Farming exposure and asthma phenotypes
Gonçalves Dias Pereira, Patricia

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Chapter 1

General introduction
ASTHMA

Asthma is a chronic inflammatory disease of the airways. It is characterized by respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough that vary over time and in intensity, together with variable expiratory airflow limitation. Symptoms and airflow limitation may resolve naturally or in response to medication, and may sometimes be absent for weeks or months. However, patients can experience episodic exacerbations of asthma that may be life-threatening and lead to hospitalizations (1). In asthma patients, stimuli such as allergens, cigarette smoke, cold air as well as exercise can trigger episodic symptoms, as a result of inflammation and structural changes in the airways (figure 1).

Figure 1: Asthma and your airways (from NIH Medline plus, http://www.nlm.nih.gov/medlineplus/magazine/issues/fall11/articles/fall11pg4.html). Lungs and airways of a normal individual (left), inflamed airway of an asthmatic (middle) and airway during and asthma attack (right). In a normal airway airflow is unobstructed, while in asthmatics the airway wall is thickened due to inflammation and edema, and increased mucus production by goblet cells in the airways also contributes to narrowing of the airways. In response to different stimuli, the smooth muscle in an asthmatic airway contracts heavily and excess mucus is produced. As a result, the diameter of the lumen is decreased, and the airway is obstructed, leading to shortness of breath.

The prevalence of asthma has increased notably over the last decades, and the World Health Organization estimates that 235 million people currently suffer from asthma worldwide (2). In The Netherlands, more than 500,000 people have asthma, generating substantial costs, both direct with medical care, and indirect by loss of productivity. Airway hyperresponsiveness and inflammation of the airways are hallmarks of asthma, but the type and severity of these parameters varies according to the form of the disease.
There is often a link between asthma and allergy and many asthma patients have allergic asthma, but other presentations of asthma are also common, such as nonallergic asthma. Different manifestations of disease, also called phenotypes, are determined by genetic and environmental factors and overlap or changes in phenotypes may occur over time. Understanding the heterogeneity of asthma is important since the long-term prognosis and responsiveness to treatment may differ with respect to the different phenotypes (3). In this thesis, the focus will be on the allergic and nonallergic/occupational phenotypes of asthma.

In the past decades, much research has been done on the mechanisms behind the development of asthma and although better understanding has been achieved, the mechanisms that underlie the disease are not yet fully understood. In general, cells of the innate and adaptive immune system act together with epithelial cells and smooth muscle to cause airway hyperresponsiveness, mucous hypersecretion and airway wall remodeling, contributing to airway obstruction, which leads to the typical asthma symptoms (4).

**Allergic asthma**

In allergic individuals, the immune system recognizes harmless compounds that are inhaled (such as house dust mites, animal dander, pollen) as a threat and elicits an immune response to these compounds, in such case called allergens. In this type of asthma, allergic sensitization is present, which is defined by the presence of serum immunoglobulin E (IgE) antibodies and/or a positive skin-prick test to proteins of commonly inhaled or ingested allergens. When an allergen is inhaled by predisposed individuals, pattern recognition receptors present on the epithelial cells lining the respiratory tract detect these stimuli, activating epithelial cells to secrete the inflammatory mediators interleukin (IL)-33, IL-25, granulocyte-macrophage-colony stimulating factor (GM-CSF) and thymic stromal lymphopoietin (TSLP) that will activate antigen presenting dendritic cells to promote T helper 2 (Th2) response (4,5). These activated dendritic cells take up the antigen and migrate to the draining mediastinal lymph nodes, where they present the antigen to naïve T cells, inducing Th2 differentiation and proliferation. Th2 cells produce the cytokines IL-4, IL-5 and IL-13 which induce the pathophysiological hallmarks of asthma. IL-4 inhibits differentiation into Th1 lymphocytes, induces differentiation of mast cells and B cells and further promotes Th2 proliferation. Moreover, IL-4 induces immunoglobulins produced by B cells to switch to IgE. IL-5 supports the development of eosinophils in the bone marrow, and induces chemotaxis of eosinophils to the lungs. IL-13 contributes to IgE production, induces goblet cell metaplasia and airway hyperresponsiveness (4). After its production, IgE binds to receptors on the surface of mast cells and remains there until the next allergen exposure. When individuals are re-exposed to the same allergen, antigens bind directly to the IgE on mast cells, inducing cross-linking of IgE receptors and subsequently the release of histamine, prostaglandins and leucotrienes. These inflammatory mediators bind to their specific receptors on airway smooth muscle cells, inducing contraction and consequent narrowing of the airways (bronchoconstriction) and shortness of breath. The chronicity of the Th2 inflammation in asthmatic patients is thought to be caused by a defect in immune tolerance, which is the mechanism that prevents excessive immune responses in healthy individuals. Specific phenotypes of dendritic cells can induce
tolerance to inhaled allergens (6,7), but the main cell type involved in down regulation of excessive immune responses is the regulatory T cell (8). Studies on numbers and functionality of regulatory T cells in asthmatics show contradictory results. While some studies show decreased numbers of Treg cells in blood and bronchial alveolar lavage fluid (BALF) of asthmatic patients as compared to healthy controls (9,10), a recent study demonstrated higher numbers of Treg cells in blood of asthmatic children, suggesting that Treg cells may be increased to control exuberant allergic responses (11).

Although asthma is typically a Th2 disease, cells from the innate immune system, such as dendritic cells, basophils and macrophages, play an important role in the disease. Macrophages are one of the most abundant cells in the respiratory tract and, according to their localization, can be divided into two populations: alveolar and interstitial macrophages, with distinct functions and possibly distinct origins (12). Nevertheless, both alveolar and interstitial macrophages are capable of quickly dealing with threats to lung homeostasis, adopting effective phenotypes based on signals from the surrounding tissue. There is an ongoing debate regarding the classification of the phenotypical changes adopted by macrophages (12,13). For the purpose of this thesis the following classification was adopted (figure 2) (12,14,15):

- Classically activated macrophages or M1 macrophages: this phenotype develops after exposure to IFNγ and TNFα, or lipopolysaccharide (LPS), under the influence of the transcription factor IRF5 (16), resulting in a macrophage population that has enhanced microbicidal or tumoricidal capacity. These cells are important in host defense against intracellular pathogens by amplifying the Th1 response (by production of IL-12, IL-1β and TNFα), and by generating reactive oxygen species (ROS) and nitric oxide (NO)(17).
- Alternatively activated macrophages or M2 macrophages: these cells are induced by IL-4 and IL-13 and are important in Th2 responses such as asthma and helminth infections and in wound healing by contributing to the production of extracellular matrix (14).
- Regulatory macrophages or M2-like macrophages: a close sibling of the M2 macrophages, these cells are induced by Toll-like receptor (TLR) stimulation in combination with a number of stimuli such as IL-10, prostaglandin E2 (PGE2) and glucocorticoids. Regulatory macrophages produce high levels of IL-10, exerting anti-inflammatory functions which are beneficial in the late stages of immune responses, limiting inflammation (14).

The involve ment of both M1 and M2 macrophages in asthma has been demonstrated. Levels of M1 inducers (IFNγ, LPS and TNFα) are higher in asthmatics, especially in those with severe forms of the disease (18,19), linking this macrophage phenotype to nonallergic and/or severe asthma. On the other hand, M2 macrophages have been shown to be related to allergic asthma. Higher percentages of macrophages expressing the M2 activation markers mannose receptor and transglutaminase 2 (TGM2) are found in bronchial biopsies of asthmatic patients in comparison to healthy controls (20,21). Furthermore, elevated levels of chitinase family members, produced by M2 macrophages were found in serum and lung tissue of asthmatics, suggesting an increased numbers and/or increased activity of these cells in asthma. In mice, these cells are recognized by
the chitinase-like protein YM1, also known as eosinophil chemotactic factor (ECF-L). Mouse studies have shown that asthmatic mice that received adoptive transfer of M2 macrophages had increased airway inflammation as compared to asthmatic mice that did not receive this macrophage phenotype (22,23), suggesting that M2 macrophages contribute to the pathogenesis of asthma. Indeed, it has been shown that M2 macrophages contribute to tissue recruitment of eosinophils (24). Treatment with M2 inhibitors have decreased airway hyperresponsiveness, airway inflammation, mucus cell proliferation and collagen deposition (25,26) suggesting that M2 macrophages could be a promising target for asthma treatment. Regulatory macrophages could play a role in resolution of asthma due to their production of anti-inflammatory cytokines IL-10 and TGFβ. Indeed, it has been shown that macrophages from asthmatic patients produce less IL-10 than macrophages from healthy subjects (27), suggesting that impairment in the activity of regulatory macrophages could contribute to the persistence of the disease.

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Figure 2: Schematic representation of the three macrophage phenotypes and their characteristics IFNγ: interferon gamma; TNFα: tumor necrosis factor alpha; LPS: lipopolysaccharide; MHC class II: major histocompatibility complex class II; IL: interleukin; NO: nitric oxide; IRF5: interferon regulatory factor 5; Fe: iron; TGM2: transglutaminase 2; YM1: chitinase-3-like protein-3; FIZZ1/Relm: resistin-like molecule-α; Arg-1: arginase-1; TGFβ: transforming growth factor beta; TLR: Toll-like receptor; PGE2: prostaglandin E2; PPARγ: peroxisome proliferator-activated receptor gamma. (from Boorsma et al (12))

Additional cardinal features of asthma are changes in the airway wall structure. Asthmatic airways often show increased thickness of the muscle layer which contributes to bronchoconstriction and increased deposition of extracellular matrix proteins, such as
collagen, resulting in thickened basal membrane. Furthermore, the increased numbers of goblet cells, and the consequent excess in mucus production, add to narrowing of the airways. These changes in lung structure altogether are called remodeling of the airway.

**Nonallergic asthma**

Nonallergic (intrinsic) asthma has, per definition, neither IgE reactivity to allergens in the serum nor obvious involvement of Th2 cells. This form of disease is often more severe and difficult to treat, being related to resistance to inhaled corticosteroids and requiring long-term treatment with systemic corticosteroids. In general, nonallergic asthma develops later in life and is more common in women, often associated with obesity (4). The mechanisms underlying nonallergic asthma are less well understood than the ones playing a role in allergic asthma. It is known that while in allergic asthma the eosinophils are the most prominent cells in the lungs, in nonallergic asthma the neutrophils predominate. This may contribute to corticosteroid resistance in nonallergic asthma, since several studies in mouse models of asthma have shown that neutrophils have a poor response to corticosteroids (28-30). The proinflammatory cytokine IL-17A has also been shown to be related to corticosteroid resistance (29,31,32). This cytokine is the signature cytokine of Th17 cells, a distinct population of T helper lymphocytes that differentiate in the presence of IL-6, IL-21, IL-23, transforming growth factor (TGF)β and IL-1β and produce the cytokines IL-17A, IL-17F, IL-21 and IL-22 (33). IL-17 contributes to asthma pathogenesis by stimulating the production of inflammatory cytokines, including CXCl chemokines capable of recruiting neutrophils (29,32,34-37), as well as by promotion of smooth muscle contraction (34), and increase of mucus production (38). Several studies suggest the involvement of IL-17 in asthma and a relation with asthma severity. IL-17 levels are increased in BALF and sputum of asthmatics (39) and correlate with asthma severity (40,41). A recent study has shown that children with nonallergic asthma have an IL-17 shifted immunity, promoting neutrophil inflammation and less functional regulatory T cells (11).

A substantial proportion of occupational asthma is known to be nonallergic, and it is highly prevalent in farmers and those in farm-related occupations. Although the pathophysiology of occupational asthma is still unclear, it is well-established that it is induced by an acute inflammatory response, involving the cytokines IL-1, IL-6, IL-17, CXCL-1 and TNFα and the subsequent massive infiltration and activation of neutrophils in the airways, which is very similar to nonallergic asthma in the general population (42-45); (44,46). Occupational asthma seems to be a sub-phenotype of nonallergic asthma. Clinicians now realize that the classification of asthma in allergic or nonallergic is an oversimplification. Different asthma phenotypes are presently being defined as asthma endotypes, differing in genetic susceptibility, age of onset, molecular mechanisms, clinic presentation, environmental risk factors, prognostic and response to treatment (4,47).

**Hygiene Hypothesis**

Although it is well-established that farmers and agricultural workers have a higher risk for development of lung diseases such as nonallergic asthma, chronic bronchitis and chronic obstructive pulmonary disease (COPD) (48,49), several studies have shown a protective effect of farming on the development of atopy and allergic asthma (50-53). This is in line
with the theory proposed by Strachan in 1989, in which he stated that the increased prevalence of hay fever and eczema, both allergic diseases, could be explained by the decrease in family size and higher standards of personal cleanliness. According to this theory, children with more siblings have more microbial exposure in early life, preventing the development of allergic diseases (54). As a consequence, children in westernized countries, protected from infectious burdens in early life that are common in the developing world, suffer an increased risk of developing allergic disease (55). The proposed immunological mechanism behind this “Hygiene Hypothesis” is that the immature immune system is Th2 biased at birth (56), and to develop a healthy balance between Th1 and Th2 responses, the Th1 response has to develop early in life. To initiate this development, certain microbial exposures are needed (57). Another proposed mechanism is that exposure to microbes induces IL-10 secretion by monocytes, macrophages and dendritic cells. Given the anti-inflammatory effects of IL-10, microbial exposure would be beneficial in dampening both Th2 and Th1 responses with the aim of achieving homeostasis (55).

Similar protection against allergic diseases is seen in children that go to daycare centers and children and adults exposed to farm environments. Epidemiological studies showing this protective effect in farmers and agricultural workers may however be biased by healthy worker survival, a phenomenon in which workers who have health problems, leave the workplace and choose a job with no or less exposures.

In this thesis we investigated both effects of farm exposures on the immune system: the protective effects against allergic asthma and the induction of nonallergic asthma. This investigation was performed in agricultural workers and in mouse models of lung inflammation. Additionally, we studied healthy worker survival effect in a five-year follow-up study of a cohort of Dutch agricultural workers.

Mouse models of asthma

To study the pathophysiology of asthma in depth, animal models are needed, since for ethical reasons options for human experimentation are very limited. In addition, investigation upfront the disease development is hardly possible in humans, making the study of the etiology of disease very difficult (58). The mouse is the most frequently used animal model for asthma, sharing many similarities with human asthma regarding the response to allergen inhalation. In both mouse models and human asthma, allergen inhalation induces Th2 cytokine production, eosinophil migration, IgE production, mast cell degranulation, airway hyperresponsiveness and, in chronic stages, remodeling of the airways (59,60). Furthermore, mice are relatively inexpensive and easy to breed and to house, and the use of inbred mice with defined immunologic and physiologic properties are important advantages of the use of this animal model (59). However, there are critical differences between mouse models of asthma and human asthma. One major difference is that mice do not spontaneously develop asthma, thus, to study asthma in mice, an asthmatic-like reaction has to be induced by allergens.

The most commonly used model for allergic asthma is the model in which mice are intraperitoneally sensitized to ovalbumin (OVA) in combination with an adjuvant (a chemical compound that activates the immune system, inducing inflammation). After sensitization, mice are exposed to aerosolized OVA to induce the allergic response. This
model has contributed greatly to the increase of knowledge of mechanisms inducing asthma in mice and humans. Nonetheless, the OVA model does not reflect the human situation, which develops by re-exposure to allergens through the airways, with no adjuvant needed. Therefore, efforts have been made to develop allergen models that reflect the human disease, by using natural relevant allergens, such as house dust mites (HDM), grass pollen, cockroach or fungi, administered intranasally or intratracheally without the use of adjuvants, in a repetitive manner over a period of time. In these models, the process of allergic sensitization includes the activation of the innate immune system in the airways, reflecting the human situation (61).

In this thesis, we used the HDM model of allergic asthma and the farm dust extract (FDE) model to induce nonallergic asthma. The FDE model has been established to mimic the exposures of farmers and agricultural workers, and basically, the dust collected in stables and barns is extracted and the extract is diluted in saline and is given intranasally to mice (62-66).

Although mouse models provide several advantages to study the mechanisms of asthma, differences between mice and men should always be considered, to avoid that lessons learned from mouse models are misleading. Potential targets discovered in mouse studies should be translated to the human situation and studying these targets in humans, first in an in vitro setting and then in experimental medicine and clinical trials.

Scope of this thesis

Farmers and agricultural workers have a higher risk of developing nonallergic lung inflammation, despite evidence of protection against allergic asthma and other atopic diseases. Asthma is a heterogeneous disease with several phenotypes and endotypes that differ in genetic susceptibility, environmental exposures, underlying mechanisms, cell types involved, severity and response to treatment.

The aim of this thesis was to investigate the mechanisms underlying allergic and nonallergic lung diseases, in: (a) a mouse model of allergic airway inflammation, (b) a mouse model of farm dust extract-induced nonallergic asthma (c) agricultural workers and (d) healthy non-exposed controls. In chapter 2 the question was addressed whether exposure to farm dust extracts from different farms induces different types of airway inflammation and T-cell polarisation in a mouse model and in agricultural workers from the same farms compared to healthy non-exposed controls. Furthermore, the protective effect of different farm dust extracts on the HDM-induced allergic airway inflammation was investigated. Chapter 3 addresses the question whether healthy worker survival could explain the finding that microbial exposures in farmers and agricultural workers are associated with less atopy. In chapter 4 the effects of fluticasone propionate on nonallergic lung inflammation and hyperresponsiveness induced by farm dust extract were studied. Next, the focus was on macrophages and their role on lung inflammation. In chapter 5 macrophage phenotypes were characterized and quantified using immunohistochemistry in three different murine models of HDM-induced allergic asthma and the question was addressed whether different macrophage phenotypes correlated with disease severity. In chapter 6, lung macrophage populations were assessed in established murine models of allergic and nonallergic lung inflammation by means of FACS and immunohistochemistry. Chapter 7 focuses on the question whether farm dust extract
exposure exerts a direct effect on macrophages, inducing phenotypical changes that could explain the protective effect of farming on the development of allergic inflammation. Chapter 8 summarizes the findings of this thesis and places these in perspective of current and future research.
References


Introduction


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