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Novel insights in occupational asthma due to persulfate salts

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DOCTORAL THESIS

Novel insights in occupational asthma due to persulfate salts

Nous coneixements en l'asma ocupacional degut a sals de persulfat

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“Panta rei kai oudén ménei” (πάντα ρεῖ καὶ οὐδὲν μένει)

Todo fluye, nada permanence

(Heráclito de Éfeso, 535 a.C. - 475 a.C.)

LIST OF ABBREVIATIONS

AHR	Airway Hyperresponsiveness
AP	Ammonium Persulfate
APCs	Antigen-presenting Cells
ASM	Airway Smooth Muscle
ATP	Adenosine Triphosphate
AUC	Area Under the Curve
BAL	Bronchoalveolar Lavage
BMMCs	Bone Marrow-derived Mast Cells
BSA	Bovine Serum Albumin
CXCR2	Chemokine Receptor-2
cys-LTs	Cysteinyl-Leukotrienes
DCs	Dendritic Cells
DELFIA	Dissociation-Enhanced Lanthanide Fluorescent Immunoassay
DMSO	Dimethylsulfoxide
ELISA	Enzyme-linked Immunosorbent Assay
FCεRI	High affinity IgE receptor
FOT	Forced Oscillation Technique
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
H&E	Haemotxilin&eosin
HDM	House Dust Mite
HMW	High Molecular Weight
HSA	Human Serum Albumin
i.p.	Intraperitoneal
ICS	Inhaled Corticosteroid
Ig	Immunoglobulin (e.g. IgE)
IL	Interleukin (e.g. IL-4)
IL-17RA	Interleukin-17 receptor A
IL-5Rα	Interleukin-5 receptor α
ILC2s	Type-2 Innate Lymphoid Cells
ILCs	Innate Lymphoid Cells
IU	International Unit
KO	Knock-out
KP	Potassium Persulfate
LABA	Long-acting beta2-agonist
LDIIA	Low Dose Irritant-Induced Asthma
LLNA	Local Lymph Node Assay
LMW	Low Molecular Weight
mAb	Monoclonal Antibody
MCP-1	Monocyte Chemoattractant Protein 1
mDCs	Myeloid Dendritic Cells
MHCII	Major Histocompatibility Complex class II

NaP	Sodium Persulfate
NF-kB	Nuclear Factor kB
OA	Occupational Asthma
OmAb	Omalizumab
ORMDL3	Orosomucoid like 3
OVA	Ovalbumin
PARs	Protease-activated Receptors
PBS	Phosphate Buffered Solution
Penh	Enhanced Pause
PRRs	Pattern Recognition Receptors
R	Resistance
RADS	Reactive Airways Dysfunction Syndrome
ROS	Reactive Oxygen Species
SAL	Saline
SD	Standard Deviation
SEM	Standard Error of the Mean
SEPAR	*Sociedad Española de Neumología y Cirugía Torácica*
SI	Stimulation Index
Tc	T Cytotoxic
TCR	T Cell Receptor
TDI	Toluene Diisocyanate
TGF-β	Tumor Growth Factor β
Th	T Helper (e.g Th2, T Helper type 2)
TLR	Toll-like Receptors
TMA	Trimellitic Anhydride
TNFα	Tumor Necrosis Factor α
Tregs	T Regulatory Cells
TRP	Transient Receptor Potential
TRPA1	Transient Receptor Potential Ankyrin 1
TRPV1	Transient Receptor Potential Vanilloid 1
TSLP	Thymic Stromal Lymphopoietin
TV	Tidal Volume
WEA	Work-Exacerbated Asthma
WRA	Work-Related Asthma

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1. INTRODUCTION

1.1 Asthma

1.1.1 Definition and prevalence

Asthma is a heterogeneous disease, usually characterized by chronic airway inflammation. It is defined by a history of 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 and associated with airway hyperresponsiveness (AHR), which may resolve spontaneously or in response to medication (1). Classically, asthma has been considered as an illness associated with atopy and/or allergic reactions, which begins in childhood and may or may not persist into adulthood (2). However, there is increasing evidence that asthma is a multifactorial disease with a heterogeneous and variable clinical expression which may manifest at any age. This indicates that, rather than being a specific disease entity, asthma may be constituted by several separate, overlapping syndromes, probably with different causes and natural histories. This heterogeneity is influenced by multiple factors including age, sex, socioeconomic status, race and/or ethnicity, and gene-environment interactions (3).

Asthma is now one of the commonest chronic diseases worldwide. Its prevalence is increasing in many countries, especially in westernized societies due to the high degree of industrial development (4,5). According to The Global Asthma Report, 334 million people suffer from asthma and, over the next two decades, the population of sufferers is likely to increase by an additional 100 million as communities adopt modern lifestyles and become urbanized (6). In Europe, about 30 million children and adults under 45 years of age have asthma; its prevalence is 8.2% in adults and 9.4% in children (7,8). The Spanish Society of Pneumology and Thoracic Surgery (SEPAR) reports similar results, with prevalence around 5% in the adult population and above 10% in children, and an increasing trend in the last 20 years in both populations (9). Although mortality rates due to asthma worldwide have fallen notably over the past 25 years, there are currently no therapeutic regimens that can cure the disease and its burden is likely to rise in line with its increasing prevalence of the disease (10).

The reasons for the growing prevalence of asthma have not been defined, although associations with a wide range of risk factors have been reported. Genetic factors by themselves cannot explain the rapidity of this epidemiological shift, as changes in populations are too slow to have such an effect (11,12). Lung, gut and skin are continuously exposed to the external environment and are directly involved in asthma development; for this reason, the environment is widely accepted as a key determinant of asthma pathogenesis (12). Although the importance of gene-environment interactions in the expression of disease has recently been highlighted, the analysis of these relationships from a functional perspective has proved to be a real challenge.

Asthma symptoms may represent a major burden, not only in terms of morbidity and reduced quality of life, but also in terms of healthcare costs (1). Like other chronic diseases, asthma has

both significant direct costs associated with medication and health care and significant indirect costs as well, especially due to reduced work productivity (13,14). The annual financial burden attributed to asthma in Spain is nearly 1.7 million Euros and the SEPAR estimates that the total cost of an adult patient ascends to 1,950 Euros per year (9). Among children, missing school days due to asthma is associated with suboptimal asthma control, urgent or emergent asthma-related healthcare utilization, mold in the home, and financial barriers to asthma-related health care (15). In adult asthma, demographic characteristics such as poverty, low educational attainment, female gender, race and urban environments are associated with greater healthcare costs and reduced quality of life and housework activities. The indirect costs of this disease are principally incurred from the complete cessation of employment or low productivity due to impairment and illness (13).

1.1.2 Inflammation and remodeling

Asthma may manifest itself as a short single attack that disappears spontaneously, as a single, more severe attack, or as successive crises over several days. Despite this marked heterogeneity, chronic inflammation is a hallmark of the disease and is regulated by the interaction of cells from both the innate immune system (dendritic cells, mast cells, eosinophils and neutrophils) and the adaptive immune system (T cells and B cells) (3). Structural cells such as lung epithelial cells have a barrier function but may also secrete an array of cytokines instructing antigen-presenting cells (APCs) to mount a specific immune response. Together, this leads to recruitment of inflammatory cells and release of inflammatory mediators (16). Classically, the cell profile of patients with asthma has been characterized by airway eosinophilia, although it may also be neutrophilic or contain only a few inflammatory cells (paucigranulocytic) (1).

Airway inflammation causes typical clinical symptoms such as recurrent episodes of wheezing, breathlessness, chest tightness and cough, particularly at night and/or in the early morning (1). These episodes are usually associated with widespread but variable airflow obstruction that often reverses, either spontaneously or with treatment. Chronic inflammation is also associated with structural changes in the airways that increase the existing AHR to a variety of stimuli, and with the development of airflow limitation as the result of bronchoconstriction, airway edema, mucus secretion and airway wall remodeling (17). Specifically, structural airway changes including airway wall thickening, increased airway smooth muscle (ASM) mass, thickened basement membranes, subepithelial fibrosis, alterations in extracellular matrix, vascular proliferation and glandular hypertrophy are collectively referred to as airway remodeling (18,19). Initially, airway remodeling is a result of chronic inflammation, but may persist independently of ongoing inflammation; its persistence correlates with persistent AHR (20).

1.1.3 Asthma phenotypes

It has been suggested that genetic factors (atopy) (21,22), environmental factors (allergens, viruses, air pollution and occupational exposures) (23-25) and life style (smoking, diet and so on) (26,27) contribute to the development of asthma. Atopy, defined as the genetic tendency toward a hyperproduction of immunoglobulin (Ig)-E antibodies against aeroallergens and the subsequent development of allergic diseases, is associated with type I hypersensitivity reactions against common environmental allergens, via the production of specific IgE antibodies (28). This is the case of allergic or atopic asthma, the most easily recognized asthma phenotype, which often commences in childhood and is associated with a past or family history of allergic disease. These patients usually respond well to inhaled corticosteroid (ICS) treatment (1).

Nevertheless, clinical guidelines reflect the heterogeneity of asthma by defining multiple levels of severity and by dividing patients into categories or asthma phenotypes. In recent years, asthma phenotypes have been defined on the basis of clinical or physiological characteristics (severity, age at onset, degree of obstruction and resistance to treatment), by asthma triggers (exercise, allergens, aspirin-induced, menstruation) or on the basis of the type of inflammation (eosinophilic, neutrophilic or paucigranulocytic) (29,30). Asthma phenotypes were initially focused on combinations of clinical characteristics, which may provide only a partial explanation of the full complexity of the condition.

In this context, little is known about triggers of disease, genetic susceptibility and interaction with the environmental factors. This situation limits the current descriptions of asthma phenotypes. A stronger system for classifying asthma considering its multidimensionality is needed to identify subgroups with consistent patterns of disease. This would help to identify novel therapeutic targets and biomarkers that meet formal diagnostic and prognostic criteria, and also better predictors of response to treatment (29).

Two strategies have been tested in recent years in order to implement this approach. The first is the identification of asthma phenotypes based on statistical analyses in order to identify single groups or clusters (31-33). This clustering approach involves a group of multivariate mathematical algorithms that broadly quantify the similarity between individuals within a population on the basis of the (multiple) specified variables (31). In this regard, Moore *et al.* identified five clusters of asthma; all the groups contained patients who met the American Thoracic Society definition of severe asthma, supporting the clinical heterogeneity of asthma and the need for new approaches to classify disease severity (32). Additionally, Siroux *et al.* distinguished four asthma phenotypes in two large epidemiological studies and found that the phenotypes clearly discriminated between populations in terms of quality of life and blood eosinophil and neutrophil counts. The authors concluded that these homogeneous phenotypes may help to better identify novel risk factors (both genetic and environmental) and may contribute to a better understanding of the disease (33).

The second strategy defines different cohorts based on the natural history of the disease. Currently, there are three cohorts in Europe (34-36) and one in America (37,38). Ongoing studies of large-scale molecularly and genetically focused and extensively clinically characterized clusters or cohorts of asthma should enhance our ability to molecularly understand these clusters and lead to more targeted and personalized approaches to asthma therapy (29). For example, U-BIOPRED is a European Union consortium of academic institutions, pharmaceutical companies and patient organizations which assesses adults with severe asthma, mild/moderate asthma and healthy controls. Recently, it has been shown that these patients with severe asthma have more symptoms and exacerbations, accompanied by worse quality of life, and more anxiety and depression than patients with mild/moderate disease. Moreover, like other severe asthma cohorts, U-BIOPRED is characterized by poor symptom control, increased comorbidity and airway inflammation (mainly eosinophilia), despite high levels of treatment (36). Nevertheless, all these asthma cohorts are mainly focused on severe or refractory adult asthma and further cohort studies involving all types of asthma are necessary.

1.1.4 Immunology of asthma

Three major types of innate and adaptive cell-mediated immunity have very recently been identified that protect the host against distinct pathogens and pathogenesis of inflammatory diseases. Within each type of response the innate lymphoid cells (ILCs), which lack the T-cell receptor, and T-helper (Th)/T-cytotoxic (Tc) cells from the adaptive immune system share transcription factor and effector cytokine expression, suggesting that these three types of immunological programs are indispensable and optimized to cope with different types of pathogenic challenges. However, the distinct activation signals, tissue location, action timing and polarizing signals during an immune response imply that T cells and ILCs do not play completely redundant roles, and it will be a challenge to elucidate the precise contribution of the innate *versus* the adaptive arms of cell-mediated effector immunity (39) (further details of effector immunity are shown in *figure 1*).

Classically, asthma has long been considered the hallmark Th2 pathologic disorder of the airways, although it is now known that this definition only applies to the allergic asthma phenotype. Currently, although a steadily increasing number of protein allergens have been sequenced, only a very small percentage of the total proteins from the environmental sources our immune system encounters elicit an allergic reaction due to molecular interactions between the allergen and its corresponding IgE antibody (40). What initiates the inflammatory process in the first place, and makes certain people susceptible to its effects, is an issue that is currently under investigation. The expression of asthma is a complex, interactive process that depends on the interplay between two major factors - host factors (particularly genetics) and environmental exposure that occur at a crucial time in the development of the immune system.

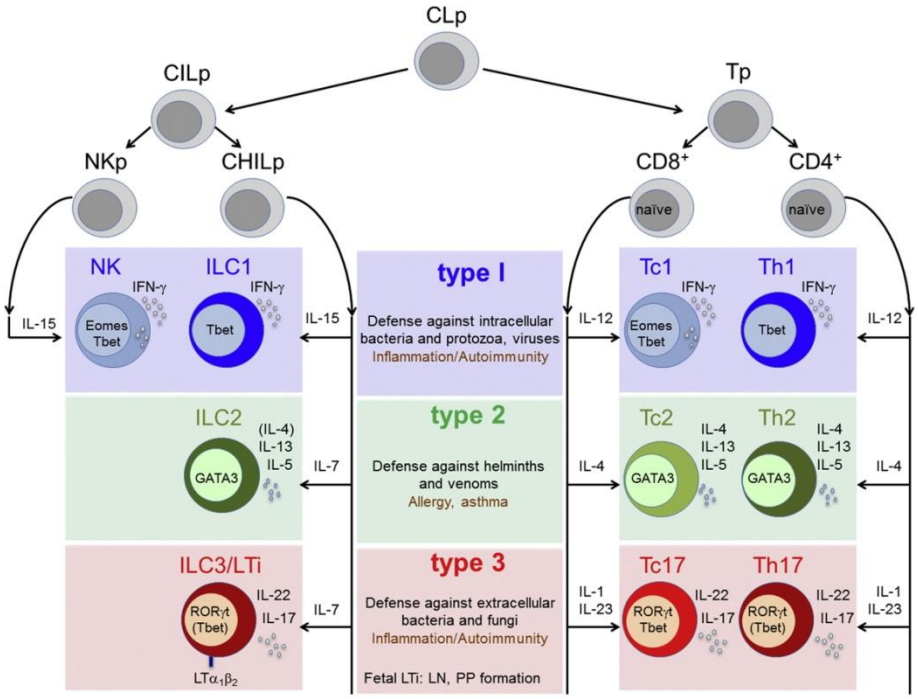

Figure 1. The three major types of innate and adaptive cell-mediated effector immunity. CILp, common innate lymphoid precursor; CLp, common lymphoid precursor; LN, lymph node; LTi, lymphoid tissue inducer; PP, peyer patch; Tp, T-cell progenitor (39).

At a first contact, allergens can disrupt the epithelial barrier function of the airways and trigger epithelial cells by allergen exposure through pattern recognition receptors (PRRs) and protease-activated receptors (PARs), and through induction of reactive oxygen species (ROS), thus activating the epithelial nuclear factor kB (NF-kB) signaling pathway. Epithelial activation leads to the production of chemokines, cytokines, and endogenous danger signals that recruit and activate innate immune cells, such as dendritic cells (DCs), type-2 innate lymphoid cells (ILC2s), eosinophils, and basophils. DCs are a specialized population of APCs (or allergen-presenting cells) which are located in association with the airway epithelium and underlying mucosa in the airways, and have the potential to take up and present inhaled allergens to $CD4^+$ and $CD8^+$ T cells when they migrate to the draining mediastinal lymph nodes (41). DCs process allergens into small peptides and then present them via the major histocompatibility complex class II (MHCII) for recognition by T cell receptors (TCR), leading to T-lymphocyte activation, division and differentiation. The T-cell side of this synapse is focused on CD3 and the TCR, which bind specifically to the peptide/MHCII complex, as well as CD4 molecules that stabilize the interaction (42). Upon recognition, the Th2 polarized cells will produce cytokines involved in asthma pathogenesis such as interleukin (IL)-4 and IL-13 which are required to drive the isotype switch of B-lymphocytes into plasmocytes producing allergen-specific IgE antibodies. Together with IL-9, these cytokines play an important role in mast cell development, mucus overproduction and AHR (43) (*figure 2*).

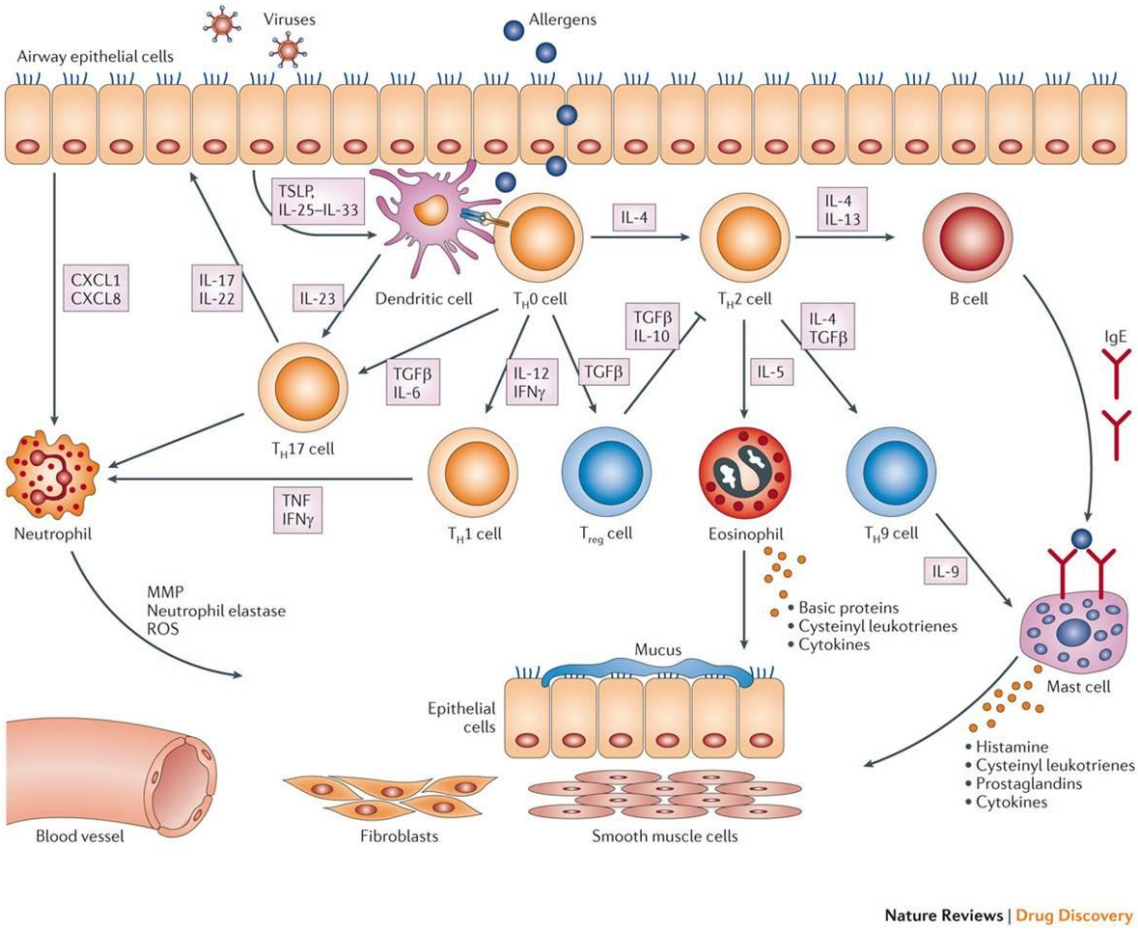

Figure 2. Overview of immune response in allergic disease. CXCL1, chemokine CXC motif ligand 1; IFNγ, interferon-γ; IgE, immunoglobulin E; IL, interleukin; MMP, matrix metalloproteinase; ROS, reactive oxygen species; Th0, T helper 0; TGFβ, transforming growth factor β; TNFα, tumor necrosis factor-α; Treg, T regulatory; TSLP, thymic stromal lymphopoietin (44).

The resulting allergen-specific IgE antibodies bind to circulating mast cells via the high affinity IgE receptor (FcƐRI). Re-exposure to a previously encountered allergen leads to its cross-linking on mast cell-bound specific IgE, resulting in the degranulation of the mast cells and subsequent release of preformed and synthesized mediators (histamine, prostaglandins and leukotrienes, enzymes, cytokines and chemokines) (45). This constitutes an acute or early-phase asthmatic response characterized by constriction of the airway smooth muscle cells and endothelial cells, mucus production and vasodilatation. A few hours later (4-6h), a secondary response is initiated by the mast cells together with Th2-lymphocytes secreting other cytokines (IL-3, IL-5 and granulocyte macrophage colony stimulating factor (GM-CSF)), attracting and activating inflammatory cells such as eosinophils and basophils. Although the main focus in asthma has been their roles as inflammatory cells, a growing body of data suggests that these cells also function as APCs to initiate or enhance Th2 responses (41). These cells release a variety of lipid mediators (leukotrienes and prostaglandin D2), cytokines and chemokines contributing to the late asthmatic response which is characterized by chronic inflammation, mucus hypersecretion and further structural airway remodeling due to repetitive cycles of tissue damage and

inflammatory cell recruitment. The excessive inflammation results in structural changes in the airway architecture: airway wall thickening, subepithelial fibrosis which contributes to the thickening of airway walls due to collagen deposition, among other factors, increased vascularity, goblet cell hyperplasia, airway smooth muscle cell hyperplasia or hypertrophy, and epithelial hypertrophy (*figure 3*) (43). The physiological consequences of these changes remain uncertain, in part because these changes are not fully reversed by current asthma therapy. However, airway remodeling is postulated to be a determinant of AHR which can be mediated through several mechanisms, including altered neural regulation or increased contractility of airway smooth muscle cells (46). Airway remodeling can also contribute to AHR through purely mechanical means (47).

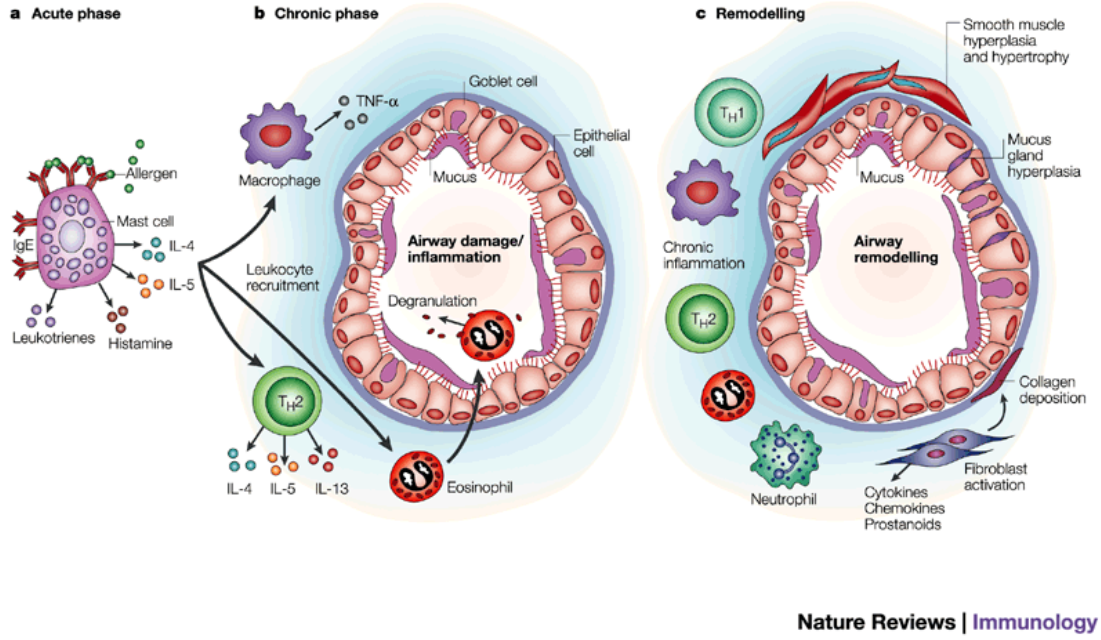

Figure 3. Airway inflammation in asthma. Asthma is a complex process involving progression from acute events (**a**) such as allergen-induced activation of mast cells to release pro-inflammatory cytokines and mediators, leading to acute bronchoconstriction and airway obstruction, to chronic inflammation (**b**) characterized by activation of Th2 cells and macrophages, and recruitment and degranulation of eosinophils. The changes in the airway cause not only airflow obstruction but also an increase in airway responsiveness. Finally, some subjects present a further progression of the inflammatory changes towards airway remodeling (**c**); this can lead to permanent alterations in the airway architecture which make obstructive events irreversible. IgE, immunoglobulin E; IL-4, interleukin 4; TH1, T helper 1 cells; TNF-α , tumor necrosis factor-α (48).

1.1.4.1 Other cells involved in asthma

Until recently, the mechanisms and the mediators involved in asthma only referred to allergic or atopic asthma. However, it is now clear that there are different asthma phenotypes, each with a distinct pathophysiology, which are defined as asthma endotypes (49). Some airway inflammation is eosinophilic in nature regardless of whether or not there is an allergic process. Nonallergic eosinophilic asthma may be controlled by ILC2 cells, which resemble Th2 cells in many ways. ILC2 cells lack antigen-specific receptors but, like Th2 cells, they react to epithelium-derived cytokines (IL-25, IL-33 and thymic stromal lymphopoietin (TSLP)) and

produce Th2 cytokines (IL-13, IL5 and IL-9) contributing to tissue eosinophilia and mucus production. As ILC2 cells produce little IL-4, there is no associated IgE response from B cells (50). Eosinophils contribute to the pathophysiology of asthma via cysteinyl-leukotrienes (cys-LTs) but are less important as a source of these mediators than mast cells. They may also act as APCs and produce several Th1 and Th2 cytokines. Besides, eosinophils express high levels of tumor growth factor (TGF)-β, which has been linked to subepithelial fibrosis in asthma (51).

There is increasing evidence that certain phenotypes of asthma, such as the late-onset and severe forms, are more likely to be associated with an influx of neutrophils in the airways (52,53), which may be enhanced by factors such as environmental pollution (54), psychological factors such as stress or anxiety (55), presence of viral exacerbations (23), smoking (56) and even occupational exposure to chemicals (57,58). The role of neutrophils in allergic diseases is still uncertain and remains poorly explored. Airway neutrophilia may be increased in asthma as a result of high doses of corticosteroids, or it may be controlled by chemotactic factors and Th17 cells (59). Th17 cells are a distinct T cell lineage comprising type 3 cell-mediated effector immunity but their role in asthma has not been fully elucidated. It has been observed that allergic sensitization followed by a challenge in the airways induces a strong Th17 response in association with airway neutrophilia and AHR, but it also attenuates the allergic response by inhibiting DCs and chemokine (CCL11 and CCL17) synthesis when there has already been sensitization to an allergen (60). Moreover, an inverse relationship between IL-25 (known also as IL-17F) and IL-17A in regulating allergic airway responses has been described; neutralization of IL-25 correlates with a decrease in IL-13 but also matches with an increase in IL-17A and the prevention of AHR (61). Nevertheless, despite this protective role, IL-17A itself also enhances the contractile force of the airway smooth muscle, favoring AHR (50).

However, other cell subsets are reported to regulate some important pathways in asthma pathogenesis. This is the case of Th9 cells producing IL-9, an essential factor for AHR (62), regulatory T cells (Tregs), which are potent suppressors of inflammation, AHR, and airway remodeling along with IL-10 and TGF-β cytokines (50), or structural cells such as epithelial cells. Epithelial cells express a wide variety of inflammatory mediators and interact with the DCs, releasing key mediators such as TSLP, IL-25, and IL-33, which promote a Th2 bias in DC precursors. Moreover, they can interact directly with the environment and may be activated by pathogens and endotoxins through PRRs such as TLRs, thus enhancing or triggering an allergic response (43).

It is now apparent that more than one process may be modulated or regulated by different cell subsets. The view of eosinophilic asthma as an exclusive type 2 immune disorder or neutrophilic asthma as an exclusive Th17 disorder is probably an oversimplification seen only at the extremes of a continuous spectrum (50). In many cases, there is an overlap in the types of cytokines and symptoms found in an asthma phenotype. This is the case of non-allergic asthma, in which the mechanisms involved in its onset and development are not completely

known. In general, this type of asthma is characterized by a cellular profile containing neutrophils, eosinophils or only a few inflammatory cells (paucigranulocytic), and often requires treatment with higher doses of inhaled and/or oral steroids (1). It usually appears in adulthood, with a higher proportion of new cases among non-atopic patients (29,63), and is associated with a variety of triggering risk factors. The presence of harmful substances such as chemical or organic compounds in the workplace constitutes one of the risk factors considered in this thesis and it will be discussed in depth in the context of work-related asthma (WRA).

1.2 Occupational asthma

Exposure to specific agents present in the workplace can induce asthma or aggravate a pre-existing condition in a relatively large proportion of workers. This exposure may account for up to 25% of all cases of adult-onset asthma (64). This percentage is likely to increase, since new substances in the workplace and new work situations that were previously unrecognized as sources of exposure have recently been described (65). The lungs and the skin are primary targets for a diverse spectrum of work-related dusts, gases, fumes and vapors, which can all cause annoyance, irritation, eczemas, corrosive changes and/or sensitization, depending on the concentration inhaled, the duration and route of exposure and on their physical-chemical properties (66).

Classically, WRA has been one of the most prevalent lung-related occupational diseases in industrialized countries. It appears to be the result of multiple factors, including genetics, environment and behavior (57). Occupational asthma (OA) describes a subset of WRA that is caused by work, in contrast to work-exacerbated asthma (WEA) which refers to a condition worsened, by work but not caused by it (64) (*figure 4*). Bernstein *et al.* defined OA as follows: "*Occupational asthma is a disease characterized by variable airflow limitation and/or hyperresponsiveness and/or inflammation due to causes and conditions attributable to a particular occupational environment and not to stimuli encountered outside the workplace"* (67). OA refers to asthma occurring *de novo* caused by exposure in the workplace or the recurrence of previously quiescent asthma (i.e., asthma as a child or in the distant past that has been in remission) induced either by sensitization to a specific substance (known as sensitizer-induced OA) or by exposure to an irritant inhaled at work (known as irritant-induced OA) (68). WEA can be observed in patients with pre-existing or concurrent asthma that is worsened by work-related factors, regardless of the frequency or duration of the worsened asthma and of whether there are permanent changes in severity, or alternatively as the development of asthma that has been present in childhood or earlier life and now recurs due to agents in the workplace (69). Differentiating between the two entities is not easy, since OA may be diagnosed in patients with asthma prior to occupational exposure and WEA in patients whose asthma is not caused by occupational exposure but begins in adulthood when the individual is working. Nevertheless, the distinction is important, because the treatment and

prognosis of the two entities may differ significantly – as may the medical-legal implications, since WEA is not supported by workers' compensation systems in all jurisdictions (65,70).

Two types of OA have been described, depending on the existence of a latency period between the first exposure to the agent and the onset of asthma symptoms. OA with latency is immunologically mediated (allergic or sensitizer-induced OA) and sensitization to a workplace agent occurs after a latency period of months to years. Immunologic OA can be divided further into the classical IgE-mediated form and the more evasive poly-immunological non-IgE-mediated form. The second type is non-immunological OA without latency (irritant-induced OA) which is caused by exposure to irritant chemicals without any prior sensitization (71). The most common form of non-immunologically mediated OA is reactive airways dysfunction syndrome (RADS), which is initiated by a single acute exposure to a high concentration of an irritant gas, smoke, fume or vapor. The onset of symptoms within 24 hours of exposure and persisting for at least three months typically occurs as the result of a workplace accident or of a setting with poor ventilation and limited air exchange without any preceding respiratory complaints (72). An individual may also develop asthma known as low dose irritant-induced asthma (LDIIA), although this entity is less well documented. Its onset is delayed after repeated moderate- and/or low-intensity exposures to irritants, but few data are available on the frequency, risk factors, pathophysiology, management, and prognosis of this exposure (65;73) (*figure 4*). Differentiating between immunologically but not IgE-mediated OA, usually induced by chemicals, and LDIIA poses a diagnostic challenge when the two types of asthma appear after a latency period (74).

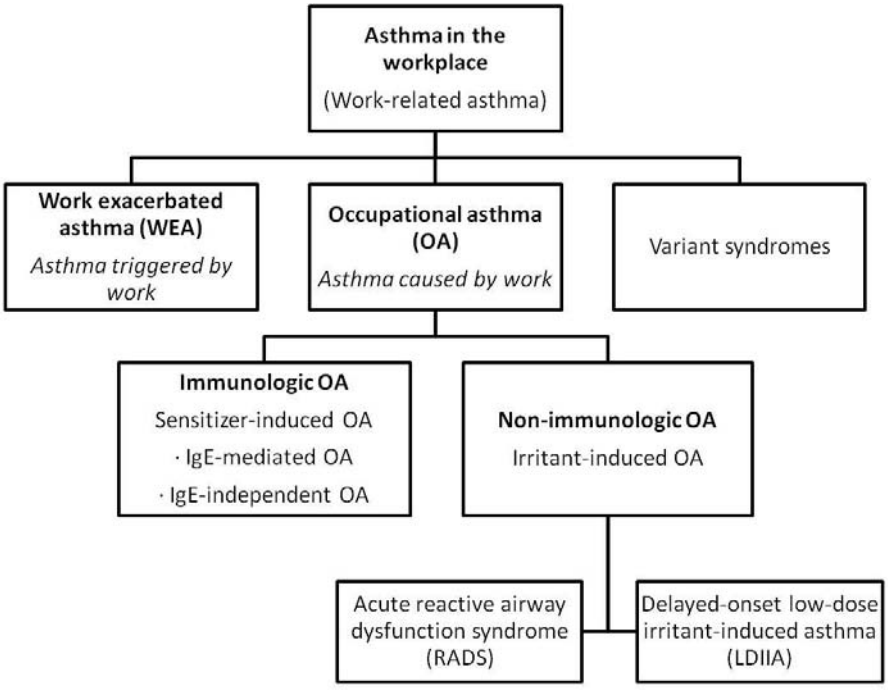

Figure 4. Definition and classification of work-related asthma (65).

Patients with OA frequently experience excessive time off work, workplace-specific severe disability, loss of income and job loss. Their asthma is frequently difficult with poor control despite high doses of treatment. Thus, proper prevention strategies are necessary to reduce the prevalence of occupational diseases and to minimize the serious health consequences and the strong socioeconomic impact of having to leave the workplace (75). Primary prevention aims to avoid sensitization to workplace agents, and thus prevent disease. Ideally, the workplace would have measures in place to ensure that workers do not inhale asthma-inducing agents, and would replace them with harmless substances. Unfortunately, though, many sensitizers cannot be replaced with non-sensitizing agents and so efforts are made to reduce exposure to respiratory sensitizers by instituting occupational hygiene measures such as containment, improved ventilation, and the use of personal protective equipment, as well as worker education to enhance adherence to recommended measures (24,76). Secondary preventive measures include screening and early identification of exposed workers by means of medical surveillance, which enables early diagnosis and removal from further exposure (77,78). At present, the most common measure for avoiding OA-induced symptoms is complete removal from the workplace (79). However, there is insufficient scientific evidence to assert that cessation of exposure improves asthma symptoms. In fact, it has been shown that, approximately 70% of affected workers with OA who completely avoid exposure still experience asthma symptoms (75,80-82). A possible alternative to full cessation of exposure is reduction, with the aim of minimizing the adverse socio-economic effects. This reduction can be achieved through relocation to less exposed jobs, improvement in workplace hygiene, modifying materials to reduce their allergenic properties, and the use of personal protective devices. Nonetheless, some studies indicate that reduced exposure seems to be less beneficial than removal of the patient from the workplace (82). Tertiary prevention acts once the disease has fully manifested itself and consists of measures aimed at softening the impact of long-term disease and disability. It may involve re-assigning a worker to a different job, supplying personal respiratory equipment, and providing anti-asthma medication (76).

In this context, it is important that employers recognize the problem and develop an appropriate policy regarding hazardous agents. It is their responsibility to educate workers about the risks of the agents used. Employers should also provide regular health surveillance and perform exposure monitoring. Often, people with low education may find it hard to find a new job, and employees working in small-sized companies have difficulty in relocating within the firm. Moreover, in several countries compensation for OA is insufficient, with the result that workers are not encouraged to report their symptoms to their employers. The most important forms of compensation include reimbursement of medication, retraining, unemployment or disability benefit (79). In Spain, a table of recognized occupational diseases appended to *Royal Decree 1299/2006* included a group caused by chemical agents, named *Occupational diseases caused by inhalation of substances and agents not included in other*

sections. In 2007, the health authorities estimated that asthma accounted for more than 50% of occupational respiratory diseases (83).

1.2.1 Immunological occupational asthma

More than 400 known causes of OA have been reported, and the list is growing continuously with the development of new technologies and the improved recognition of the diagnosis by physicians (67). Specific occupations associated with asthma include animal handlers, bakers, grain handlers, detergent and pharmaceutical industry workers, metal-refining workers, among others. It has been shown that an estimated 16.3% of all cases of adult-onset asthma are caused by occupational exposure within the population with attributable risk of occupational asthma (84). The prevalence and onset of OA depends mainly on the causative agent to which a worker is exposed and the route and intensity of the exposure, besides predisposing host factors such as atopy or other genetic factors, and environmental factors such as changes in diet or smoking status (85). Nevertheless, none of these factors have sufficient predictive accuracy to determine the ability of a worker to participate in a job that carries a risk of sensitization.

The agents causing immunological OA can be divided into two categories, depending on their molecular weight: biological agents of high molecular weight (HMW) (>5 KDa), such as proteins, glycoproteins and polysaccharides, and chemical agents of low molecular weight (LMW) (< 5 KDa) such as synthetic chemicals, natural compounds, drugs and metals (76). Although HMW agents predominate among occupational respiratory sensitizers, the number of LMW agents is constantly growing and constitutes an important subset of etiologic agents, a situation which emphasizes the need to continually update our knowledge through literature reviews. Important chemicals reported as causes of OA include isocyanates, acid anhydrides, western red cedar, persulfate salts, metals and some acrylates (86).

The recognition of the underlying immunological basis of OA will be important for the identification of suitable biomarkers that meet formal diagnostic and prognostic criteria and will allow the development of novel strategies for treatment, as they may differ depending on the nature of the causal agent (76). As in the case of non-work-related allergic asthma, HMW agents (mostly proteins) act as complete antigens, although they may possess functional characteristics (e.g., proteolytic activity) which promote their allergenicity or present PRRs which enable them to stimulate innate immune responses via TLRs which may enhance their sensitizing capacity. HMW agents are recognized by APCs which mount a type2 immune response inducing OA via production of specific IgE antibodies from B cells stimulated by cytokines IL-4 and IL-13. Re-exposure to the allergen can then cause cross-reactivity with the high-affinity FcεRI on tissue mast cells, leading to degranulation and immediate bronchoconstriction, as well as to subsequent recruitment of eosinophils and late-phase inflammatory response (87).

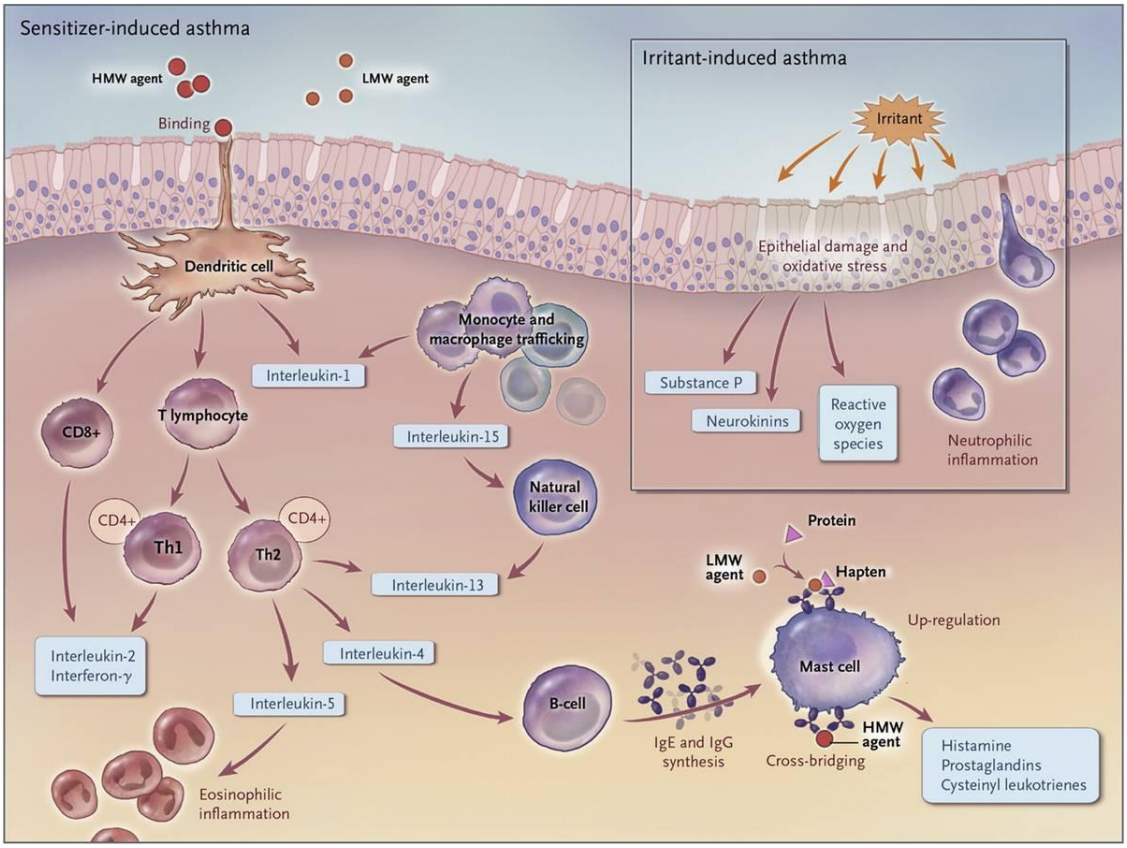

Figure 5. Mechanisms involved in sensitizer-induced asthma and irritant-induced asthma. HMW, high-molecular-weight; LMW, low-molecular-weight; Th1, Type 1 helper (76).

The pathophysiology involved in the case of OA induced by LMW agents is more controversial. It is uncertain whether the immune responses induced by chemical respiratory allergens and the important immunologic effector mechanisms are IgE-mediated. Clinical features, patterns of Th2 cytokines, pathological features and the finding that treatment with anti-IgE humanized antibody was effective in some patients with OA due to LMW agents (88) may suggest the presence of an IgE-mediated mechanism. Nevertheless, the failure to detect specific IgE antibodies against most of the LMW agents, the shorter but overlapping latency periods for sensitization and disease, and the mixed inflammatory cell pattern argue against an IgE-mediated explanation (89). For certain LMW agents (e.g., chlorinated platinum salts, trimellitic anhydride (TMA), and other anhydrides), the development of OA is accompanied by the production of specific IgE antibodies. Since they are non-immunogenic in their native state, it is assumed that they probably act as haptens and react with body proteins (albumin, keratin and tubulin) to produce a functional antigen to induce a type2 immune cascade (71). However, the presence of a specific IgE for most other LMW agents has not been proven. It is believed that the mechanisms are related to a specific immune response but do not necessarily imply an IgE-associated immunity, although they may include cell-mediated and mixed reactions. In this case, a mixed type2/1 immune response or induction of γδ-specific CD8 may play a role (76). Moreover, LMW agents can also stimulate the human innate immune response by up-regulating the immune PRR receptors of monocytes and increasing chemokines that regulate

monocyte and macrophage trafficking (90). Whether or not the mechanism involved is IgE-mediated, the binding of IgE to their receptors, Th2 (IL-5) and Th1 (IFN-γ) cytokines, and other proinflammatory chemokines (monocyte chemoattractant protein 1 [MCP-1]; tumor necrosis factor [TNF-α]) induce the recruitment and activation of inflammatory cells. These cells (mast cells, eosinophils, macrophages, and, in some instances, neutrophils) characterize airway inflammation, which contributes to the functional alterations of OA (71,76,89). As regards irritant-induced asthma, oxidative stress is likely to be one of the causes of airway epithelium damage, leading to a release of ROS by the epithelium, in addition to the increased release of neuropeptides from the neuronal terminals. This results in a neurogenic inflammation with the release of substance P and neurokinins (*figure 5*) (76).

1.3 Asthma treatments

The aim of asthma management is to achieve control and to prevent exacerbations. According to international guidelines, ICS are the mainstay of asthma control and maintenance (1). Many patients are well controlled with ICS treatment, although long-acting β_2 agonists (LABA) should be added if the symptoms and/or exacerbations persist (91). Furthermore, if patients remain uncontrolled, increasing doses of ICS in addition to LABA or the addition of extra-controllers such as leukotriene receptor antagonist and anti-cholinergic tiotropium might be considered (92,93). In the case of severe asthma, patients remain symptomatic despite the currently available maximum inhaled medication, and therefore additional treatment alternatives are necessary. Macrolides have been proposed as a potential treatment due to their immunomodulatory and anti-inflammatory effects, but ERS/ATS guidelines do not recommend their use due to the development of microbial resistances and the uncertain clinical benefits (94). In recent years, the most promising strategies are targeted therapies that encourage the improvement of certain clinical features and of certain immunological pathways (95). Blocking a single mediator in the biological pathways forming the basis of asthma endotypes is unlikely to be very effective in this complex disease but some promising results have been reported in selected patients. These novel targeted therapies are mainly based on the Th2/non-Th2 immunological pathways (*figure 6*) (96).

Several monoclonal antibodies (mAb) against the cytokine IL-5 have been developed such as mepolizumab and reslizumab as well as against its IL-5 receptor α (IL-5Rα) (e.g., benralizumab). This cytokine is known to mediate eosinophil differentiation, proliferation and activation and is therefore a suitable target for treating asthma with high eosinophilia (97). Early trials which targeted the biological activity of IL-5 did not consider evidence of eosinophilia and did not seem to provide clinical benefit, except for improving rates of exacerbations (98-100). Nevertheless, these studies evidenced the importance of choosing the correct population and the correct outcome parameters for study (101,102). Mepolizumab has recently been approved by the European Medicines Agency for patients with severe eosinophilic asthma (103).

Other biological treatments targeting specific immunological pathways have been developed, focusing in particular on the Th2 response (91). Inhibition of IL-4/IL-13 (with lebrikizumab, tralokinumab or dupilumab) in patients with a Th2-inflammatory profile has achieved encouraging results in the form of fewer exacerbations, improved lung function, and improved symptoms (104). Another interesting target for reducing inflammation is TSLP, which induces the activation of ILCs to stimulate Th2 cytokine production. A recent study reported an attenuation of the early and late asthmatic responses and also an attenuation of the markers of systemic and airway inflammation by anti-TSLP action (105). However, few effective strategies are available for non-eosinophilic asthma. Some strategies have focused on chemokine receptor-2 (CXCR2), the main IL-8 receptor expressed by neutrophils, with promising results in patients with airway neutrophilia (106) but no therapeutic effect has been achieved with the mAb against the IL-17 receptor A (IL-17RA) (brodalumab) (107). Thus, further research is required for the treatment of this phenotype.

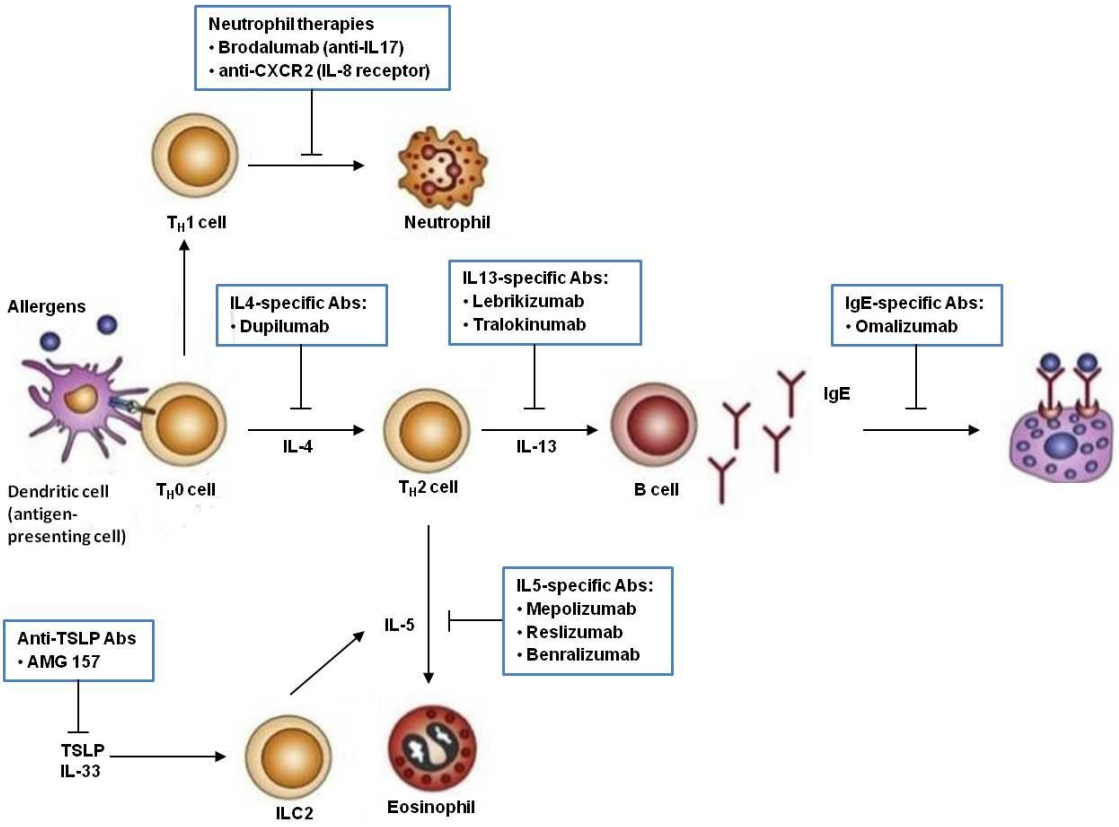

Figure 6. Targeted therapies acting on different pathways. Abs, antibodies; CXCR2, chemokine receptor-2; IgE, immunoglobulin E; IL, interleukin; ILC2, innate lymphoid cells; Th, T-helper cell.

In this instance, the only biological agent available in clinical practice was the anti-IgE monoclonal antibody omalizumab (OmAb). Its clinical efficacy has been well established in several clinical trials including adults and children with diagnoses ranging from moderate to severe allergic asthma (108). Despite the wide clinical experience with OmAb, not all patients respond equally and certain controversial issues remain. In addition, the exact mechanisms by

which OmAb may act are only partially known as it seems to act on IgE regardless of antigen specificity (39,109). Current indications for OmAb treatment are confined to severe allergic asthma, although there is increasing evidence that this antibody may have some new indications apart from allergic asthma, such as non-allergic asthma, chronic urticaria and even anaphylaxis (110). In addition, an increasing number of OmAb studies are providing new information on the role of IgE-mediated mechanisms in various diseases; their results are sometimes totally unexpected because it was not thought that IgE was involved in their pathogenesis.

In the case of OA, only one study has evaluated the effects of OmAb treatment even in patients with OA due to chemical agents. That study found that three patients were able to continue in their workplace when anti-IgE was administered, suggesting a possible alternative for these patients who remain uncontrolled with conventional treatment (88). This issue will be dealt with in detail in this thesis (See *Chapter 3*).

1.4 Experimental animal models of asthma

Despite the improved understanding of the pathophysiology of asthma obtained from clinical studies, certain processes remain poorly understood. In this regard, experimental animal models have proven invaluable for investigating the mechanisms underlying the airway pathophysiology involved in the onset and development of asthma both *in vivo* and *ex vivo*. There is no generally accepted model of the human disease, and the differences in species and strains, in addition to the variations in sensitization and challenge protocols, may influence the outcomes of studies. Nonetheless, these models afford us the opportunity to design and conduct studies using intact immune and respiratory systems and have been instrumental in understanding some of the mechanistic bases of the syndrome and in providing insights into pathways linked to the interaction in the lung (111). Experimental animal models also make it possible to test the safety and efficacy of new drugs and therapeutic agents (112).

With the exception of cats and horses, most animals used in the study of asthma do not spontaneously develop the disease. In order to develop asthmatic-like immune reactions, they have to be sensitized and provoked with allergens (113). Mice are widely used in asthma research because they are relatively inexpensive and easy to handle, there are specific reagents and equipment available, and the development of transgenic and knockout (KO) mice has opened up new possibilities for *in vivo* studies. The variability between mouse strains helps to identify the different mechanisms involved in asthma physiopathology as it allows the enhancement or inhibition of specific molecular pathways in order to study their importance in the development of an asthma phenotype. The BALB/c mouse is the most commonly used strain for allergen challenge models, as these mice are more likely to develop a type 2 immune response upon allergic sensitization and challenge. As transgenic mice have evolved, the

C57BL/6 strain has been increasingly used. This strain is prone to develop a type 1 immune response, and in contrast, decreased levels of eosinophilia are observed (114).

Most research has focused on HMW agents that are frequently associated with allergic asthma, especially house dust mites (HDM) (115,116), *Aspergillus* (117) or species with no clinical relevance such as chicken egg ovalbumin (OVA) (118). Few research groups have investigated LMW-induced OA. Most animal studies of LMW-induced OA have been done with diisocyanates (119-121), although other chemicals such as TMA (122), glutaraldehyde (123) and persulfate salts (124) have also been assessed.

Most of these models involve a first phase of sensitization followed by a provocation (or challenge) phase. The outcome of the parameters studied may differ substantially from other models, depending on variables such as mouse strain (as reported above), sensitization and challenge schedules (i.e., route of application, timing and dosage) and the endpoint of physiological, inflammatory and immunologic measures. This means that it is difficult to compare them. Nevertheless, the main objective when developing and validating a mouse model of asthma is to reproduce the most characteristic features of human asthma such as airway obstruction after exposure to the causal agent, non-specific AHR (methacholine), airway inflammation and even airway remodeling (113).

In addition, strategies other than sensitization and challenge protocols have been used to study the possible mechanisms involved in OA due to LMW agents. For instance, blocking agents have been used in some animal models to study their possible impact on asthma regulation. These agents are able to inhibit a certain pathway either partially or completely by blocking specific molecules or receptors. For example, some agents and mAbs have been used to selectively deplete certain types of leukocyte such as neutrophils and eosinophils in order to study their role in the regulation of inflammatory processes in an animal model of asthma due to isocyanates (125). As previously observed, new asthma treatments based on mAb targeting particular molecules involved in asthma pathogenesis (i.e., IgE and some cytokines) also provide valuable information about the immune mechanisms involved in asthma (101,109). Other options focus on targeting ion channels (126) and transient receptor potential channels (TRP) which function as intracellular calcium release channels. The two major proinflammatory TRP ion channels in sensory neurons (TRPV1, the capsaicin receptor, and TRPA1, the stress receptor) can be stimulated by chemicals and other agonists when causing acidification and oxidation and may lead to chronic inflammation, hyperreactivity, cough or pain (5,25). When a TRP antagonist is used in an animal model, inflammation and acute airway responses to chemical exposure are substantially decreased, suggesting that the activation of TRPA1 and TRPV1 on airway sensory fiber terminals by hazardous irritants could evoke noxious respiratory sensation, sensitization or respiratory reflexes, and the local release of proinflammatory neuropeptides, which can lead to OA or irritant-induced asthma (127,128).

The study of transgenic mice has also provided valuable insights into asthma pathogenesis and many animal models have been developed. As far as TRP channels are concerned, KO mice for TRPA1 have been developed and have been used to study its role in a mouse model of chemical-induced asthma (128). Moreover, other models have focused on orosomucoid-like 3 (ORMDL3) which has been strongly linked with severe asthma and is expressed predominantly in airway epithelial cells (129). Transgenic mice overexpressing human ORMDL3 show an upregulation of airway inflammation and remodeling by changing the levels of calcium within the endoplasmic reticulum (130,131). Mast cells are also an important target in asthma and their possible role in non-allergic asthma is currently being investigated via the study of mast cell-deficient mice (132,133). At present, as transgenic techniques evolve, the number of experimental animal models used to study asthma pathogenesis is increasing rapidly.

1.4.1 Experimental outcomes

1.4.1.1 Lung function measurements

In human patients, pulmonary function tests are important tools for describing the phenotype of a respiratory disease. It is important to reproduce these tests in small rodents. Several noninvasive methods are available for this kind of assessment in experimental animals, including unrestrained whole-body plethysmography which allows the monitoring of various parameters, including respiratory frequency, tidal volume (TV), peak flows, and enhanced pause (Penh), in conscious mice at repeated moments in time. Penh, calculated from the chamber pressure signal (134), has been reported to be an unreliable measure for airway responsiveness as it is highly dependent on respiratory frequency, and changes inside the camera (such as humidity) may affect the pressure (135,136). Invasive methods such as forced pulmonary maneuvers and forced oscillations are believed to be more sensitive and specific, as they determine true physiological parameters, and they should be considered as the gold standard for assessments of pulmonary function in mice (136). In this context, the *FlexiVent System* (Scireq; Montreal, Canada) has been used to assess reactivity to methacholine. When equipped with an integrated nebulizer, the *FlexiVent* can be used both to deliver aerosol challenges to a mouse's lungs and to follow the developing bronchoconstriction through automated data collection. The system can calculate and display detailed dose-response curves which can be computed and charted in real time to demonstrate AHR.

1.4.1.2 Airway inflammation

Eosinophilia is the hallmark of allergic asthma, as in the case of asthma induced by HMW agents. However, asthma induced by LMW agents has been associated with an influx mainly of neutrophils found in bronchoalveolar lavage (BAL) (58,124,125,137). The type of inflammation may also differ depending on the duration of the exposure to the causal agent, and a small influx of eosinophils along with neutrophilia may be observed with a multiple challenge protocol (138). Moreover, the influx of inflammatory cells is mediated by several cytokines and

chemokines which can also be detected in BAL fluid, homogenates of lung tissue, and supernatant of stimulated lymphocyte cultures.

Most animal models are based on acute asthma and the inflammation found in the airways. However, long-term structural changes can also be observed in lung tissue using histological staining methods. In sensitized mice, the response to an allergen provocation usually causes some degree of peribronchiolar and perivascular inflammation, epithelial shedding, and mucus hypersecretion due to the proliferation of the goblet cells. In some cases perivascular remodeling is also observed (139).

1.4.1.3 Serum immunoglobulins

As already mentioned, the role of IgE in chemical-induced asthma remains uncertain. The specific-IgE antibodies have not been detected for most LMW agents and this makes it difficult to elicit their possible role in the immunological pathways involved in OA due to chemical agents. Most chemical-specific IgE are not detectable because of assay detection limits or because of the variable (and sometimes long) time intervals between the last exposure and serology testing, as well as the lack of antigen forms used for immunoassays or the use of wrong forms such as conjugates with albumin (140). For this reason, determination of total serum IgE is usual in animal testing, and increased levels of total IgE in serum samples of mice treated with chemicals have been found (119,122,123). Similarly, total serum IgG can also be detected in these samples, and total serum IgG1 and IgG2a are often detected in these mice (141).

1.4.2 Animal model of persulfate-induced asthma

This thesis will mainly focus on findings concerning immunological OA induced by LMW agents, with particular attention to persulfate salts. These chemical compounds are widely used in different manufacturing processes in the chemical, pharmaceutical, metallurgic, textile, photographic, food, and particularly cosmetic industries. They may be present in hair bleaching products at concentrations up to 60% (142) and they are capable of causing immunological sensitization and subsequent allergic disease (such as contact dermatitis and bronchial asthma): these salts are the main cause of OA among hairdressers (143-145). The dermal sensitization capacity of three persulfate salts ($(NH_4)_2S_2O_8$, ammonium persulfate [AP]; $Na_2S_2O_8$, sodium persulfate [NaP]; $K_2S_2O_8$, potassium persulfate [KP]) (*figure 7*) was determined by local lymph node assay (LLNA), a method measuring the function of induced proliferative responses in the auricular lymph nodes draining the site of topical application. In the LLNA, chemicals were defined as biologically relevant dermal sensitizers, and can be classified when the test concentrations yield at least a threefold increase in stimulation index (SI) (EC3 value) compared with vehicle-treated controls. The EC3 was derived by linear interpolation of dose–response data and was calculated by interpolating between two points on the SI axis, one immediately above and the other immediately below the SI value of 3. The relationship of EC3

value to relative potency is inverted; hence, the lower the EC3 value, the higher the potency. The results classified AP and KP as moderate sensitizers and NaP as a strong sensitizer (146). For the experimental studies in this thesis, AP was used for sensitization and provocation in an animal model.

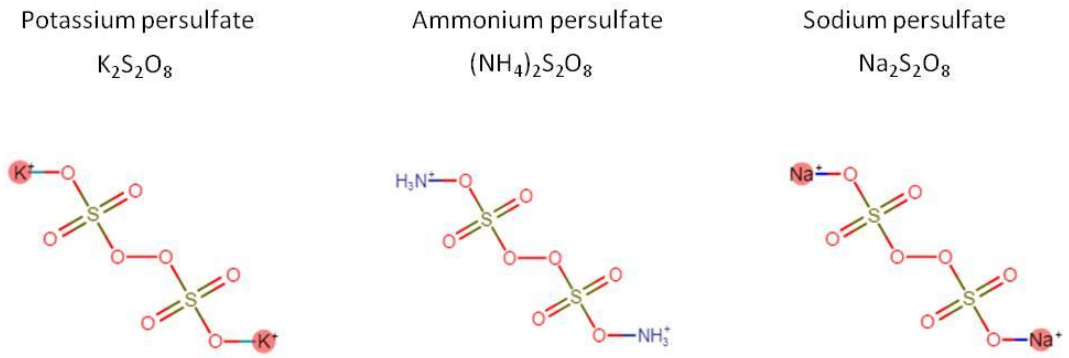

Figure 7. Chemical structures of the three most commonly used persulfate salts.

The fate of inhaled persulfate salts in the human body and the nature of the antigen that is eventually produced are largely unknown. In fact, this is true of most chemicals that induce OA. Diisocyanates present two highly reactive isocyanate groups (-N=C=O) which have a high affinity for the hydrogen atoms of –OH, –SH or $–NH_2$ on endogenous proteins. It is known that isocyanates can bind albumin, keratin, tubulin and glutathione, which are all abundant in the two primary sites of exposure, the respiratory tract and/or the skin (147). To date, no AP adduct with a body protein such as albumin has been detected, although AP has been reported to be able to oxidize some single amino acids. The oxidation of cysteine residues within proteins was already reported for H_2O_2 and these redox changes can concern key regulatory proteins in airway hyperreactivity (148). In this respect, several proteins involved in inflammatory responses, oxidative stress, epithelium integrity, and dermatological disorders have been reported after the persulfate challenge, suggesting that this challenge affects proteins associated with oxidative stress and induces an inflammatory response along with tissue damage and tissue remodeling (149). Nevertheless, this possible role in the oxidative stress needs to be evaluated further.

No consensus has been reached regarding the details of the immune response involved in persulfate-induced OA. To date, some authors have proposed an IgE-mediated mechanism (150) and positive skin prick tests against persulfates have been reported in some patients (143,145) although these results are not borne out in all studies (151,152). Moreover, despite evidence of a type 2 immune response, the involvement of type 1 has also been suggested (152). In this regard, a mouse model of persulfate-induced asthma can provide valuable knowledge about the immunological pathways which may be involved in this type of OA.

For some years now, the Pneumology Unit at our hospital has long focused on OA induced by persulfate salts (124,143,146,153,154) and has demonstrated that AP is able to induce an asthma-like response after dermal sensitization and a single intranasal instillation validating an animal model of persulfate-induced asthma (124). In these studies, the results of *in vitro* lymphocyte proliferation tests showed a clear dose response curve when stimulating lymphocytes from AP-sensitized mice with different concentrations of AP, but no proliferation after stimulating lymphocytes of *naïve* or dimethylsulfoxide (DMSO)-treated mice, suggesting that the activation of these lymphocytes was AP-specific. Furthermore, several features of human OA were induced in BALB/C mice sensitized and challenged with AP. This group presented increased reactivity to methacholine 24h after AP challenge (AHR) which was accompanied by neutrophilic inflammation, increased levels of total serum IgE, T and B cell proliferation in auricular and cervical draining lymph nodes, and increased levels of IL-4, IL-10 and IL-13 in supernatants of auricular lymph nodes. This thesis will mainly focus on the physiopathology of OA induced by persulfate salts, using this previously validated mouse model of persulfate-induced asthma (124).

2. HYPOTHESIS AND OBJECTIVES

Occupational asthma is one of the most common lung-related occupational diseases, and occupational exposure represents up to 25% of all cases of adult-onset asthma (64). Persulfate salts are the main cause of OA amongst hairdressing professionals, although the mechanisms by which these substances yield an immune response resulting in OA are still unclear (155). At present, the most commonly used approach for avoiding asthma symptoms is definitive cessation of exposure to the offending agent, which is usually achieved in practice by removal of the employee from the workplace (79), although there is no scientific evidence to assert that cessation of exposure actually improves asthma symptoms (82). Thus, it is important to know how patients evolve once they avoid their exposure to causal agents, so as to minimize both the serious health consequences and the strong socioeconomic impact derived from leaving the workplace.

In the present thesis, the asthmatic response was analyzed in the context of chemical-induced asthma due to persulfate salts using a validated mouse model previously developed by our research group. In this model, the dermal exposure to ammonium persulfate (AP) can result in systemic sensitization, which may lead to OA after airway exposure to the chemical. Therefore, AP induces an asthma-like response in this animal model based on dermal sensitization followed by a single intranasal challenge (124).

The specific objectives of each study are:

2.1 Chapter 1. Persistence of dermal sensitization

Hypothesis

The respiratory and inflammatory responses decrease with increasing delay between dermal sensitization and intranasal instillation, although they do not disappear completely.

Objectives

1. To examine how long the asthmatic response to ammonium persulfate can be induced after dermal sensitization, by assessing the airway hyperresponsiveness, lung inflammation and immune response induced by a single intranasal instillation administered at different time points.

2.2 Chapter 2. Persistence of the asthmatic response after persulfate inhalation

Hypothesis

In persulfate-induced occupational asthma, the asthmatic response decreases progressively over time after cessation of exposure to the causal agent.

Objectives

1. To examine the persistence of the asthmatic response after a specific ammonium persulfate challenge in sensitized mice.

2. To assess the airway hyperresponsiveness and to evaluate the lung inflammation and immune response at different time points after intranasal instillation.

3. To determine the immune response profile induced in occupational asthma due to persulfate salts.

2.3 Chapter 3. Effect of anti-IgE in occupational asthma due to low molecular weight agents (persulfate salts)

Hypothesis

The administration of the mouse precursor of Omalizumab (anti-IgE) decreases the asthmatic response by improving the response associated with occupational asthma due to persulfate salts.

Objectives

1. To develop a multiple challenge protocol simulating a chronic asthma phenotype.

2. To evaluate the role of IgE and the mechanisms involved in the development of the immune response in this type of occupational asthma due to persulfate salts.

3. To assess the effect of anti-IgE treatment in occupational asthma induced by persulfate salts.

3. CHAPTER 1

Persistence of respiratory and inflammatory responses after dermal sensitization to persulfate salts in a mouse model of non-atopic asthma.

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3.1 Manuscript I.

Persistence of respiratory and inflammatory responses after dermal sensitization to persulfate salts in a mouse model of non-atopic asthma

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Abstract

Background

Exposure to ammonium persulfate (AP) has been reported to be the main cause of occupational asthma in hairdressers. The aim of this study is to assess how long the asthmatic response to AP can be induced after dermal sensitization in a mouse model.

Methods

BALB/c mice received dermal applications of AP or dimethylsulfoxide (DMSO) (control) on days 1 and 8. They then received a single nasal instillation (challenge) of AP or saline on days 15, 22, 29, 36, 45, 60 and 90. Airway hyperresponsiveness (AHR) was measured 24 hours after the challenge using a non-specific methacholine provocation test. Pulmonary inflammation was analysed in bronchoalveolar lavage (BAL), and total serum immunoglobulin (Ig) E, IgG1 and IgG2a were measured in serum samples. Histological analysis of lung slides was performed.

Results

Mice dermally sensitized and intranasally challenged with AP showed airway hyperresponsiveness to methacholine as long as 45 days after initial sensitization, as well as increased percentage of neutrophils in BAL compared with the control group. At day 60, dermally sensitized mice still presented bronchial hyperresponsiveness, while the percentage of neutrophils returned to baseline levels similar to those of controls. Total serum IgE remained high in AP-sensitized mice until 22 days after dermal sensitization. Total serum IgG1 and IgG2a increased from 45 days after dermal sensitization and remained high at 90 days.

Conclusions

Both respiratory responsiveness to methacholine and airway inflammation responses decrease with increasing time between sensitization and challenge. Respiratory responsiveness to methacholine tends to persist longer than inflammation.

Keywords: Occupational asthma, Airway hyperresponsiveness, Lung inflammation, Ammonium persulfate

Background

Persulfate salts are highly reactive low molecular weight (LMW) chemical compounds which are present in considerable proportions (10-20%) in the bleaching powders used by hairdressers during hair-bleaching procedures [1]. Exposure to these salts has been identified as the main cause of immunological sensitization and subsequent allergic diseases such as contact dermatitis and bronchial asthma, and it has been associated with a high risk of occupational asthma (OA) in hairdressers [2-4].

Nevertheless, the mechanisms by which persulfate salts induce sensitization and OA are not well established [5]. An immunologic mechanism has been postulated; various authors have suggested an IgE-driven mechanism, based on skin prick test positivity to persulfate salts and the finding of high levels of total serum IgE in hairdressers with OA [4,6]. However, other data seem to suggest that persulfate salts act through an immunological mechanism without driving an IgE response [7]. Therefore, studies of OA using suitable animal models may be able to shed light on the processes involved in the onset and persistence of bronchial hyperresponsiveness and airway inflammation and remodeling.

In a previous study using local lymph node assays [8], our research group identified ammonium persulfate (AP) as a moderate dermal sensitizer. In later work we developed and validated a mouse model of chemical-induced asthma using AP. In this model, mice were dermally sensitized with AP and then underwent a single airway challenge with AP, which triggered the responses typical of human OA [8,9].

It has been reported that asthma symptoms and nonspecific airway hyperresponsiveness persist even after cessation of exposure. The reason for this is not clear. In the present study, we examined how long the asthmatic response to AP persists after dermal sensitization. The aim of the study was to compare the airway responses, lung inflammation, and immune responses induced by a single intranasal AP challenge administered at variable intervals (between 1 and 90 days) after dermal sensitization to AP.

Methods

Mouse model of chemical-induced asthma

On days 1 and 8, male BALB/c mice (~20 g, 6 weeks old; Harlan, The Netherlands) received dermal applications of 5 % ammonium persulfate (AP, [$(NH_4)_2S_2O_8$], Sigma-Aldrich, Steinheim, Germany) or vehicle (dimethylsulfoxide (DMSO), Sigma-Aldrich, Steinheim, Germany) on the dorsum of both ears (20 µl). On days 15, 22, 29, 36, 45, 60 and 90, under light anesthesia with isoflurane (Forane®, Abbott Laboratories, Madrid, Spain), mice received an intranasal instillation (40 µl) of 1 % AP or vehicle (saline, 0.9 %NaCl). The experimental groups were DMSO/SAL and AP/AP: the first abbreviation identifies the agent used for dermal applications on days 1 and 8 (sensitization) and the second identifies the agent administered via intranasal

instillation on days 15, 22, 29, 36, 45, 60 and 90 (challenge). Each group of mice (vehicle or AP) consisted of five to eight animals at each time point. The experiments were repeated twice per group.

Respiratory responsiveness to methacholine

One day after intranasal challenge, reactivity in response to methacholine was measured using a forced oscillation technique (FlexiVent, SCIREQ, Montreal, Canada). As previously described, mice were anesthetized with pentobarbital (70 mg/kg body weight) (Nembutal®, Abbot Laboratories) and airway resistance (R) was measured using a "snapshot" protocol. For each mouse, R was plotted against methacholine concentration (0-20 mg/ml) and the area under the curve (AUC) was calculated [10].

Total serum immunoglobulins

After functional airway measurements, mice were deeply anesthetized by an intraperitoneal injection of pentobarbital (90 mg/kg body weight). Blood was taken from the retro-orbital plexus and centrifuged (14,000g, 10 min) and serum samples were stored at −80 °C for further analyses. The mouse ELISA kits (Bethyl Laboratories Inc., Montgomery, USA) were used to measure total serum immunoglobulin (Ig)-E, IgG1 and IgG2a (diluted samples 1/5, 1/12,500 and 1/5000 respectively). Measurements were performed according to the manufacturer's instructions, using biotinylated anti-mouse IgE, IgG1 or IgG2a detection antibodies and horseradish peroxidase conjugate.

Pulmonary inflammation in bronchoalveolar lavage

Once blood samples were collected, bronchoalveolar lavage (BAL) was performed in situ. The lungs were lavaged three times with 0.7 ml sterile saline (0.9 % NaCl), and the fluid recovered was pooled. Cells were counted using a Bürker hemocytometer (total cells) and the bronchoalveolar lavage (BAL) fluid was centrifuged (1000g, 10 min). For differential cell counts, 250 µl of the resuspended cells (100,000 cells/ml) were spun (300g, 6 min) (Cytospin 3, Shandon, Thermo Scientific, Cheshire, United Kingdom) onto microscope slides, air-dried and stained [May-Grünwald, 5 min (QCA; Tarragona, Spain) and Giemsa, 15 min (Merck, Darmstadt, Germany)]. For each sample, the numbers of macrophages, eosinophils, neutrophils and lymphocytes were counted in 500 cells.

Lung pathology

After BAL, lungs were instilled with formaldehyde 3.7-4.0 % until all lobes were deemed to be fully inflated by visual inspection. Instillation was always performed by the same person and in homogeneous conditions. Evaluation of lung injury on slides stained by haematoxylin and eosin (H&E) and Masson's trichrome was performed by an experienced pathologist in a blinded manner. A semi-quantitative scoring system was used to grade the severity and extent of inflammation, as well as bronchiolar epithelium hyperplasia on H&E stained sections. The thickness of the infiltrate in the interalveolar septa and hyperplasia was graded as follows: 0

(normal) = absence of inflammatory cells; 1 (mild) = 1-2 layers of inflammatory cells; 2 (moderate) = 3-5 layers; 3 (severe) = more than 5 layers [11].

Data analysis

All data are presented as mean ± standard deviation (SD) and were analysed using the non-parametric Kruskal-Wallis test and Mann-Whitney U-test (Graphpad Prism 4.01, Graphpad Software Inc, San Diego, USA). A level of $p < 0.05$ (two-tailed) was considered significant.

Results

Respiratory responsiveness to methacholine

The non-specific total respiratory resistance to methacholine increased 24 h after the intranasal instillation with AP in the groups of mice which received dermal treatment with AP and were also intranasally challenged with AP (the AP/AP group) at time points 15, 22, 29, 36, 45 and 60 days (*Fig.1a,b*), compared to the control groups which received dermal treatment with DMSO and were intranasally challenged with saline (the DMSO/SAL group), At the last time point, after receiving a single challenge with AP on day 90, no changes in non-specific total respiratory resistance to methacholine were found in the AP/AP mice (*Fig.1a,b*).

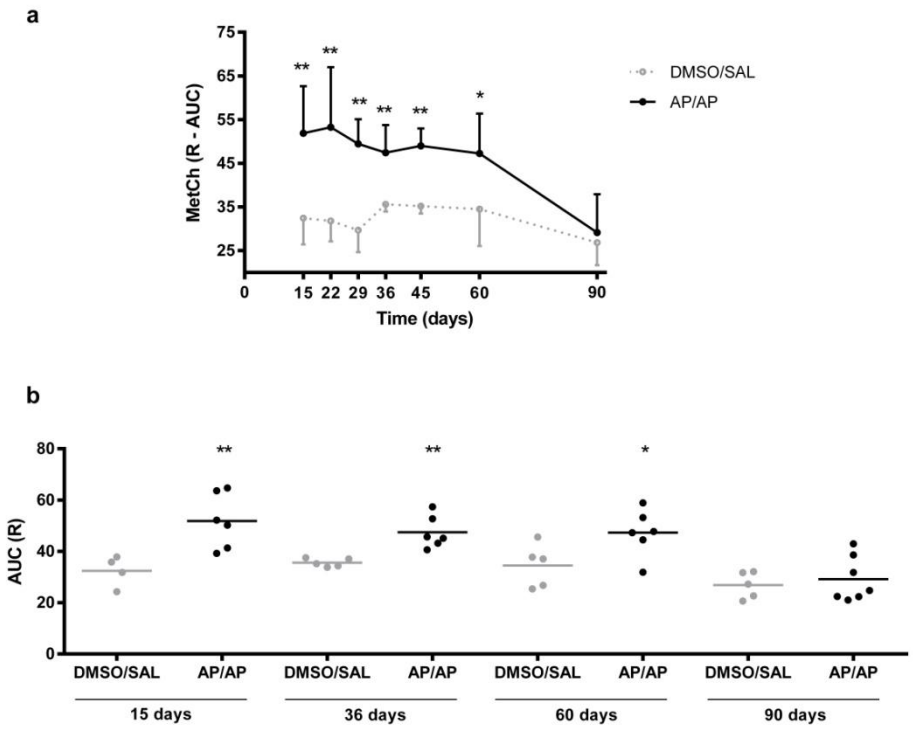

Fig. 1 Respiratory responsiveness to methacholine expressed as area under the curve (AUC) of the resistance (R) 24h after intranasal instillation of AP or vehicle (saline). Experimental groups were DMSO/SAL and AP/AP and were consisted in 5-8 mice per group. First abbreviation refers to dermal sensitization (day 1 and 8), and the second to the agents administered via intranasal instillation (day 15, 22, 29, 36, 45, 60, 90). **a** Mean ± SD of AUC of R against methacholine concentrations (0 to 20 mg/ml). **b** Mean individual values of AUC at 15, 36, 60 and 90 days after challenge. *$p < 0.05$, **$p < 0.01$ compared with DMSO/SAL. AP, ammonium persulfate; AUC, area under the curve; DMSO, dimethylsulfoxide; SAL, saline.

Total serum immunoglobulins

Total serum IgE levels showed a trend towards an increase on day 15 (p=0.083), and increased significantly on day 22 in the AP/AP group compared with the control mice (*Fig.2a*). Total serum IgG1 and IgG2a levels in AP-treated mice started to increase later than total serum IgE. In the case of IgG2a, the increase became significant 60 days after the first dermal sensitization, and was maintained after 90 days; in the case of IgG1 there was a trend towards an increase, although it did not reach significance (p=0.076) (*Fig.2b,c*).

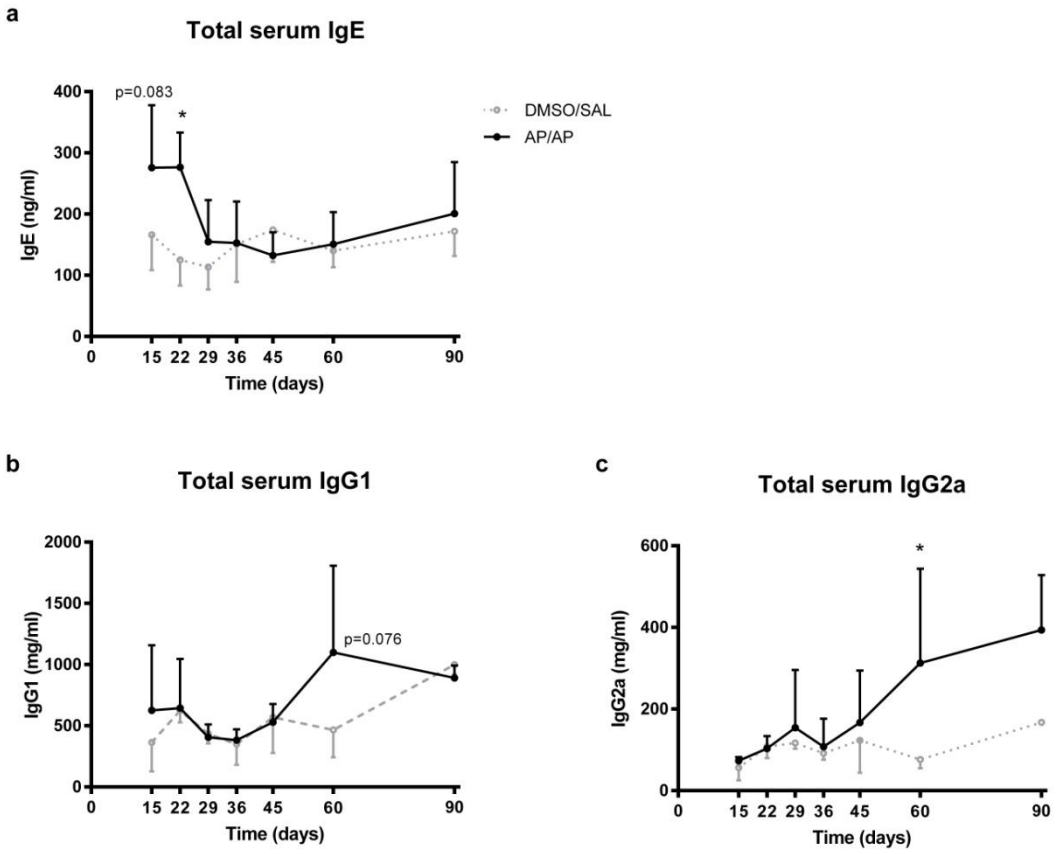

Fig. 2 Total serum immunoglobulin (Ig)-E, IgG1 and IgG2a. Blood was collected 24h after intranasal instillation of AP or vehicle (saline). Total serum IgE, IgG1 and IgG2a were measured using a standard ELISA. Experimental groups are the same as in Fig. 1 and were consisted in 4-6 mice per group. **a** Mean ± SD of total serum IgE. **b** Mean ± SD of total serum IgG1. **c** Mean ± SD of total serum IgG2a. *p < 0.05 compared with DMSO/SAL control group. AP, ammonium persulfate; DMSO, dimethylsulfoxide; SAL, saline.

Pulmonary inflammation (bronchoalveolar lavage)

Figure 3 shows the BAL neutrophil count 1 day after a single challenge. AP-treated mice (AP/AP) showed significantly higher percentages of BAL neutrophils at time points 15, 22, 29, 36 and 45 days than the DMSO/SAL control group (*Fig.3*). There were no significant differences in the percentages of eosinophils and lymphocytes in BAL samples between the groups (data not shown).

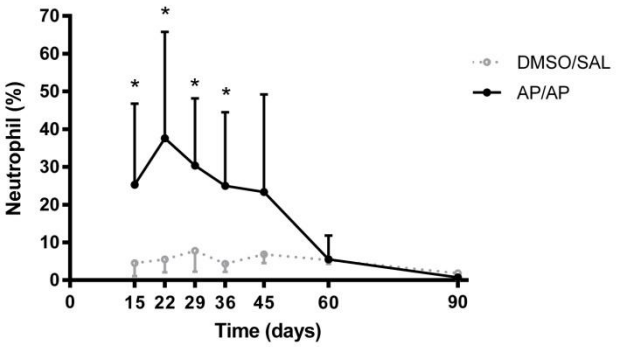

Fig. 3 Percentage of neutrophils in BAL obtained 24h after intranasal instillation of AP or vehicle (saline). Experimental groups are the same as in Fig. 1 and were consisted in 5-8 mice per group. Mean ± SD of percentage of neutrophils in BAL. *p<0.05 compared with DMSO/SAL control group. No significant differences were found in the other groups studied at different time points. AP, ammonium persulfate; BAL, bronchoalveolar lavage, DMSO, dimethylsulfoxide; SAL, saline.

Airway histopathology

A blinded histopathological examination of lung tissue sections from the AP-treated mice assessed as long as 60 days after sensitization revealed an increase in inflammatory cell infiltration (grade 1-2, mild to moderate) and the presence of alveolar macrophages (grade 1, mild) (*Fig.4a,b*) compared with control groups (*Fig.4d,e*). At 90 days, the stained sections of AP/AP mice presented reductions in inflammatory cell infiltration (grade 0-1, normal to mild) (*Fig.4c,f*). Selectively, at 15 days some moderate peribronchiolar epithelium hyperplasia was observed in the AP/AP group (grade 2, moderate) (*Fig.4a*) compared with controls (*Fig.4d*). In this acute single challenge model, no collagen deposition was found in the lung sections stained with Masson's trichrome (data not shown).

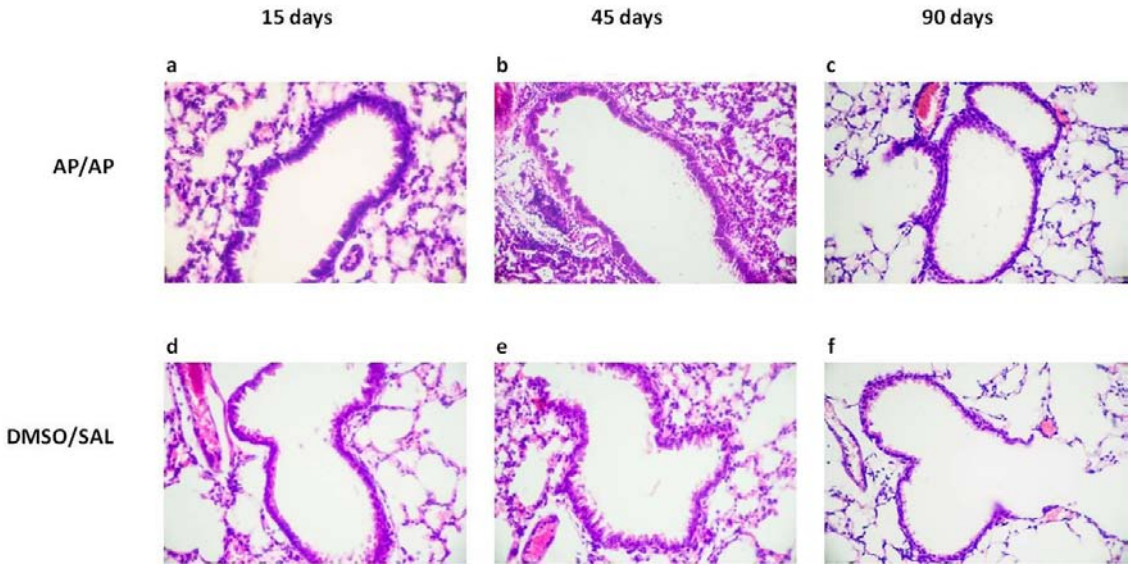

Fig. 4 Lung histopathology. Representative images of haematoxylin and eosin stained histological lung sections. Experimental groups in this figure are represented with sections from DMSO/SAL, and AP/AP groups assessed 15 (**a** and **d**), 45 (**b** and **e**) and 90 (**c** and **f**) days after AP sensitization. AP, ammonium persulfate, DMSO, dimethylsulfoxide; SAL, saline.

Discussion

We investigated the time course of immunologic and respiratory responses after dermal sensitization in a validated mouse model of OA due to persulfate salts [9]. We were able to induce both respiratory responsiveness to methacholine and pulmonary inflammation in AP-sensitized mice with a single intranasal challenge with AP up to 40 days after initial AP sensitization. Even 60 days after initial AP sensitization, a single challenge could still induce respiratory responsiveness (without neutrophilic inflammation), while 90 days afterwards, a single challenge with AP no longer induced these asthma-like symptoms. In terms of the immune response, there was evidence of systemic sensitization (with an increase in IgE) at early stages, while high IgG levels appeared later.

Exposure to persulfate salts is associated with a high risk of developing OA, although the mechanisms by which these substances induce sensitization and OA are not well understood [2,4]. It has been suggested that persulfate-induced OA is mediated by an immunological mechanism [2,4,12]. Positive skin prick tests to persulfates have been reported, suggesting an IgE-mediated mechanism [4,12-14]. An increase in total IgE levels has been described in studies with human patients and with mouse models [4,9]. In this connection, in a mouse model of chemical-induced asthma previously developed by our group using AP [9], we showed that AP, after two dermal applications and only one airway challenge, can induce features of human occupational asthma in mice, including respiratory responsiveness to methacholine, neutrophil inflammation in BAL, T- and B cell proliferation and a Th2 cytokine profile in the auricular lymph nodes (the site of sensitization), and also increased total serum IgE levels.

Recent animal and human data collectively support a central role for skin barrier function and skin exposure in the development of Th2-like sensitization and the subsequent development of asthma. Mouse models have also shown that chemicals can induce mixed Th1/Th2 responses [15,16]. The skin may play a role both as an important route of exposure and as an immunological organ that can contribute to pulmonary immune diseases [17]. The epidermis contains keratinocytes and Langerhans cells, a major dendritic cell in the skin which can acquire antigen, migrate to draining lymph nodes, and initiate immune responses [18]. The activation of dendritic cells with subsequent T-lymphocyte transformation in the lymph nodes draining the skin produces activated effector T-lymphocytes or memory cells in the systemic circulation [19]. Once in the lung, effector T-cells will produce cytokines and chemokines or undertake cytotoxic functions. Repeated lung exposure to the irritant by inhalation doses may synergistically amplify this allergic inflammation and asthma. These cells are able to express high-affinity receptors for IgE and, upon re-exposure, binding of the allergen to IgE orchestrates the immune system to initiate a more aggressive and rapid memory response [20].

In this study, we found a trend towards an increase in total serum IgE levels already at 15 days after initial dermal application of AP. IgE levels remained high until 22 days. A previous study by our group [21] described the course of bronchial hyperresponsiveness and immunologic test results in patients with OA due to persulfate salts, and found that total IgE levels remained increased even in patients who ceased exposure. On the other hand, levels of total serum IgG1 tended to increase from day 60, when total serum IgE levels had returned to baseline values. This is compatible with a Th2 immunological response, despite the unexpected increases in levels of total serum IgG2a (characteristic of a Th1 stimulation in mice) from day 60. These results are consistent with other studies carried out by our group with the same animal model and other mouse models of asthma using LMW agents [9,11,20-23], which suggested a mixed Th2-Th1-type immune response in sensitized mice. It has also been proposed that an increase in IgG levels may have a protective effect in this animal model [24], as the percentage of neutrophils decreased at the same time point that IgG started to increase.

Nevertheless, respiratory responsiveness to methacholine still persisted at 45 days despite the increased levels of IgG. Thus, inflammation and respiratory responsiveness were not associated after AP exposure. These results suggest that the presence of abnormal airway smooth muscle function is determinant for respiratory responsiveness to LMW agents in this OA model, while the presence of mucosal airway inflammation may aggravate the situation but is not the cause. Swedin *et al*. [25] also reported dissociation between airway inflammation and airway hyperresponsiveness (AHR) in an ovalbumin allergic mouse model, suggesting that inflammatory cells in BAL do not change in parallel with AHR. Regarding LMW agents, Vanoirbeek *et al*. [22] observed the same pattern of dissociation in a mouse model of OA due to isocyanate. These results are consistent with previous observations in subjects with asthma in whom BAL inflammation was not a predictive surrogate marker of AHR [26], suggesting that other factors such as airway wall remodeling, the activation state of inflammatory cells, T-cell activation or autonomic dysfunction may play a more important role in the development of AHR. Recently, in a mouse model of severe asthma, Raundhal *et al*. [27] demonstrated a role for IFN-γ in the induction of AHR, whereas IL-17 promotes neutrophilic airway inflammation. These observations suggest that IFN-γ is the predominant cytokine associated with AHR in severe asthma and that airway inflammation and AHR may not always be linked.

The progressive reduction in responsiveness over time also occurs in patients with OA who cease exposure, although the results of the present study only partially reflect this situation. It is well established that patients with OA generally become less responsive to the sensitizer after complete exposure removal [4]. Nevertheless, there is insufficient scientific evidence to assert that cessation of exposure improves asthma symptoms, and many patients do not become completely unresponsive to the sensitizer [28]. In our study, asthma symptoms and functional airway abnormalities had disappeared after 90 days. However, our results did not

prove that these mice would not become responsive if repeated exposures to the causal agent were given.

In conclusion, we show that both AHR and airway inflammation responses decrease with increasing time between sensitization and challenge. These findings suggest that dermal contact with a chemical can cause long-term sensitization and may lead to asthmatic symptoms. Moreover, many days after sensitization, exposure to the causal agent may produce various responses of which AHR is the most persistent; for its part, the inflammation response may be decreased. In any case, the mechanisms underlying the process remain undefined and more studies in this direction are needed.

Authors' contributions

Conception and design: XM, MJC, and JV; Analysis and interpretation: XM, MO, SGO, AA and MJC. Drafting the manuscript for important intellectual content: all. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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4. CHAPTER 2

Persistence of asthmatic response after ammonium persulfate-induced occupational asthma in mice.

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4.1 Manuscript II.

Persistence of asthmatic response after ammonium persulfate-induced occupational asthma in mice

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Abstract

Introduction

Since persulfate salts are an important cause of occupational asthma (OA), we aimed to study the persistence of respiratory symptoms after a single exposure to ammonium persulfate (AP) in AP-sensitized mice.

Material and methods

BALB/c mice received dermal applications of AP or dimethylsulfoxide (DMSO) on days 1 and 8. On day 15, they received a single nasal instillation of AP or saline. Airway hyperresponsiveness (AHR) was assessed using methacholine provocation, while pulmonary inflammation was evaluated in bronchoalveolar lavage (BAL), and total serum immunoglobulin E (IgE), IgG1 and IgG2a were measured in blood at 1, 4, 8, 24 hours and 4, 8, 15 days after the single exposure to the causal agent. Histological studies of lungs were assessed.

Results

AP-treated mice showed a sustained increase in AHR, lasting up to 4 days after the challenge. There was a significant increase in the percentage of neutrophils 8 hours after the challenge, which persisted for 24 hours in AP-treated mice. The extent of airway inflammation was also seen in the histological analysis of the lungs from challenged mice. Slight increases in total serum IgE 4 days after the challenge were found, while IgG gradually increased further 4 to 15 days after the AP challenge in AP-sensitized mice.

Conclusion

In AP-sensitized mice, an Ig-independent response is induced after AP challenge. AHR appears immediately, but airway neutrophil inflammation appears later. This response decreases in time; at early stages only respiratory and inflammatory responses decrease, but later on immunological response decreases as well.

Introduction

Occupational asthma (OA) is one of the most common forms of lung-related occupational diseases in Europe, and its annual incidence is increasing. It is estimated that 10% to 25% of all adult onset asthma cases are work-related or caused by occupational exposure [1,2]. More than 400 agents have been reported to cause asthma in the workplace [3]. These agents can be divided into two groups according to their molecular weight: high-molecular-weight (HMW) or low-molecular-weight (LMW) [4]. Persulfate salts are LMW chemicals widely used in various manufacturing processes [5], especially in bleaching hair products, and are capable of causing immunological sensitization and subsequently allergic diseases such as contact dermatitis and asthma. Persulfate salts are acknowledged as the main cause of OA amongst hairdressing professionals [6-10].

However, the mechanisms by which these substances induce sensitization and OA are not yet clear as the processes seem to differ from the typical IgE-mediated allergic response. Previously, our research group demonstrated that AP is able to induce an asthma-like response in a validated mouse model of chemical-induced asthma. In these studies, several features of human OA were induced, such as airway hyperresponsiveness (AHR), neutrophilic inflammation, increased levels of total serum immunoglobulin-E (IgE), along with T and B cell proliferation and increased levels of IL-4, IL-10 and IL-13, one day after intranasal instillation of ammonium persulfate (AP) [11,12].

At present, the measure most commonly implemented to avoid OA-induced symptoms is complete removal from workplace exposure [13]. However, there is insufficient scientific evidence to assert that cessation of exposure improves asthma symptoms [14]. It has been shown that in the case of complete avoidance of exposure, fewer than 1/3 of workers with OA recover from their symptoms [15-17]. Reduced exposure has been suggested as a possible alternative to full cessation, with the aim of minimizing the adverse socio-economic effects. However, a recent systematic review reports that reduced exposure seems to be less beneficial than removal of the patient from the workplace [15].

In the case of persulfate salts, it is not known how patients evolve once they avoid exposure to the causal agent. Only one study has described the course of AHR and immunological outcome parameters in patients with OA due to persulfate salts. Despite the persistence of asthma symptoms and AHR in these patients, the study reported an improvement in their condition if exposure was ceased [18].

The aim of the present study was to examine the persistence of the asthmatic response after a specific AP challenge in AP-sensitized mice [11]. AHR, lung inflammation and immune response were evaluated at different time intervals after intranasal instillation of AP in dermally sensitized mice.

Materials and methods

Animals

Male BALB/c mice (~20 g, 6 weeks old) were obtained from Harlan (Horst; The Netherlands). The mice were housed in filter top cages in a conventional animal house with 12 h dark/light cycles and received slightly acidified water and pelleted food (Teklad 2014, Harlan Laboratories, Indianapolis, IN) *ad libitum*. All experimental procedures were approved by the Ethical Committee for Animal Experiments of Hospital Universitari Vall d'Hebron.

Mouse model of persulfate salt-induced asthma

On days 1 and 8, all groups of mice received dermal applications of 5% ammonium persulfate (AP, $[(NH_4)_2S_2O_8]$, Sigma-Aldrich, Steinheim, Germany) or vehicle (dimethylsulfoxide (DMSO), Sigma-Aldrich, Steinheim, Germany) on both ears (20 µl). On day 15, under light anesthesia with isoflurane (Forane, Abbott Laboratories, Madrid, Spain), they received 40 µl of 1% AP or vehicle (saline, 0.9%NaCl) via intranasal instillation (challenge). The experimental groups were DMSO/SAL and DMSO/AP, identified as control groups, and AP/AP identified as the treatment group: the first abbreviation referring to dermal sensitizations (days 1 and 8) and the second to the agent administered via intranasal instillation (day 15). Each group of mice (controls and treatment) consisted of 4-7 animals for each period of time after intranasal instillation: 1 hour, 4 hours, 8 hours, 24 hours (day 16), 4 days (day 19), 8 days (day 23) and 15 days (day 30). The experiments were repeated twice per group.

Pulmonary function measurement

Airway hyperresponsiveness. After intranasal instillation, reactivity to methacholine was assessed invasively using a forced oscillation technique (FOT) with FlexiVent system (Flexivent, SCIREQ; Montreal, Canada) at each time point (1 hour, 4 hours, 8 hours, 24 hours, 4 days, 8 days and 15 days). Mice were deeply anaesthetized by an intraperitoneal injection of pentobarbital (70 mg/kg) (Nembutal, Abbot Laboratories). The trachea was exposed and tracheotomised, and connected to a ventilator controlled by computer. Airway resistance (R) was measured with a ''snapshot'' protocol and plotted against methacholine concentration (from 0 to 10 mg/ml) and the Area under the curve (AUC) was calculated [19].

Total serum immunoglobulins (IgE, IgG1, IgG2a)

After the methacholine test was assessed, blood was taken by cardiac puncture and pooled (before BAL). Serum samples were obtained and stored at -80ºC for further analyses. The Mouse ELISA kits (Bethyl Laboratories, Inc., Montgomery, USA) were used to measure total serum IgE, IgG1 and IgG2a (diluted samples 1/5, 1/12500 and 1/5000, respectively). Measurements were performed according to the manufacturer's instructions, using biotinylated antimouse IgE, IgG1 and IgG2a detection antibodies and horseradish peroxidise conjugate.

Bronchoalveolar lavage

After blood sampling, bronchoalveolar lavage (BAL) was performed. The lungs were lavaged three times with 0.7 ml of sterile saline (0,9% NaCl) and the recovered fluid was pooled. Total cells were counted using a haemocytometer and the BAL fluid was centrifuged (1000 g, 10 minutes). The supernatant was frozen (-80ºC) until further analyses. For differential cell counts, 250 µl of the resuspended cells (100000 cells/ml; 1400 g, 6 minutes) were spun (Cytospin 3, Shandon, Thermo Scientific, Cheshire, United Kingdom) onto microscope slides, air-dried and stained [May-Grünwald, 5 min (QCA; Tarragona, Spain) and Giemsa, 15 min (Merck, Darmstadt, Germany)]. Counts for the number of macrophages, eosinophils, neutrophils and lymphocytes were performed in 500 cells from each sample.

Levels of interferon-gamma (IFN-γ) and interleukins-2 (IL-2), IL-4, IL-5, IL-10, IL-13 and IL-17A were measured in the first fraction of undiluted BAL fluid by a mouse cytokine magnetic bead panel according to the manufacturer's instructions (Bio-Plex Pro Mouse Cytokine Group I 7-plex Assay, Bio-Rad Laboratories S.A.; Madrid, Spain). Lower limits of detection were 1.56, 3.41, 6.11, 1.85, 1.26, 4.00, 3.02 pg/mL for IFN-γ, IL-2, IL-4, IL-5, IL-10, IL-13 and IL-17A, respectively.

Measurement of Th2 related cytokines in homogenized lung tissue

After performing BAL, the left lung was removed and homogenized with 500 ml of BSA/PBS 5%. The homogenate was centrifuged (3000 g, 10 min) and levels of cytokines were measured in the supernatant. The pellet was dried and weighed. Concentrations of IL-2, IL-4, IL-5, IL-13, IL-10, and IL-17A were measured using a Cytometric Bead Array Plex (Bio-Plex Pro Mouse Cytokine Group I 7-plex Assay, Bio-Rad Laboratories S.A.; Madrid, Spain). Measured concentrations were corrected for the lung dry weight. Lower limits of detection were 3.41, 6.11, 1.85, 1.26, 4.00, 3.02 pg/mL for IL-2, IL-4, IL-5, IL-10, IL-13 and IL-17A, respectively.

Lung pathology

After BAL, lungs were instilled with formaldehyde 3.7-4.0% until all lobes were deemed to be fully inflated by visual inspection. Evaluation of lung injury on slides stained by haematoxylin and eosin (H&E) and Masson's trichrome was performed by an experienced pathologist in a blinded manner. A semi-quantitative scoring system was used to grade the severity and extent of inflammation on haematoxylin-eosin stained sections. We graded the thickness of the infiltrate in the interalveolars septa using as follows: 0 (normal) = absence of inflammatory cells; 1 (mild) =1-2 layers of inflammatory cells; 2 (moderate) = 3-5 layers; 3 (severe) =more than 5 layers.

Data analysis

All data are presented as mean ± standard error of the mean (SEM) and were analyzed using the non-parametric Kruskal-Wallis test and Mann-Whitney U-test (Graphpad Prism 4.01,

Graphpad Software Inc, San Diego, USA). A level of $p<0.05$ (two-tailed) was considered significant.

Results

Airway hyperresponsiveness to methacholine

To assess the course of airway hyperresponsiveness (AHR) to methacholine, AUC was calculated for each individual mouse in each experimental group. The airway resistance to methacholine assessed 1 hour after the challenge was significantly increased in AP/AP mice compared with control groups assessed at the same time. This response remained increased until 4 days after inhalation (*figure 1*). Additionally, significant differences in early AHR (1-8 hours) were found in DMSO/AP groups compared with DMSO/SAL groups. At the later time points (8 and 15 days after the challenge) no significant increases in AHR to methacholine were found.

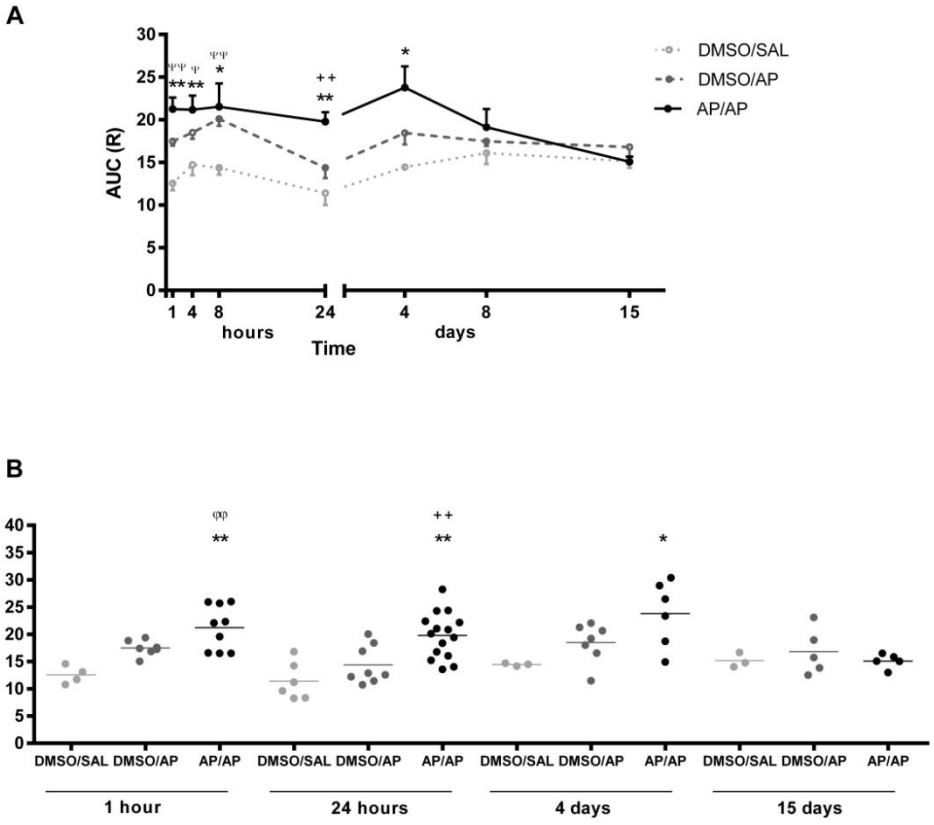

Figure 1. Airway hyperresponsiveness (AHR) to methacholine expressed as resistance (R) was measured 1 hour, 4 hours, 8 hours, 24 hours, 4 days, 8 days and 15 days after intranasal instillation by the forced oscillation technique to increasing concentrations of methacholine. Experimental groups were DMSO/SAL, DMSO/AP and AP/AP. First abbreviation refers to dermal sensitization (day 1 and 8), and the second to the agents administered via intranasal instillation (day 15). **A)** Mean ± SEM of AUC of R against methacholine concentrations (0 to 10 mg/ ml) for all periods of time. **B)** Mean individual values of AUC 1 hour, 24 hours, 4 days and 15 days after challenge. *p<0.05, **p<0.01 compared with DMSO/SAL, ++p<0.01 compared with DMSO/AP, Ψp<0.05 and ΨΨp<0.01 when DMSO/SAL is compared with DMSO/AP. No significant differences were found in the other groups studied at different time intervals. AP, ammonium persulfate; AUC, area under the curve; DMSO, dimethylsulfoxide; SAL, saline.

Pulmonary inflammation (bronchoalveolar lavage)

No differences were found in the total cell count in any of groups assessed at any time point. There was a quick response in the total number of neutrophils found 8 hours after the AP challenge in AP-treated mice compared with the control group (DMSO/SAL), which persisted until 24 hours post-inhalation (*figure 2B*). There were no eosinophils in BAL samples from any of the groups.

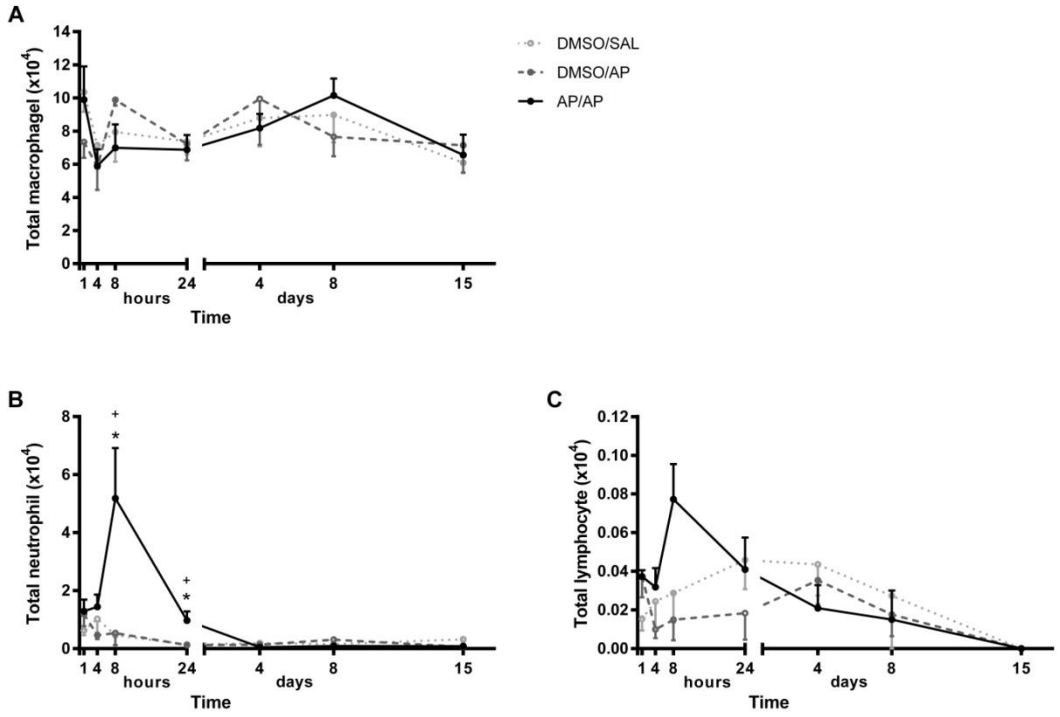

Figure 2. Total number of macrophages (A), neutrophils (B) and lymphocytes (C) in BAL obtained 1, 4, 8 and 24 hours, and 4, 8 and 24 hours, and 4, 8 and 15 days after AP challenge. Experimental groups are the same as figure 1. Mean ± SEM of total number of neutrophils in BAL. *p<0.05 compared with DMSO/SAL, +p<0.05 compared with DMSO/AP. No significant differences were found in the other groups studied at different time points. AP, ammonium persulfate, BAL, bronchoalveolar lavage, DMSO, dimethylsulfoxide; SAL, saline.

Measurement of the cytokines mentioned in BAL fluid revealed increases in IL-10 levels 4 h after AP challenge and increases in IL-2 and IL-13 levels 4 days after AP challenge in the group of AP-sensitized mice, although statistical significance was not reached (p = 0.053, p= 0.076 and p = 0.083, respectively) (*figure 3*).

Neither levels of IL-4, IL-5, nor IL-17A were detected in BAL samples. Cytokine levels were not detectable in tissue homogenate except for IL5, although no significant differences were observed between the groups.

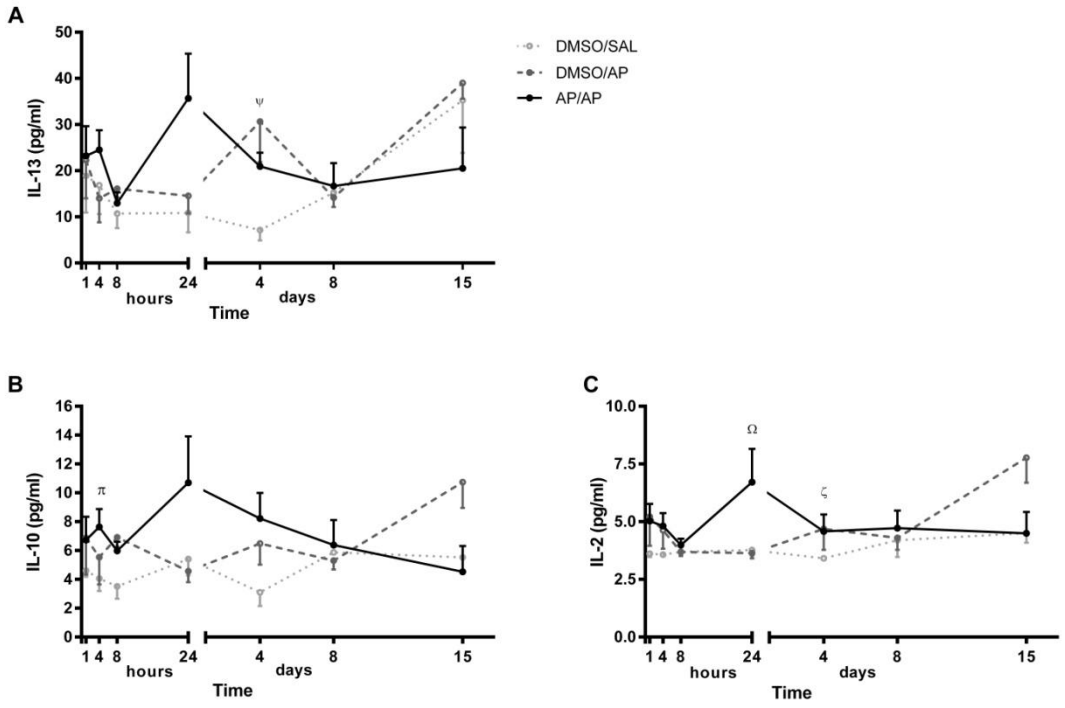

Figure 3. Levels of interleukin (IL)-2, IL-10 and IL-13 in BAL fluid. BAL samples were collected 1, 4, 8 and 24 hours, and 4, 8 and 15 days after AP challenge. Experimental groups are the same as figure 1. **A)** Mean ± SEM of IL-13 concentration. **B)** Mean ± SEM of IL-10 concentration. **C)** Mean ± SEM of IL-2 concentration. Ψ: p = 0.083 compared with DMSO/SAL, π: p = 0.053 compared with DMSO/SAL, Ω: p = 0.055 compared with DMSO/AP, ζ: p = 0.076 compared with DMSO/SAL. No significant differences were found in the other groups studied at different time intervals. AP, ammonium persulfate; BAL, bronchoalveolar lavage; DMSO, dimethylsulfoxide; IL, interleukin; SAL, saline.

Total serum immunoglobulins (IgE, IgG1 and IgG2a)

Figure 4A shows the levels of total serum IgE at the different time points assessed. AP/AP-treated mice showed significant increases in total serum IgE 4 days after the challenge compared with control groups. However, 8 and 15 days after the challenge no significant differences were found compared with controls, and IgE levels returned to baseline values. Total serum IgG1 increased significantly from 4 to 15 days after the AP challenge (*figure 4B*), while total serum IgG2a was significantly increased in AP-treated mice 4 days after the challenge (*figure 4C*).

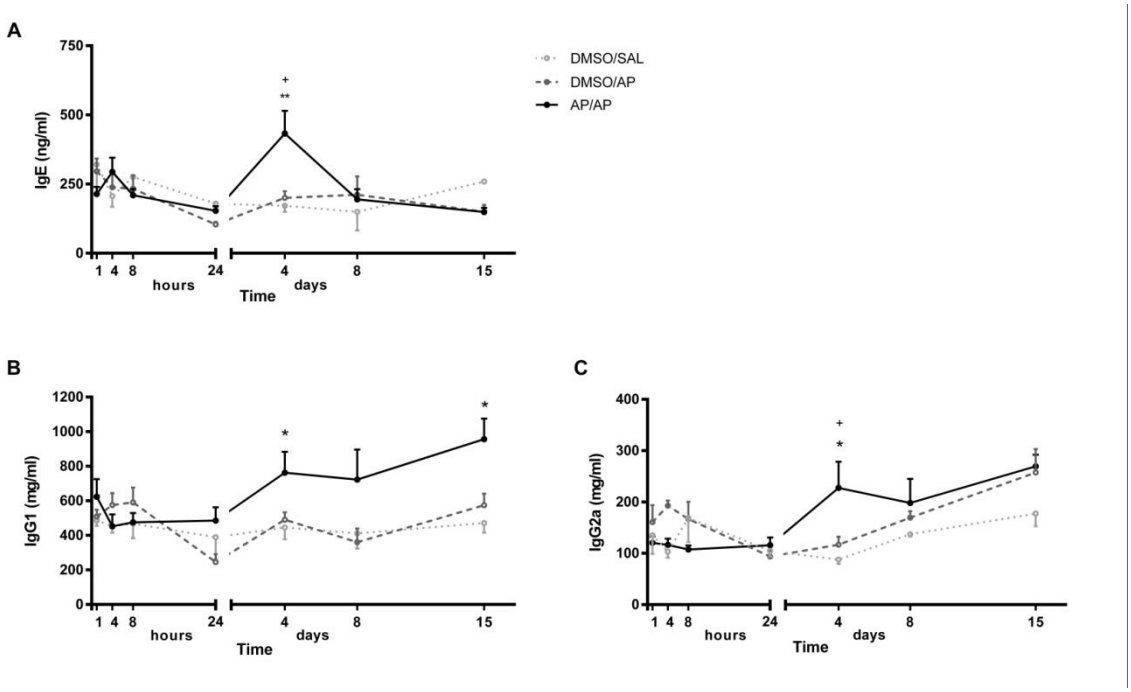

Figure 4. Total serum immunoglobulin (Ig)-E, IgG1 and IgG2a. Blood was collected 1, 4, 8 and 24 hours, and 4, 8 and 15 days after AP challenge. Total serum IgE, IgG1 and IgG2a were measured using a standard ELISA. Experimental groups are as in figure 1. **A)** Mean ± SEM of total serum IgE. **B)** Mean ± SEM of total serum IgG1. **C)** Mean ± SEM of total serum IgG2a. *p<0.05, **p<0.01 compared with DMSO/SAL, +p<0.05 compared with DMSO/AP. No significant differences were found in the other groups studied at different time intervals. AP, ammonium persulfate; DMSO, dimethylsulfoxide; SAL, saline.

Airway histopathology

A blinded histopathological examination of lung tissue sections from the AP/AP mice assessed 8 hours after AP challenge revealed mild to moderate inflammatory cell infiltration and presence of alveolar macrophages compared with control groups. Selectively, at 4 days after the challenge, some moderate peribronchiolar epithelium hyperplasia was observed in the AP/AP group compared with control groups (*figure 5A*). In this acute single challenge model, no collagen deposition was found, as shown in the lung sections stained with Masson's trichrome (*figure 5B*). Scoring of stained lung sections illustrates that in AP/AP mice there was an increase in inflammatory cells between 1 and 24 h after challenge (Grade 1, mild), with a maximum at 4 days (Grade 2, moderate). In the DMSO/AP group there was an increase in inflammatory cells 1 hour after challenge (Grade 1, mild) that disappear 4 hours after challenge. No inflammation was observed in the control group.

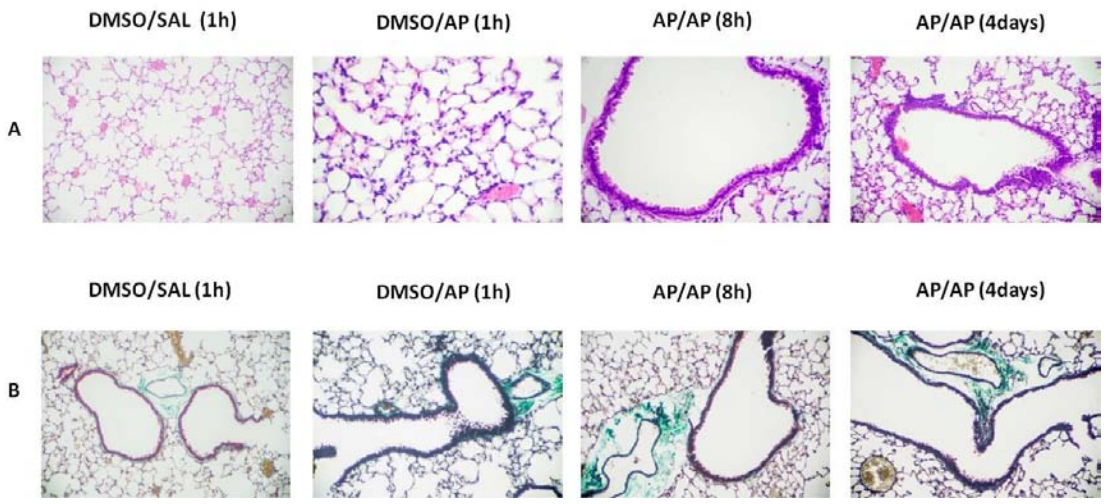

Figure 5. Lung histopathology. Representative images of lung sections are shown at low and high magnification. **A)** Haematoxylin and eosin stained histological lung sections. **B)** Massons's trichrome stained histological lung sections. Experimental groups in this figure are represented with sections from DMSO/SAL, DMSO/ DMSO/AP groups and AP/AP groups assessed 8 hours and 4 days after AP challenge. AP, ammonium persulfate, DMSO, dimethylsulfoxide; SAL, saline.

Discussion

The present study shows that, in dermally sensitized mice, after exposure to persulfate salts the asthmatic response peaks early after the challenge, and then decreases gradually over time. A reduction in the inflammatory response is observed early, while decreases in airway hyperresponsiveness (AHR) and immunological response occur later on.

Our data show a persistent AHR up to 4 days after intranasal challenge with ammonium persulfate (AP). However, although AHR persisted for several days, a significant increase in pulmonary inflammation was only found within 24 hours after the challenge, with inflammatory cells reaching their peak after 8 hours as shown in both BAL samples and histopathological sections. So there was a clear dissociation in time between AHR and inflammatory response. This type of dissociation between inflammation and AHR has been described in previous studies of patients with asthma. In a study in which the effects of anti-IL5 were evaluated in patients refractory to high doses of inhaled corticosteroids, a reduction in the number of eosinophils in sputum was observed, but no changes in pulmonary function or AHR were found [20]. Kariyawasam *et al.* [21] studied the involvement of inflammation and airway remodelling in the pathogenesis of AHR. After antigen challenge, in asthmatic patients with a late response, the increased airway inflammation 24 hours after challenge had returned to baseline values after seven days, while increases in AHR and remodeling biomarkers like RBM procollagen III, procollagen I and expression of HSP-47 persisted at this time point. This dissociation in time between AHR and lung inflammation has also been reported in several animal models [22,23]. The results of these studies suggest that AHR may be the result of independent factors in which inflammation does not have such a relevant direct role. Recently, Hox *et al*. [24], observed AHR without bronchial inflammation after an intranasal challenge

with ClO- followed by an ovalbumin challenge in mice. The authors concluded that this AHR is independent of the classic adaptive immunity mechanism. They showed that the induction of AHR may depend on a neuroimmune interaction involving both mast cell activation and the transient receptor potential ankyrin (TRPA) 1-dependent stimulation of sensory neurons. This mechanism could explain why the group of non-sensitized mice which received the AP challenge (DMSO/AP) showed early AHR in the present study. In this group, this AHR was not accompanied by an increased number of neutrophils or increased levels of total serum IgE, as happened with the asthmatic groups (AP/AP), demonstrating a possible regulation by a nonspecific irritant mechanism in this case [24–26]. It is known that LMW agents elicit an asthmatic response later than HMW agents. Consequently, the early AHR in the previously sensitized asthmatic groups may also be due to this possible irritant effect of the causal agent.

In this model of OA, peak levels of total serum IgE were found 4 days after AP challenge. In some studies in patients with OA due to persulfate salts, the latent period between the exposure and the onset of symptoms and the type of response observed when the challenge test is assessed suggest that OA induced by persulfate salts is mediated by an immunological mechanism [8–10]. Positive skin-prick tests for persulfate salts have been reported, suggesting that this immunological mechanism may be mediated by IgE [8,9,27,28]. Nevertheless, the possible role of IgE in persulfate salt-induced OA has not been well established.

Increased levels of IL-2, IL-10 and IL-13 in BAL fluid and IL-5 in tissue homogenate in AP-treated mice were observed after AP exposure, which suggests a mixed Th1-Th2-type immune response in sensitized mice. IL-13 is known for its central role in both IgE production and induction of AHR in allergic humans and mice [29] a finding that is borne out by the results obtained in this study. IL-10 is a cytokine with broad anti-inflammatory properties and has an important role in the regulation of Th2 responses [30]. In an experimental study of allergen exposure in sensitized asthmatic patients, spontaneous increases in the levels of IL-10 produced by *ex vivo* sputum cells were reported [31]. Consequently, the increase observed in the concentration of IL-10 in BAL samples in this study may be due to a compensatory mechanism for the allergic response which occurs after exposure. Finally, IL-2 a typical Th1 cytokine is also linked to the maintenance of Th2 cells, among other activities [32].

A mixed Th1-Th2 response was found not only in BAL cytokines, but also in serum. In this model, both IgE and IgG1 were increased at selected time points. This finding was already reported in another model of chemical-induced asthma [11,19,33]. In this study, levels of total serum IgG2a showed the same trend as IgE and remained increased 4 days after AP challenge. While IgE is a typical Th2 response, IgG2a is characteristic of a Th1 immune response. Other animal models using LMW agents to induce asthma have shown similar results in the form of increased levels of serum IgG2a [33,34]. There is also evidence of this mixed Th1-Th2 immune response in these animals in view of the cytokine profile in cells in the local draining lymph nodes, since the sensitizer compound caused an increase in both Th1-Th2 cytokines [11,33,35].

As reported above after the initial peak response, total serum IgE returned to baseline values one week after the challenge. Changes in the levels of total serum IgG1 did not follow the same pattern: total serum IgG1 increased significantly from the fourth day post-challenge and persisted over the two weeks of the experiment. These results are consistent with other studies with animal models of asthma induced by LMW agents, which showed increased total levels of serum IgG (IgG1 and IgG2a) [33,36,37].

The role of IgG in response to occupational agents is even more complex. Immunological sensitization to LMW agents is often for life and levels of specific IgG may persist for many years [4]. This IgG persistence was also observed by Vanoirbeek *et al*., based on animal models of OA due to LMW agents [33]. It has been suggested that IgG1 may be important for monitoring the effect of exposure to LMW agents, and particularly to isocyanates, before the onset of the condition [38], although we did not confirm this possible role in our study. Furthermore, it has been reported that an increase in levels of serum IgG, which matches with the decrease of the AHR and inflammatory response, may have a protective effect in this model of OA [39]. Recent studies with asthmatic patients showed a progressive increase in IgG levels with prolonged exposure to allergens [40,41].

To our knowledge, this is the first study to assess the persistence of systemic and ventilatory responses in an animal model of OA due to persulfate salts after the end of exposure to the causal agent. Our experiments show that the progressive decrease in the asthmatic response over time observed in mice may mirror that in patients with OA when exposure to the causal agent ceases [14]. However, many of these patients do not completely recover from their asthmatic symptoms [17], supporting the notion that complete removal from the workplace is not more likely to avoid symptoms than continued exposure [14]. In this context, the mouse model described in this study shows evidence that animals exhibit systemic sensitization which makes them susceptible to developing a new asthmatic response when they are re-exposed to the causal agent. This finding has implications for the recurrence of asthma symptoms.

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Author Contributions

No additional contributions. Conceived and designed the experiments: MJC XM JAV. Performed the experiments: MOM SGO MJC. Analyzed the data: MOM SGO MJC XM, Contributed reagents/materials/analysis tools: MOM SGO MJC XM. Wrote the paper: FM MOM XM MJC JAV.

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5. CHAPTER 3

Effect of anti-IgE in occupational asthma caused by exposure to low molecular weight agents.

Submitted

5.1 Summary of the study

Omalizumab (OmAb) is indicated for the treatment of allergic diseases as it blocks the free serum IgE reducing mediator release from mast cells and consequently, allergic inflammation (110). However, the role of IgE and the immunological mechanisms involved in OA due to LMW agents are not well established. Previous studies with the experimental animal model of OA due to persulfate salts proved an immunological mechanism for AP sensitization and showed high levels of total serum IgE, suggesting a key role for IgE. For this reason, we propose to evaluate the effects of anti-IgE mouse precursor of OmAb in the established experimental animal model of OA due to persulfate salts and further explore the role of IgE in chemical-induced asthma.

Firstly, a multiple challenge protocol was developed in order to improve the mouse model of chemical-induced asthma and yield a more sustained asthmatic response. These protocol outcome parameters were analyzed 24, 48 and 96 hours after the last challenge. Asthmatic response observed was similar to that obtained in previous experiments with a single intranasal instillation, although there was a small influx of eosinophils and lymphocytes in BAL along with airway neutrophilia.

Complete neutralization of serum IgE was achieved in all AP-treated mice receiving anti-IgE mAb. Moreover, treatment with anti-IgE mAb removed AHR at the same time as levels of IL-13 were reduced compared with non-treated mice. These findings are consistent since IL-13 has reported to be essential in the induction of AHR. Actually, some reports have proved that IL-13 alone induced many pathophysiological features of asthma (156). Additionally, anti-IgE mAb lowered levels of other cytokines such as IL5, demonstrating its capacity to decrease the high-affinity receptor FcεRI-mediated production of cytokines involved in the asthmatic response (110).

As regards cellular airway inflammation, anti-IgE mAb administration caused a decrease in the total number of eosinophils and neutrophils 48h and 96h after the last challenge. To our knowledge, the present study is the first report of an OmAb effect on neutrophils, and this is an important issue to be further studied. Reversed inflammatory cell infiltration was also observed in the histological analysis of the lung sections from anti-IgE-treated mice, which also revealed normal bronchial epithelial hyperplasia similar to that of control groups 48h after the last challenge. This OmAb effect on airway structures has already been reported; specifically, OmAb may reduce or even reverse airway remodeling, conferring a persistence of this mAb effects on asthmatic patients (109).

In conclusion, the administration of OmAb precursor improved asthmatic response in the experimental animal model of OA due to persulfate salts by preventing AHR and reducing airway inflammation patterns. In this regard, OmAb may result in positive effects in the treatment of OA caused by persulfate salts. Additionally, these results suggest that an

immunologic mechanism is involved and that IgE may play an important role in the development of the immune response in OA caused by LMW agents.

6. GENERAL DISCUSSION

Occupational asthma (OA), a common cause of work-related lung disease in the industrial world, is new-onset asthma attributable to exposures in a particular work environment (67). As explained in the introduction, agents causing OA can be subdivided into HMW and LMW agents (76). HMW and some LMW agents lead to an immune response mediated by an IgE-dependent response, while other LMW agents seem to act via non-IgE mediated mechanisms. The onset and development of OA induced by chemicals are still not fully understood, as more than one immunological mechanism may be involved.

Persulfate salts are reported to be the main cause of OA among hairdressers (143,144). The symptoms of asthma can be observed months or even years after the first exposure and during this period of time patients become immunologically sensitized. Once sensitized, these patients can exhibit a marked reversible airway obstruction when exposed to persulfate salts, as well as non-specific bronchial hyperresponsiveness to methacholine and inflammation of the airways, characterized by T-lymphocytes, neutrophils and eosinophils (151,153). Accordingly, this thesis has focused on OA caused by LMW agents and more specifically, on OA caused by persulfate salts, in order to evaluate the asthmatic response and provide insights in this context. For this purpose, we used a mouse model of chemical-induced asthma based on a previous model developed by our research group. This mouse model has been repeatedly validated by testing different chemical agents such as toluene diisocyanate (TDI), TMA, toluene diamine and dinitrochlorobenzene (122,124,157,158).

6.1 Management of occupational asthma

As explained in the introduction to this thesis, OA may lead to employment disability and may have a significant impact in terms of work loss, increasing the relative burden of indirect health care costs (89). Despite the large number of reviews evaluating both dermatitis and asthmatic response when exposure to the causative agent persists or ceases, the data available are insufficient to identify the optimal measure for avoiding asthma symptoms (75,82,89,150,159). It has been shown that asthma symptoms and non-specific AHR may persist even years after cessation of exposure to the causal agent, although the reasons for this are not clear. In the case of OA due to persulfate salts, only one clinical study has described the time course of AHR and immunological outcome parameters. Although asthma symptoms and AHR persisted in these patients, their condition seemed to improve if the exposure was avoided (153). Prospective and randomized studies are required to further assess the efficacy of environmental interventions in treating OA and thus to confirm these findings. In this context, the aim of the first part of this thesis was to examine how long the asthmatic response to AP persists after dermal sensitization (*Chapter 1*) and after specific AP challenge in sensitized mice (*Chapter 2*) in our mouse model of persulfate-induced asthma. The results of these studies revealed that although sensitized mice became progressively less responsive over time and the asthma symptoms even disappeared, there were clear signs of systemic sensitization.

The progressive decrease observed in the asthmatic response of AP-challenged mice may partly corroborate literature reports. Specifically, complete removal from exposure to the sensitizing agent has frequently been suggested to be the most efficient therapeutic approach in immunological-mediated OA (79). Nevertheless, regarding the clinical outcomes of OA, fewer than one-third of the patients recovered from their asthma symptoms when the exposure ceased (with a complete symptomatic recovery of 32%) (80). Accordingly, Muñoz *et al.* concluded that avoidance of the causative agent was not related to the improvement of the disease (81). Moreover, because of socioeconomic considerations it may be difficult to remove the patient from the workplace and a reduction of exposure might constitute an alternative approach, although it does not seem as effective as complete removal (75,82).

The systemic sensitization observed in our studies with the animal model occurred with an increase in IgE at early stages and a subsequent return to baseline values, while IgG levels turned up later on and persisted during the study period. The presence of elevated IgE and IgG1 is consistent with a Th2 immune response, whereas IgG2a is compatible with Th1 stimulation. These results have been previously reported in other experimental models of chemical-induced asthma (120,141,160). Despite evidence that an immunological mechanism may be involved (143-145), the exact pathways by which persulfate salts induce sensitization and OA are mainly unknown, given that prior clinical studies have provided controversial information. Although a positive prick test does not by itself indicate sensitization, it provides a complementary tool for the diagnosis of OA (161). On the one hand, some studies found negative skin prick test reactions in hairdressers with OA caused by persulfate salts (151,152). Conversely, Helaskoski *et al.* found fifteen hairdressers with positive prick test reactions to persulfates (161), while other authors have suggested an IgE-mediated mechanism based on positive skin prick tests and increased levels of total serum IgE. Nevertheless, the possible role of IgE in persulfate-induced OA has not been well established. Additionally, specific-IgE to persulfate salts has been reported only occasionally (150) as it is difficult to prepare human serum albumin (HSA) conjugates of these salts. To date, no persulfate conjugate with a body protein like HSA has been found. As mentioned in the introduction, AP can oxidize some single amino acids and modify some key regulatory proteins involved in the sensitization process; it has also been associated with the induction of proteins controlling inflammatory responses, epithelium integrity, and oxidative stress (149). Further studies are needed to broaden our understanding of the role of oxidative stress.

As seen above, persistently high levels of IgG have been observed in our studies and in other experimental work (120,141,160). A possible protective role for IgG in this animal model of OA has been postulated (162) as our results showed that increased levels of IgG matched with decreased respiratory and inflammatory responses; when asthmatic groups of mice presented high serum IgG, both AHR and neutrophilia were attenuated. Moreover, this progressive increase in IgG with sustained exposure to the causal agent has been reported in clinical studies, and it has even been suggested that IgG1 may be a useful biomarker for monitoring

the effect of exposure to LMW agents (163,164). Nevertheless, we did not confirm this possible role in our study. In any case, as in our studies, there is evidence that immunological sensitization can endure throughout the lifetime and that IgG levels may persist for many years after removal of the causal agent (76) with major implications for the recurrence of asthma symptoms. Despite the decline in asthmatic response exhibited in the animal model, we did not test whether these mice would respond to a possible new exposure to the causal agent because of the persistence of systemic sensitization.

The mechanisms associated with asthma recovery or persistence have rarely been explored. Apart from our studies of chemical-induced OA in the animal model, few reports have evaluated the persistence of asthma following avoidance of the causal agent. The longest follow-up after complete suppression of exposure reported that higher levels of IFN-γ were associated with better improvement of asthma, but this has not been confirmed by other studies (165). In addition, Froidure *et al.* (166) proposed that myeloid dendritic cells (mDCs) from patients with asthma and persistent disease present proTh2 features related to asthma activity despite avoidance of the causal agent. In any case, these studies were mainly related to IgE-mediated asthma and studies focused on OA due to LMW agents are now necessary.

6.2 Dissociation of airway hyperresponsiveness and inflammation

Dissociation between AHR and inflammation was observed in the studies included in this thesis. In *Chapter 1*, AHR was the most persistent response and even 60 days after initial AP sensitization, a single challenge could still induce AHR without neutrophilic inflammation. Similarly in *Chapter 2*, although AHR persisted up to four days after AP challenge, airway neutrophilia was only found within 24 hours after challenge. Common knowledge has traditionally linked AHR to airway inflammation (1,167) but many recent studies have proved that inflammatory cells do not necessarily change at the same time as AHR in experimental animal models (141,168,169), and some even showed that asthma can exist in the absence of an influx of bronchial inflammatory cells. In fact, airway remodeling or complex regulations based on neuroimmune interactions seem to be actively involved in the AHR. This point will be discussed later in this section.

As already explained, although the airway granulocytic infiltrate in the lungs of most asthmatics is mainly dominated by eosinophils, in many cases neutrophilic airway inflammation is a hallmark of OA caused by LMW agents (49). Many clinical studies have reported neutrophilic inflammation in patients with chemical-induced asthma (58,170) as have experimental studies with animal models of chemical-induced asthma (122,125). The results from our animal model of persulfate-induced OA have repeatedly shown a marked airway neutrophilia in BAL but no increase in eosinophils or lymphocytes. Histology also revealed only a minor perivascular infiltrate of eosinophils and lymphocytes. Nevertheless, factors such as the time course of the disease and the pattern of exposure, besides the own genetic

susceptibility, may affect the nature of the pulmonary inflammation. This is the case of the outcomes in *Chapter 3*, in which a multiple challenge protocol led to a small infiltration of eosinophils and lymphocytes, despite the fact that neutrophilia was still predominant.

Although neutrophils seem to be involved in both the lymphocyte activation and the effector phase (AHR, lung inflammation and epithelial damage) (59), their role in the development of AHR is controversial. Some studies have reported that neutrophil depletion does not affect the development of AHR (171,172), whereas others have found AHR suppression when neutrophils were depleted (125,173). Moreover, some studies have focused on the Th17 pathway to explain the airway neutrophilia. These studies reveal that airway exposure to allergen in sensitized individuals causes the release of adenosine triphosphate (ATP) and uric acid, activating the inflammasome complex and leading to the production of mature interleukin (IL)-1β, which creates a pro-inflammatory milieu with the production of chemokines that mobilize neutrophils and enhance Th17 cell differentiation in the lung. IL-17A plays a critical role in neutrophil and Th2-mediated eosinophil recruitment to the lung by regulating the expression of various pro-inflammatory mediators such as cytokines, chemokines, and adhesion molecules (174,175). Nevertheless, in our studies we could not prove the involvement of this Th17 pathway as levels of IL-17A were not detected. In any case, assessment of the role of neutrophils in OA due to LMW agents remains an area of active research.

As regards airway remodeling, Kariyawasam *et al.* also showed persistence of AHR along with remodeling biomarkers without any sign of cellular inflammation, demonstrating a similar pattern of dissociation (176) to that found in other studies with patients (98,177). It has been suggested that airway remodeling, as well as the activation state of involving cells or an autonomic dysfunction, may affect the development of AHR rather than airway inflammation, which probably aggravates the condition (177). In airway remodeling, the main structural changes observed in asthma are loss of epithelial integrity (178), thickening of basement membrane (179), subepithelial fibrosis (180), goblet cell and submucosal gland enlargement (181,182), increased smooth muscle mass (181), decreased cartilage integrity (183), and increased airway vascularity (184,185). This remodeling process has been proposed as an explanation of the characteristics of asthma, as an attempt to protect against allergen-induced airway inflammation and AHR. For example, increased airway wall stiffness could protect from further narrowing of the airways, and subepithelial fibrosis, a result of increased deposition of extracellular matrix proteins including collagens (186), could reduce the amount of allergen exposure by shielding the immune system from these substances (187). In our studies, we found no clear indications of airway remodeling, only mild to moderate inflammatory cell infiltration and selective moderate peribronchiolar epithelium hyperplasia. These small changes in histological lung sections may be a consequence of an acute protocol of asthma induction. Moreover, it should be borne in mind that a LMW agent was used, while most other studies have used HMW agents.

Overall, the results of these studies showing dissociation between AHR and inflammation suggest that AHR may be the result of independent factors in which inflammation does not have such a relevant direct role. It has also been postulated that a nonspecific irritant mechanism may be involved in AHR. Once the chemical is taken up, APCs may stimulate both the immune system and sensory pathways that activate the central nervous system, leading to neuroimmune interactions with a key involvement of mast cells and transient receptor potential channels (TRPA1 and TRPV1) which stimulate the sensory nerves (128). In this regard, $TRPA1^{-/-}$ knock-out (KO) mice, or even TRPA1 antagonists, have provided valuable information as these mice did not show AHR after chemical exposure. These data suggest that activation of these TRP channels on airway sensory fiber terminals by hazardous irritants could trigger the local release of proinflammatory neuropeptides, leading to OA or irritant-induced asthma (127,128). In any case, there is no doubt that the adaptive immune system also plays an important role since, after the early response, the induction of AHR only occurs in a context of specific immune sensitization. For example, Devos *et al.* (133) observed that $Rag2^{-/-}$ KO mice, which lack mature lymphocytes, did not present AHR, indicating that properly functioning lymphocytes are necessary for the asthmatic response development. However, these studies involved isocyanate-induced asthma and the role of this neuroimmune mechanism should be confirmed in the case of persulfate salts in the near future.

6.3 Effects of anti-IgE administration

Given the high risk of job loss and the benefits of symptom improvement, optimal management of OA consists in personalizing the therapeutic approach. The role of IgE in the asthmatic response has been extensively studied; its contribution to allergic asthma is relatively clear (28,188), and it has become a good therapy target. As explained above, OmAb is becoming widely used as a potential therapy in allergic disease. Many clinical trials have shown that OmAb seems to have beneficial effects in terms of both reducing asthma exacerbations and decreasing, or even removing the need for, ICS treatment (108). In fact, the suitable condition for treatment with OmAb is severe allergic asthma not controlled by conventional drug treatment, with total IgE levels ranging between 30 and 1,500 international unit (IU)/mL. Recently, the drug has been approved for therapeutic use in children aged 6–12 years (110).

However, the role of IgE in OA due to LMW agents has been repeatedly questioned, since no direct relation between IgE and the immunological response has been established. As mentioned above, the results of *Chapter 1* and *Chapter 2* suggest that persulfate salts are dermal sensitizers which cause long-term sensitization in our animal model of persulfate-induced asthma, with increases in total serum IgE at early stages and in IgG at a later time point. For this reason, we aimed to evaluate the role of IgE and the mechanisms involved in the development of the immune response. By neutralizing the IgE, we wanted to study effects of anti-IgE mAb treatment in the established mouse model of persulfate-induced OA. Only one

clinical study has demonstrated benefits with OmAb therapy in OA caused by chemical agents (88); the results of *Chapter 3* corroborate these observations.

Specifically, anti-IgE mAb abolished the AHR, reduced inflammatory cells both in BAL and histological lung sections, lowered levels of cytokines and reversed bronchial epithelial hyperplasia compared with non-treated groups. These findings confirmed previous results observed when this drug was tested (189,190). Results of experimental studies using a murine model of OVA-induced chronic allergic airway inflammation had already shown that AHR, inflammatory cell counts and cytokines such as IL-5 and IL-13 in BAL fluid decreased after receiving OmAb therapy (191). In parallel, reductions in T-lymphocytes producing IL-13 after OmAb administration were observed in patients with moderate-to-severe allergic asthma (189). The fact that treated mice showed decreased AHR is consistent with the reduced levels of IL-13 in the same groups, as this cytokine has been reported to be essential for the development of AHR. Additionally, the results presented in *Chapter 2* support the notion that IL-13 plays an important role in the induction of AHR, since AP-treated mice showed increased levels of IL-13 compared with vehicle groups. (192). In fact, studies performed in mouse models of asthma have proven that IL-13 alone is capable of inducing many of the pathophysiological features characteristic of asthma (156). Alternatively, IL-13 may also induce AHR via direct effects on resident airway cells such as epithelial cells, airway smooth muscle cells or alveolar macrophages (193). For example, IL-13 has been associated with enhanced airway smooth muscle contraction through its effects on calcium signaling, confirming that this cytokine is widely involved in the development of AHR (194).

In our third study, the fold-increase in IL-5 in asthmatic mice may partially explain the low level of infiltration of airway eosinophils observed. No reduction in IL-5 could be observed directly as a consequence of therapy, although anti-IgE mAb tended to reduce slightly IL-5 slightly levels, demonstrating once again its capacity to decrease the FcεRI-mediated production of cytokines involved in the asthmatic response (190). When OmAb is administered, the expression of the FcεRI receptor is lowered, which may contribute to the lower antigen-presenting ability and, overall, the weakened immune response (195).

The effects on IL-10 levels observed in *Chapter 3* may be attributable to the anti-inflammatory properties of this cytokine (196). At early stages, IL-10 levels in asthmatic groups were lower than those in control groups, showing that the inflammatory airway homeostasis was imbalanced through a Th2 response; this was confirmed by the presence of the asthmatic response at this time point (i.e., 24 hours after the last AP challenge). We showed that anti-IgE mAb administration resulted in a decline in both AHR and cellular inflammatory infiltration, which could explain why IL-10 levels were grown 48 hours after the last AP challenge. Additionally, the increases in IL-10 observed both in *Chapter 2* and in previous results validating the animal model (124) suggest that Treg cells were stimulated. It has also been observed that mice deficient in IL-10 producing cells exhibited excessive airway inflammation

(196). In the case of sputum cell cultures from sensitized asthmatic patients who underwent a bronchial allergenic challenge, a spontaneous production of IL-10 has been reported (197).

Classically, the cytokine IL-2 exerts both stimulatory and regulatory functions in the immune system with a central role in immune homeostasis, and has been associated with Th1 lymphocytes although it is produced by all Th cells at an early stage during its activation (198). The increased levels of IL-2 in BAL reported in *Chapter 2* may be connected to Th2 cell preservation (199). However, decreased levels of IL-2 were found when lymphocytes from AP groups were stimulated *in vitro,* suggesting an inhibition of the Th1 pathway. Conversely, levels of IFNγ were slightly increased, which is typical of Th1 stimulation (124). Previously, there was evidence of increases in both IL-4 and IFNγ derived from cells of the local draining lymph nodes in the same validated mouse model of OA induced by TDI or TMA, suggesting a mixed Th1/Th2 immune response (119,122,124). In fact, this mixed immune response has been postulated on the basis not only of the cytokine profile but of the IgE-IgG pattern as well, as mentioned above.

Currently, anti-inflammatory drugs constitute the basis of asthma therapy although their effects on airway remodeling remain to be elucidated. This is why new treatments should be directed not only against the inflammation itself but also against these chronic changes in asthmatic lungs. In this regard, *Chapter 3* demonstrated that anti-IgE mAb could act on the airway structures and reversed the bronchial epithelial hyperplasia and the inflammatory cell infiltration. It has been proposed that the persistence of OmAb effects may be due to this activity reducing or revoking airway remodeling changes (109). A recent study has also provided experimental evidence that the benefits of OmAb for airway remodeling may be due to its direct action on IgE-bound airway smooth muscle cells (200). Overall, anti-IgE mAb not only neutralized the circulating levels of serum IgE but also prevented AHR and reduced airway inflammation patterns, probably due to its proven effects in reducing the cell-bound IgE and, as a result, mediator release and allergic inflammation (110,201). In addition, it is worth noting that this drug also downregulates FcЄRI expression on basophils and mast cells by lowering or even blocking allergic reactions. OmAb also seems to act on IgE regardless of antigen specificity (109). A recent study reported a novel mechanism via which OmAb may reduce the phosphorylation status of some kinases and modulators of the signaling pathways involved in mast cell and basophil activation, leading to a decrease in pre-existing and newly synthesized mediators (202). This may explain why OmAb appears to have some new indications in settings other than allergic asthma, such as non-allergic asthma, chronic urticaria or even anaphylaxis (110). Further studies are needed to determine the role of this drug in an IgE-independent context. Nevertheless, the improved asthmatic condition of anti-IgE therapy observed in our mouse model suggests that an immunologic mechanism is involved and that IgE may play an important role in the pathophysiology of this entity.

In summary, the studies included in this thesis indicate that the development of OA due to persulfate salts does not completely bias the immune response toward a Th2 response. Rather, they suggest a complex interaction of an innate and a mixed type1-type2 adaptive immune response.

6.4 Future perspectives

The studies that comprise this doctoral thesis, based on experimental animal models, shed light on the persistence of the asthmatic response in OA due to persulfate salts and also propose the possible involvement of pathways in the immune response. In this regard, the administration of anti-IgE mAb has proved to be a useful tool for unraveling some of these mechanisms and has provided new alternative approaches for the management of OA. Further studies with other mAb directed at specific molecules such as anti-IL5 (mepolizumab) or anti-IL13 (lebrikizumab, dupilumab) in the experimental model of chemical-induced asthma should also provide useful information for the treatment of this entity.

Despite this evidence of the involvement of immunological mechanisms, however, a great deal of work remains to be done. The development of an accurate persulfate antigen which reflects these forms *in vivo* in order to have access to suitable methods for detecting persulfate-specific IgE, is an area of active research. The lack of specific IgE in certain types of LMW-induced asthma has raised questions about the mechanisms involved in its pathophysiology. Persulfate-specific IgE might not be detected in immunoassays because of the use of the wrong antigen, thus resulting in false negative tests. Persulfate-self protein reaction products are extremely diverse, and their antigenicity may differ significantly depending upon the reaction conditions under which they are formed.

The results derived from this thesis raise some new questions and open up the opportunity to further explore the immunological interactions underlying the development of chemical-induced asthma. For this purpose, transgenic mice and even some agents blocking certain immunological pathways either partly or completely have become a valuable tool for understanding some of the pathophysiological processes in OA due to LMW agents. In our mouse model, neutrophils are the predominant airway inflammatory cells present in AP-sensitized mice undergoing the AP challenge. This is not entirely typical of an immunologically mediated asthmatic response, since airway inflammation is classically characterized by eosinophils and lymphocytes in atopic asthma. Nevertheless, as has been mentioned, airway neutrophilia is also common in patients with chemical-induced asthma (58). The specific role of neutrophils (and also of eosinophils) in this mouse model of persulfate-induced asthma will be assessed by neutrophil depletion in experiments using non-specific leukocyte blockers such as cyclophospamide, or selective blockers of granulocytes such as Ly6G-specific mAb. Depletion before challenging the mice with AP will offer more information about the role of neutrophils in the development of chemical-induced asthma.

Mast cells are also essential elements of the asthmatic response, even though their role in non-allergic asthma is partially unknown. As persulfate salts can dissolve in aqueous solutions, it will be possible to perform *in vitro* experiments with mouse bone marrow-derived mast cells (BMMCs) or LAD2 human mast cell cultures. Additionally, mast cell-deficient mice should also contribute to clarify the possible involvement of these cells in this type of asthma. Since it has been proposed that mast cells communicate directly with sensory nerve fibers in the airways (203), another interesting pathway to be studied is neuroimmune interaction with the crucial involvement of TRP channels. It has previously been reported in an animal model that the use of a TRP antagonist reduced both inflammation and acute airway responses to chemical exposure (127). By using TRP channel antagonists and even KO mice devoid of TRPA1 and TRPV1, we can further evaluate the neurogenic mechanisms involved in the development of the asthmatic response in persulfate-induced asthma.

Finally, other strategies have focused on genes associated with some inflammatory diseases, as in the case of ORMLD3 encoding a calcium channel that is highly expressed in airway epithelial cells (129). Transgenic mice overexpressing ORMLD3 have shown spontaneous AHR and airway remodeling patterns, suggesting an important role in asthma (130). This experimental model is likely to provide useful information on the susceptibility to asthma, and on the mechanisms via which calcium may regulate some inflammatory processes.

Taken together, these proposals for further studies have an important contribution to make in unraveling the mechanisms involved in the development of chemical-induced asthma and are likely to improve the treatment of asthma and the quality of life of these patients.

7. CONCLUSIONS

Overall, the studies included in this doctoral thesis propose that the development of occupational asthma due to persulfate salts does not completely bias the immune response toward a Th2 response, but involves a complex interaction between an innate and a mixed type1-type2 adaptive immune response.

The main conclusions obtained are presented separately for each study.

7.1 Chapter 1. Persistence of dermal sensitization

1. Both respiratory responsiveness to methacholine and airway inflammation responses decrease with increasing time between sensitization and challenge, although respiratory responsiveness to methacholine is more persistent.

2. The evidence of systemic sensitization implies that dermal contact with a chemical can cause long-term sensitization and lead to a reoccurrence of asthmatic symptoms.

7.2 Chapter 2. Persistence of the asthmatic response after persulfate inhalation

3. In dermally sensitized mice, after the exposure to persulfate salts the asthmatic response peaks early after the challenge and then decreases gradually over time. At early stages only the inflammatory response decreases, but later on airway hyperresponsiveness and immunological response decrease as well.

4. This mouse model of persulfate-induced asthma shows once again evidence of systemic sensitization which increases the mice's susceptibility to developing a new asthmatic response after re-exposure to the causal agent.

7.3 Chapter 3. Effect of anti-IgE in occupational asthma due to low molecular weight agents (persulfate salts)

5. The outcome parameters with a multiple challenge protocol lead to increased airway hyperresponsiveness and a predominantly neutrophilic inflammation along with a small influx of eosinophils and lymphocytes in non-treated asthmatic mice.

6. Anti-IgE treatment completely neutralizes serum IgE and improves asthma features related to airway hyperresponsiveness and both inflammatory cell infiltration and levels of cytokines involved in the immunological mechanisms.

7. The efficacy of anti-IgE therapy observed in the mouse model of occupational asthma induced by persulfate salts suggests that an immunologic mechanism is involved and that IgE may play an important role in the pathophysiology of this entity. Nevertheless, the exact pathways via which IgE interacts to regulate the asthmatic response are not well established.

8. Anti-IgE treatment may be an effective therapeutic approach in occupational asthma due to persulfate salts.

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