springer J, Geppetti P, Fischer A, Groneberg DA. Calcitonin gene-related peptide as inflammatory mediator. Pulm Pharmacol Ther 2003; 16:121-30.

88. Dinh QT, Cryer A, Dinh S, Peiser C, Wu S, Springer J, Hamelmann E, Klapp BF, Heppt W, Fischer A. Transcriptional up-regulation of histamine receptor-1 in epithelial, mucus and inflammatory cells in perennial allergic rhinitis. Clin Exp Allergy 2005; 35:1443-8.

89. Ding W, Stohl LL, Wagner JA, Granstein RD. Calcitonin gene-related peptide biases Langerhans cells toward Th2-type immunity. J Immunol 2008; 181:6020-6.

90. Davies D, Medeiros MS, Keen J, Turner AJ, Haynes LW. Eosinophil chemotactic peptide sequences in rat alpha-CGRP. Activation of a novel trophic action by neutral endopeptidase 24.11. Ann N Y Acad Sci 1992; 657:405-11.

91. Kawanami Y, Morimoto Y, Kim H, Nakamura T, Machida K, Kido T, Asonuma E, Yatera K, Yoshii C, Kido M. Calcitonin gene-related peptide stimulates proliferation of alveolar epithelial cells. Respir Res 2009; 10:8.

92. Altmayr F, Jusek G, Holzmann B. The neuropeptide calcitonin gene-related peptide causes repression of tumor necrosis factor-alpha transcription and suppression of ATF-2 promoter recruitment in Toll-like receptor-stimulated dendritic cells. J Biol Chem 2010; 285:3525-31.

93. Liu J, Chen M, Wang X. Calcitonin gene-related peptide inhibits lipopolysaccharide-induced interleukin-12 release from mouse peritoneal macrophages, mediated by the cAMP pathway. Immunology 2000; 101:61-7.

94. Wang F, Millet I, Bottomly K, Vignery A. Calcitonin gene-related peptide inhibits interleukin 2 production by murine T lymphocytes. J Biol Chem 1992; 267:21052-7.

95. Boudard F, Bastide M. Inhibition of mouse T-cell proliferation by CGRP and VIP: effects of these neuropeptides on IL-2 production and cAMP synthesis. J Neurosci Res 1991; 29:29-41.

96. Williams CM, Galli SJ. The diverse potential effector and immunoregulatory roles of mast cells in allergic disease. J Allergy Clin Immunol 2000; 105:847-59.

97. He S, Walls AF. Human mast cell chymase induces the accumulation of neutrophils, eosinophils and other inflammatory cells in vivo. Br J Pharmacol 1998; 125:1491-500.

98. Bradding P, Arthur G. Mast cells in asthma - state of the art. Clin Exp Allergy 2016; 46:194-263.

99. Wernersson S, Pejler G. Mast cell secretory granules: armed for battle. Nat Rev Immunol 2014; 14:478-94.

100. Xiang Z, Nilsson G. IgE receptor-mediated release of nerve growth factor by mast cells. Clin Exp Allergy 2000; 30:1379-86.

101. Hatanaka K, Imakita M, Go S, Yamamoto A. Effects of degranulation of mast cells on proliferation of mesenchymal cells in the mesentery of mice. Cell Tissue Res 1986; 246:53-6.

102. Carter RJ, Bradding P. The role of mast cells in the structural alterations of the airways as a potential mechanism in the pathogenesis of severe asthma. Curr Pharm Des 2011; 17:685-98.

103. Robinson DS. The role of the mast cell in asthma: induction of airway hyperresponsiveness by interaction with smooth muscle? J Allergy Clin Immunol 2004; 114:58-65.

104. Pawankar R, Ra C. Heterogeneity of mast cells and T cells in the nasal mucosa. J Allergy Clin Immunol 1996; 98:S248-S262.

105. Tchougounova E, Pejler G, Abrink M. The chymase, mouse mast cell protease 4, constitutes the major chymotrypsin-like activity in peritoneum and ear tissue. A role for mouse mast cell protease 4 in thrombin regulation and fibronectin turnover. J Exp Med 2003; 198:423-31.

106. Caughey GH. Mast cell proteases as pharmacological targets. Eur J Pharmacol 2015.

107. Balzar S, Chu HW, Strand M, Wenzel S. Relationship of small airway chymase-positive mast cells and lung function in severe asthma. Am J Respir Crit Care Med 2005; 171:431-9.

108. Caughey GH. Mast cell tryptases and chymases in inflammation and host defense. Immunol Rev 2007; 217:141-54.

109. Kulka M, Sheen CH, Tancowny BP, Grammer LC, Schleimer RP. Neuropeptides activate human mast cell degranulation and chemokine production. Immunology 2008; 123:398-410.

110. Dong X, Han S, Zylka MJ, Simon MI, Anderson DJ. A diverse family of GPCRs expressed in specific subsets of nociceptive sensory neurons. Cell 2001; 106:619-32.

111. Lee MG, Dong X, Liu Q, Patel KN, Choi OH, Vonakis B, Undem BJ. Agonists of the MAS-related gene (Mrgs) orphan receptors as novel mediators of mast cell-sensory nerve interactions. J Immunol 2008; 180:2251-5.

112. Frieling T, Cooke HJ, Wood JD. Histamine receptors on submucous neurons in guinea pig colon. Am J Physiol 1993; 264:G74-G80.

113. Forsythe P, Bienenstock J. The mast cell-nerve functional unit: a key component of physiologic and pathophysiologic responses. Chem Immunol Allergy 2012; 98:196-221.

114. Tani E, Senba E, Kokumai S, Masuyama K, Ishikawa T, Tohyama M. Histamine application to the nasal mucosa induces release of calcitonin gene-related peptide and substance P from peripheral terminals of trigeminal ganglion: a morphological study in the guinea pig. Neurosci Lett 1990; 112:1-6.

115. Dinh QT, Cryer A, Dinh S, Trevisani M, Georgiewa P, Chung F, Geppetti P, Heppt W, Klapp BF, Fischer A. Protease-activated receptor 2 expression in trigeminal neurons innervating the rat nasal mucosa. Neuropeptides 2005; 39:461-6.

116. Nakasaki T, Masuyama K, Fukui H, Ogino S, Eura M, Samejima Y, Ishikawa T, Yumoto E. Effects of PAF on histamine H1 receptor mRNA expression in rat trigeminal ganglia. Prostaglandins Other Lipid Mediat 1999; 58:29-41.

117. Jarvikallio A, Harvima IT, Naukkarinen A. Mast cells, nerves and neuropeptides in atopic dermatitis and nummular eczema. Arch Dermatol Res 2003; 295:2-7.

118. Alving K, Sundstrom C, Matran R, Panula P, Hokfelt T, Lundberg JM. Association between histamine-containing mast cells and sensory nerves in the skin and airways of control and capsaicin-treated pigs. Cell Tissue Res 1991; 264:529-38.

119. Stead RH, Tomioka M, Quinonez G, Simon GT, Felten SY, Bienenstock J. Intestinal mucosal mast cells in normal and nematode-infected rat intestines are in intimate contact with peptidergic nerves. Proc Natl Acad Sci U S A 1987; 84:2975-9.

120. Gill MA. The role of dendritic cells in asthma. J Allergy Clin Immunol 2012; 129:889-901.

121. Bulloch K, Miller MM, Gal-Toth J, Milner TA, Gottfried-Blackmore A, Waters EM, Kaunzner UW, Liu K, Lindquist R, Nussenzweig MC, Steinman RM, McEwen BS. CD11c/EYFP transgene illuminates a discrete network of dendritic cells within the embryonic, neonatal, adult, and injured mouse brain. J Comp Neurol 2008; 508:687-710.

122. Steinman RM, Banchereau J. Taking dendritic cells into medicine. Nature 2007; 449:419-26.

123. Vermaelen KY, Carro-Muino I, Lambrecht BN, Pauwels RA. Specific migratory dendritic cells rapidly transport antigen from the airways to the thoracic lymph nodes. J Exp Med 2001; 193:51-60.

124. Lambrecht BN, Hammad H. The role of dendritic and epithelial cells as master regulators of allergic airway inflammation. Lancet 2010; 376:835-43.

125. Rochlitzer S, Veres TZ, Kuhne K, Prenzler F, Pilzner C, Knothe S, Winkler C, Lauenstein HD, Willart M, Hammad H, Muller M, Krug N, Lambrecht BN, Braun A. The neuropeptide calcitonin gene-related peptide affects allergic airway inflammation by modulating dendritic cell function. Clin Exp Allergy 2011; 41:1609-21.

126. Braun A, Lommatzsch M, Mannsfeldt A, Neuhaus-Steinmetz U, Fischer A, Schnoy N, Lewin GR, Renz H. Cellular sources of enhanced brain-derived neurotrophic factor production in a mouse model of allergic inflammation. Am J Respir Cell Mol Biol 1999; 21:537-46.

127. Noga O, Peiser M, Altenahr M, Schmeck B, Wanner R, Dinh QT, Hanf G, Suttorp N. Selective induction of nerve growth factor and brain-derived neurotrophic factor by LPS and allergen in dendritic cells. Clin Exp Allergy 2008; 38:473-9.

128. Hosoi J, Murphy GF, Egan CL, Lerner EA, Grabbe S, Asahina A, Granstein RD. Regulation of Langerhans cell function by nerves containing calcitonin gene-related peptide. Nature 1993; 363:159-63.

129. Kim S Suvarna MBFFCLPaJDB. Bancroft's Theory and Practice of Histological Techniques, 7th Edition. 2012. Churchill Livingstone. Ref Type: Edited Book

130. Skipper R, Destephano D. A rapid stain for campylobacter-pylori in gastrointestinal tissue-sections using diff-quik. JOURNAL OF HISTOTECHNOLOGY 1989; 12:303-4.

7 Abbreviations

AR	Allergic rhinitis
APC	Antigen-presenting cell
APES	3-Aminopropyltriethoxysilane
BALF	Bronchoalveolar lavage fluid
CGRP	Calcitonin gene-related peptide
CGRPR	Calcitonin gene-related peptide receptor
DC	Dendritic cells
DAPI	4', 6-diamidino-2-phenylindol dihydrochloride
EdU	5-ethynyl-2'-deoxyuridine
FP	Fluticasone propionate
GFAP	Glial fibrillary acidic protein
GS	Glutamine synthetase
HDM	House dust mite
H&E	Hematoxylin and eosin
Iba1	Ionized calcium binding adapter molecule 1
IR	Immunoreactive
JNC	Jugular-nodose ganglia complex
LPS	Lipopolysaccharide
MC	Mast cells
MC_t	Tryptase expressing mast cells
MC_{tc}	Tryptase and chymase expressing mast cells
MHC II	Major histocompatibility class II
NK1R	Neurokinin-1 receptor
PAS	Periodic Acid Schiff
PBS	Phosphate buffered saline
PGP 9.5	Protein gene product 9.5
SGC	Satellite glia cell
SP	Substance P
VIP	Vasoactive intestinal peptide

8 Publications

Original papers and manuscripts

1. Wiege K, **Le DD**, Syed SN, Ali SR, Novakovic A, Beer-Hammer S, Piekorz RP, Schmidt RE, Nürnberg B, Gessner JE. Defective macrophage migration in Gαi2- but not Gαi3-deficient mice. **J Immunol**. 2012 Jul 15;189(2):980-7.

2. **Le DD**, Rochlitzer S, Fischer A, Heck S, Tschernig T, Sester M, Bals R, Welte T, Braun A, Dinh QT. Allergic airway inflammation induces the migration of dendritic cells into airway sensory ganglia. ***Respir Res.*** *2014 Jun 30;15:73.*

3. **Le DD***, Funck U*, Wronski S, Heck S, Tschernig T, Bischoff M, Sester M, Herr C, Bals R, Welte T, Braun A, Dinh QT. Steroid Treatment Reduces Allergic Airway Inflammation and Does Not Alter the Increased Numbers of Dendritic Cells and Calcitonin Gene-Related Peptide-Expressing Neurons in Airway Sensory Ganglia. ***Neuroimmunomodulation.*** *2016;23(1):18-26.*

4. **Le DD**, Schmit D, Heck S, Omlor AJ, Sester M, Herr C, Schick B, Daubeuf F, Fähndrich S, Bals R, Frossard N, Al Kadah B, Dinh QT. Increase of Mast Cell-Nerve Association and Neuropeptide Receptor Expression on Mast Cells in Perennial Allergic Rhinitis. ***Neuroimmunomodulation.*** *2016 Dec 29. [Epub ahead of print]*

5. Omlor AJ, **Le DD**, Schlicker J, Hannig M, Ewen R, Heck S, Herr C, Kraegeloh A, Hein C, Kautenburger R, Kickelbick G, Bals R, Nguyen J, Dinh QT. Local Effects on Airway Inflammation and Systemic Uptake of 5 nm PEGylated and Citrated Gold Nanoparticles in Asthmatic Mice. ***Small.*** *2016 Dec 23. doi: 10.1002/smll.201603070]*

6. Schmit D*, **Le DD*,** Heck H, Bischoff M, Tschernig T, Herr C, Beisswenger C, Kobelt P, Lepper MP, Chung KF, Bals R and Dinh QT. Allergic airway inflammation induces migration of mast cell populations into the mouse airway. ***Cell Tissue Res*** *(2017). doi:10.1007/s00441-017-2597-9*

7. Heck S, Al-Shobash S, Rapp D, **Le DD**, Omlor A, Bekhit A, Flaig M, Al-Kadah B, Herian W, Bals R, Wagenpfeil S and Dinh QT. High probability of co-morbidities in bronchial asthma in Germany. *Accepted for publication in* ***npj Primary Care Respiratory Medicine***

8. **Le DD,** Schmit D, Heck S, Schick B, Bals R, Frossard N, Al Kadah B anh Dinh QT. Increased expression of MrgX1 receptor in human nasal mucosa by perennial allergic rhinitis. ***(in preparation)***

*These authors contributed equally

Reviews

9. Dinh QT, Heck S, **Le DD**, Bals R, Welte T. Pathophysiology, diagnostics and therapy of chronic cough: neuronal reflexes and antitussiva. ***Pneumologie*** *2013; 67:327-34.*

10. Heck S, Nguyen J, **Le DD**, Bals R, Dinh QT. Pharmacological Therapy of Bronchial Asthma: The Role of Biologicals. ***Int. Arch. Allergy Immunol****. 2016; 168:241-52.*

Oral presentations

11. **Le DD**, Rochlitzer S, Funck U, Suhling H, Braun A, Welte T, Dinh QT. Vorkommen von Dendritischen Zellen im Jugulare/Nodose Ganglion im chronischen Hausstaubmilben-Mausmodell für allergische Atemwegsentzündung. Herbsttreffen der Sektion Zellbiologie der **DGP 2011** in Homburg.

12. **Le DD**, Rochlitzer S, Funck U, Suhling H , Bals R, Braun A, Welte T, Dinh QT . Dendritic cells-nerve interaction in allergic airway inflammation. **European Respiratory Society (ERS) 2012** in Wien.

13. **Le DD**, Rochlitzer S, Funck U, Heck S, Bals R, Braun A, Welte T, Dinh QT. Allergic airway inflammation induces dendritic cell phenotypes in airway sensory ganglia. **European Academy of Allergy and Clinical Immunology (EAACI) 2013** in Mailand.

14. **Le DD**, Funck U, Rochlitzer S, Heck S, Bals R, Braun A, Welte T, Dinh QT. Veränderung von MHC-II positiven Zellen der Atemwegganglien bei chronischem Hausstaubmilben-Mausmodell und HRV1B Infektion. Herbsttagung der Sektionen Infektiologie & Tuberkulose und Zellbiologie der **DGP 2013** in Marburg.

15. **Le DD**, Rochlitzer S, Funck U, Heck S, Bals R, Braun A , Welte T, Dinh QT. Allergische Atemwegsentzündung induziert dendritische Zellen Phänotyp in Atemwegsganglien. **DGP 2014** in Bremen.

Posters

16. **Le DD**, Schmit D, Heck S, Bals R, Schick B, Al Kadah B, Dinh QT. Mast cells-nerve interaction in allergic rhinitis. **European Academy of Allergy and Clinical Immunology (EAACI) 2015** in Barcelona.

17. Q.T. Dinh, D. Quarcoo, H. Suhling, **DD. Le**, A. Scholze, D. A. Groneberg, C. Peiser, B.F. Klapp, T. Welte, A. Braun, A. Fischer. Alteration of Sensory Airway Nerves in Transgenic Mice overexpressing NGF in Clara Cells of the Airways. **5. Deutscher Allergiekongress 2010** in Hannover.

18. Q.T. Dinh, H. Suhling, D. Quarcoo, **DD. Le**, D.A. Groneberg, C. Peiser, B.F. Klapp, T. Welte, A. Braun, A. Fischer. Pan-neurotrophin receptor p75 contributes to the substance P induction in vagal sensory neurons of mouse airways. **5. Deutscher Allergiekongress 2010** in Hannover.

19. A. Scholze, **DD. Le**, R. Joachim, D.A. Groneberg, C. Peiser, B.F. Klapp, A. Fischer, T. Welte, Q.T. Dinh. The TRPV1 receptor contributes to the mediation of allergic airway inflammation. 5. Deutscher Allergiekongress 2010 in Hannover.

20. Q.T. Dinh, A. Scholze, **DD. Le**, R. Joachim, H. Suhling, J. Springer, D. A. Groneberg, C. Peiser, A. Fischer, T. Welte, B.F. Klapp. The TRPV1 -/- knockout mice show less allergic airway inflammation in a mouse model of allergic airway inflammation. **5. Deutscher Allergiekongress 2010** in Hannover.

21. **Le DD**, Rochlitzer S, Funck U, Suhling H, Braun A, Welte T, Dinh QT. Dendritic cells-nerve interaction in allergic airway inflammation. **American Thoracic Society International Conference 2012** in San Francisco.

22. S. Heck, S. Al-Shobash, D. Rapp, **DD. Le**, M. Flaig, W. Herian, R. Bals, S. Wagenpfeil, Q.T. Dinh. Prevalence and comorbidities of bronchial asthma in Germany – a population based study of 653.955 patient cases in Saarland. **European Respiratory Society (ERS)** 2014 in München.

23. Funck U, **Le DD**, Rochlitzer S, Heck S, Bals R, Welte T, Braun A, Dinh QT. HRV1B infection and steroid-treatment do not affect the number of dendritic cells in vagal sensory ganglia under allergic conditions. **European Respiratory Society (ERS)** 2014 in München.

24. Schmit D, **Le DD**, Heck S, Beisswenger Ch, Herr C, Bals R, Dinh QT. Changes of mast cell populations during allergic airway inflammation in mice using HDM model. **European Respiratory Society (ERS)** 2015 in Amsterdam.

9 Acknowledgements / Danksagung

An dieser Stelle möchte ich allen Coautoren danken, die zum Gelingen dieser Arbeit beigetragen haben.

Herrn Prof. Dr. Quoc Thai Dinh danke ich ganz besonders für die Möglichkeit, diese Dissertation an der Abteilung für Experimentelle Pneumologie und Allergologie durchführen zu können und für die vielseitige Unterstützung bei der Bearbeitung dieses außerordentlich interessanten Forschungsthemas. Bei Frau Prof. Dr. Martina Sester bedanke ich mich für die Betreuung des kooperativen Promotionsverfahrens vonseiten der Abteilung für Transplantations- und Infektionsimmunologie.

Herrn Prof. Dr. Tobias Welte, Herrn Prof. Dr. Armin Braun, Frau Dr. Sabine Wronski, Herrn Prof. Dr. Dr. Robert Bals, Herrn Dr. Christian Herr, Herrn PD. Dr. Basal Al Kadah danke ich für die enge Zusammenarbeit.

Ein großes Dankeschön gebührt aber auch allen Mitarbeiterinnen und Mitarbeitern der Abteilung für Experimentelle Pneumologie und Allergologie, die mich immer unterstützt und fortwährend durch fachliche Gespräche in meiner Arbeit inspiriert haben.

Bei meiner Familie und meinem Onkel möchte ich mich besonders herzlich bedanken für die liebevolle Unterstützung während der letzten Jahre.

10 Curriculum vitae / Lebenslauf