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DIPLOMARBEIT

CHARACTERIZATION OF LONG-LASTING LUNG RESIDENT CELLS TAKING UP AND RETAINING ANTIGEN FOR EXTENDED PERIOD OF TIME

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ABSTRACT

Allergic asthma is a chronic disorder of the respiratory tract characterized by airway inflammation, mucous hyperproduction and bronchial hyperresponsiveness. Clinical manifestation in patients includes coughing, wheezing, shortness of breath and chest tightness. The gravity of symptoms is perceptible particularly during the night or in the morning.

Despite the progress in understanding the underlying mechanisms and nature of asthma-drivers, the prevalence of the disease has continuously increased over the last decades.
On a biological level, it is an immunological reaction to certain invading agent that is orchestrated by diverse elements including immune as well as structural cells as well as their products. Raising evidence suggest the key role of antigen presenting cells (APC), primarily of dendritic cells (DC), in the pathobiological process of asthma. Indeed, APCs are the first players upon encounter to an antigen and thus they are essential for the sensitization and subsequent immune responses. In other words, APCs are pivotal for initiation and development of pulmonary inflammation.

In this study, a novel cell population with attributes of APC that picks up Ag and stores it for prolonged period of time was characterized. Naïve mice and those rechallenged with Ag to induce exacerbations vs mice recovered from a single episode of allergic asthma were used in these studies. The naïve group were healthy animals that had never encountered OVA, whereas rechallenged and recovered mice were those with only one experimentally induced episode of allergic asthma. Animals in latter two groups were systemically immunized with OVA on 2 separate occasions and subsequently exposed to aerosolized OVA. In the further course of the project, mice belonging to naïve as well as the rechallenged group received intranasal instillation of OVA conjugated to a red fluorochrome TexasRed (TR-OVA). Recovered rodents, by contrast, were excluded from i.n. treatment with TR-OVA and had to rest for few months in order to recuperate from the disease. Ag capture and retention in lung cells of naïve and rechallenged mice was visualized with methods of immunofluorescence. Further immunohistochemical analysis provided specification concerning their morphology, localization and possible

function. According to cell surface markers they either do or do not express, a phenotype of this cell subset was established.

ZUSAMMENFASSUNG

Allergisches Asthma ist eine chronische Erkrankung der Atemwege, gekennzeichnet durch Entzündung, Überproduktion von Schleim und bronchiale Hyperreagibilität.
Klinisch manifestieren sich diese Prozesse in Form von Husten, Niesen, Atemlosigkeit sowie beklemmendem Gefühl in der Brust.

Auch der kontinuierliche Fortschritt und die Gewinnung neuester Erkenntnisse im Zusammenhang mit den zugrunde liegenden Mechanismen der Krankheit konnten dem Ausbreiten von Asthma nicht halt bieten.
Biologisch gesehen handelt es sich um eine Überreaktion des Immunsystems auf gewisse körperfremde, ansonsten oft harmlose Substanz. Die immunologische Antwort wird dabei sowohl von Immun- als auch von Gewebszellen samt ihren Produkten vermittelt. Bedeutende Evidenzen weisen in diesem Sinne auf die zentrale Rolle der antigen-präsentierenden Zellen hin, insbesondere richtet sich das Augenmerk der Forscher auf die dendritischen Zellen. Denn, die antigen-präsentierenden Zellen sind die ersten, die die eingedrungenen Allergene abfangen. Daher bilden sie die notwendige Basis für die Sensibilisierungsprozesse und die darauf folgenden Immunantworten, sprich, sie sind ausschlaggebend für die Entstehung und Entwicklung der Atemwegsentzündung.

In dieser Studie wurde eine neue Lungenzellpopulation untersucht, die Antigene aufnimmt und sie gewisse Zeit bewahrt, eine Population also, die Eigenschaften der antigen-präsentierenden Zellen aufweist. Naive Mäuse und solche mit der Erkrankung, sowie die von den Asthmasymptomen erholten Mäuse dienten als Probanden. Die naiven „Modelle“ wurden von den Tieren repräsentiert, die bis dato keinerlei Kontakt mit dem OVA-Antigen hatten. In den erkrankten und erholten Tieren hingegen wurde auf experimentellem Wege allergisches Asthma induziert. Dazu wurden sie einer systemischen Immunisierung mit OVA zu zwei verschiedenen Zeitpunkten unterzogen. Anschließend wurden sie mit aerosolisiertem OVA behandelt. Im weiteren Verlauf wurde den naiven sowie erkrankten Mäusen das mit rotem Fluorochrom (Texas Red) konjugierte OVA-Antigen intranasal verabreicht (TR-OVA). Erholte Tiere waren

hievon ausgeschlossen und bildeten somit die Kontrollgruppe. Das rote TR-OVA Antigen konnte letztendlich in den Lungen nachgewiesen werden. Die Zellen, die es aufgenommen hatten, wurden mithilfe von Immunfluoreszenz-Techniken visualisiert. Die daraufhin folgende immunohistochemische Analyse führte zu einer umfassenden Charakterisierung hinsichtlich ihrer Morphologie, Lage in der Lunge und der möglichen Funktion. Aufgrund dieser Daten und entsprechend ihres Oberflächenmarker-Profils wurde der Phänotyp dieser Zellpopulation erstellt.

ABBREVIATIONS

Ab	Antibody
Ag	Antigen
AHR	Airway hyperresponsiveness
Alum	Aluminium sulfate
APCs	Antigen presenting cells
AR	Alexa Red
BAL	Bronchoalveolar lavage
BHR	Bronchial hyperresponsiveness
CD	Cluster of differentiation
CTLs	Cytotoxic T lymphocytes
DC	Dendritic cells
DT	Diphtheria toxin
DTR	Diphtheria toxin receptor
ECM	Extracellular matrix proteins
FACS	Fluorescence Activated Cell Sorter
FITC	Fluorescein isothiocyanate
GFP	Green fluorescent protein
GM-CSF	Granulocyte macrophage colony-stimulating factor
HDM	House dust mite
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
i.n.	intranasal
i.p.	intraperitoneal
LC	Langerhans cells
LPS	Lipopolysaccharide
MHC	Major Histocompatibility Complex
mDC	Myeloid dendritic cells
NK	Natural killer (cell)
NO	Nitric oxide

OVA	Ovalbumin
PAF	Platelet-activating factor
PAMPs	Pathogen Associated Molecular Patterns
PAS	Periodic acid-Schiff
PBS	Phosphate buffered saline
pDC	Plasmacytoid dendritic cells
PGE_2	Prostaglandin E_2
PMNs	Polymorphonuclear leukocytes
PRR	Pattern Recognition Receptor
TCR	T cell receptor
TG	transgenic
TGF	Transforming growth factor
Th	T helper (cell)
TNF	Tumour necrosis factor
TR	Texas Red
Tregs	Regulatory T cells
WBC	White blood cells
WBP	Whole-body plethysmography
WT	Wild-type

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1. INTRODUCTION

1.1 INTRODUCTION TO THE IMMUNE SYSTEM

Understanding of the human immune system has significantly advanced in the past few decades.

The immune system is defined as a composition of mechanisms coordinated by cells, tissues and molecules that all together mediate resistance to infections, and its physiologic function is to prevent those infections and eliminate the established ones. This operation is expressed as the immune response and it is a corresponding reaction of the organized immune system components to infectious microorganisms.

The significance of the immune system for health is well-founded by the fact that people with defective immune responses are susceptible to serious, often life-threatening illnesses. Paradoxically, the process of vaccination in order to stimulate immune responses and develop resistance against foreign pathogens is the most effective method for protecting individuals against infections.

On the one hand, we have an irreplaceable and incomparable defence system, but on the other hand, the immune response is the major barrier to successful organ transplantations.

Apart from that, the intention to treat cancers by stimulating immune responses against cancer cells are being implemented in different malignancies therapies (Abbas and Lichtman, 2009).
Therefore, the awareness concerning the wide field of immunology is constantly increasing among both, scientists as well as general public.

1.1.1 Innate immunity

The defence system is organized in two major subdivisions, the innate or nonspecific immune system, which mediates the initial protection against infections, and the adaptive or specific immune system, which develops more slowly and enables also protection against re-exposure to the same pathogen. The innate immune system is our first line of defence against invading organisms while the adaptive immune system acts as a second line of defence. Each of these subdivisions of the immune system has both, cellular and humoral components by which they carry out their protective function. In addition, the innate immune system also has anatomical features that behave as barriers to infection (Abbas and Lichtman, 2009; Janeway et al., 2002).

Although these two branches of the immune systems both operate to protect against invading organisms, they differ in several ways. Innate, also called native immunity, is characterized by its constant presence in healthy individuals, ready to blockade the entry of pathogens and to rapidly eliminate those who do manage entering the organism. Unlike the adaptive, the innate immunity is not particularly specific and its components recognize structures that are represented by diverse classes of microbes. It reacts equally well to a variety of organisms and in case of repeated encounter with foreign pathogen the innate immunity responds in the same extent, what means it does not demonstrate immunological memory. And finally, the innate immune system does not react against itself or against nonmicrobial/non-infectious substances, in contrast to adaptive immunity (Abbas and Lichtman, 2009). For this reason people used to believe the innate immunity is not effective enough in eradicating most infections. In the meantime it is clear that there is interplay between these two systems and that innate host defence not only provides immediate protection but also instructs the adaptive defence mechanisms to respond effectively to various pathogens (Abbas and Lichtman, 2009; Janeway et al., 2002).

Components of innate immunity

Epithelia, cells in tissue and circulation and different plasma proteins, these elements and their complementary manner represent the innate immune system (Abbas and Lichtman, 2009; Janeway et al., 2002).

Epithelial barriers

The major portals of entry of microbes are the skin, gastrointestinal tract and respiratory tract. These openings are very tempting for microorganisms to invade the host, therefore they are surrounded by epithelia that forms a physical barrier which is impermeable to most pathogens. Thus, the skin acts as our first line of defence (Abbas and Lichtman, 2009; Goldsby et al., 2001; Janeway at al., 2002). Furthermore the flushing action of tears, saliva and urine helps prevent infections of eyes, mouth and urogenital tract. Coughing and sneezing help our lungs keep the airways free from microbes. The trapping effect of mucus secreted by respiratory and gastrointestinal tract serves to protect lungs and digestive system from infections (Roitt et al., 1989).

Another way to defend our body openings are chemical barriers. Fatty acids in sweat for example inhibit the growth of bacteria. Enzymes like lysozyme and phospholipase found in tears, saliva and nasal secretions are able to break the cell wall of bacteria and stop their multiplying. Respiratory tract produces peptides such as defensins, that have antimicrobial activity. In the stomach low pH due to gastric acid makes it impossible for many ingested pathogens to survive (Roitt et al., 1989).
In the end, the normal flora of the skin and within the gastrointestinal and genitourinary tracts acts as a biological barrier by secreting toxic substances and competing with pathogenic agents for nutrients or attachment to cell surfaces (Roitt et al., 1989).

Cells of innate immunity

Cellular barriers, here leukocytes, are the main line of defence in the non-specific immune system. They are white blood cells (WBCs) and amongst others they include phagocytes, eosinophils, basophils, mast cells and natural killer cells, and they all are derived from common precursor cells: haematopoietic stem cells in bone marrow (Abbas and Lichtman, 2009; Janeway et al., 2002).
Phagocytes themselves are classified into monocytes/macrophages, neutrophils and DC. All of them serve to recognize, identify and eliminate infectious agents, and additionally

they play a role in activation of the adaptive immune response (Abbas and Lichtman, 2009; Janeway et al., 2002).

Neutrophils and monocytes belong to circulating phagocytes and are recruited to the site of infection where they recognize and phagocytose microorganisms and kill them intracellularly. Neutrophils, so-called polymorphonuclear leukocytes (PMNS), are the most abundant leukocytes in the bloodstream. Their production is stimulated by cytokines, which are produced by many cells as a reaction to infection. They are usually the first cells to reach the place of infection. Neutrophils ingest pathogens in the bloodstream as well as in the extravascular tissues, where they survive for only few hours.

Monocytes are not as numerous as neutrophils. In contrast to neutrophils, monocytes that migrate through blood vessel walls into extravascular tissues survive for longer periods and there, these monocytes differentiate into macrophages. In addition, the body has macrophages already stationed throughout the tissues and organs, thus these macrophages and blood monocytes are two families of the same cell lineage, namely mononuclear phagocytic system. Macrophages have a number of very important functions in body defence including: killing of microbes, infected cells and tumor cells by phagocytosis and processing antigens so they can be recognized by T-lymphocytes during the adaptive immune response. Because of this function they are often referred to as antigen-presenting cells (APCs). Furthermore macrophages produce and secrete diverse proteins called cytokines, growth factors and enzymes, which are significant for non-specific body defence (Janeway at al., 2002; Roitt et al., 1989).

DCs are phagocytes located throughout the skin, respiratory and gastrointestinal tract as well as lymphoid tissues. The principal task of immature DCs is to capture antigens through pinocytosis and phagocytosis, leave their initial site, enter lymph vessels and migrate to lymph nodes. By the time they reach the lymph nodes, they have matured and are now able to present antigen to T-lymphocytes. Hence, DCs belong to APCs (Janeway at al., 2002).

Mast cells are most often associated with allergy and anaphylaxis and they carry out many of the same inflammatory-mediating functions as basophils. There are two types of mast cells in the body: mast cells found in the connective tissue and those found

throughout mucus membranes. The granules of mast cells contain such mediators as histamine, eosinophil chemotactic factor, heparin, leukotrienes, platelet activating factor and cytokines. Apart from that they also synthetize prostaglandins, chemicals which promote inflammation (Roitt et al., 1989).

Basophils make up a very small percentage of WBCs in the bloodstream. Like mast cells, basophils release histamine, leukotrienes, heparin and prostaglandins which boost inflammation by causing vasodilatation, increasing capillary permeability and increasing mucous production (Roitt et al., 1989).

Eosinophils belong to leukocytes that play a significant part in allergic responses. The number of eosinophils in blood often rise above the normal range not only with allergic reactions but also upon parasitic infections like worms. They act as scavengers by destroying ingested microbes, however primarily they set their contents free in order to kill huge microorganisms extracellularly. Amongst others they release destructive enzymes for infectious organisms such as: acid phosphatase, peroxidases, major basic protein, RNase, DNases and other chemicals like leukotrienes and cytokines (Janeway et al., 2002; Roitt et al., 1989).

Natural killer cells (NK cells) build up a small fraction of the lymphocytes in the blood that, unlike T- and B-lymphocytes, do not express antigen-specific receptors (Janeway et al., 2002). Nevertheless their function is to recognize and kill cells/tissues that have been infected by viruses or cancer cells. This mechanism of identification is associated to expression of MHC-class I molecules. Healthy cells express MHC-class I on their surface and this act prevents NK cells from killing them, but virus-infected and malignant cells reduce their expression, hence they will be eliminated by NK cells. Furthermore they produce IFN-γ (interferon-γ), a cytokine which activates macrophages so they can kill ingested microbes (Roitt et al., 1989).

Complement system is an enzymatic system of serum proteins that are sequentially activated during two different pathways, the “classical” and the “alternative” pathway, resulting in a variety of antimicrobial defence. Complement components play a role in phagocytic chemotaxis, opsonization and the inflammatory response, and may be

involved in the lysis of certain bacteria, some viruses and other microorganisms (Roitt et al., 1989).

1.1.2 Adaptive immunity

When our first line of defence, the innate immunity, is not sufficient to protect the host against an invading pathogen and fails, the pathogen may yet be detected and attacked by the mechanisms of adaptive immunity. This second line of defence however takes some time to develop, as it has to accommodate to attendance of invading organism.

Adaptive immune system is also called acquired or specific immune system due to the fact that its response is antigen-specific. This specificity is demonstrated by the cells of adaptive immunity when some of them express membrane receptors that are capable of discriminating fine differences in structure between diverse antigens (Abbas and Lichtman, 2003). An antigen is defined as a substance that reacts with antibody molecules and antigen receptors on lymphocytes (Janeway et al., 2002). They may be products of microorganisms as well as molecules of non-infectious nature (Abbas and Lichtman, 2003). Moreover, acquired immunity also distinguishes among individual's own (self) and foreign (non-self) antigens, however lymphocytes that are competent of identifying self antigens are destroyed. This property is essential for preventing reactions against host's own cells and tissues (Abbas and Lichtman, 2003).
Another feature of acquired immunity is the immunological memory, what means that adaptive immune system "remembers" that it has encountered an antigen and consequently it responds more rapidly and more effective to subsequent exposure to the same antigen. Accordingly, the reaction to the prior exposure to antigen is managed by naïve lymphocytes, and repeated encounters with same antigen stimulate memory lymphocytes (Abbas and Lichtman, 2003).

In this context, it is appropriate to name the components of adaptive immune system, these are the lymphocytes and their products, e.g. antibodies. Like innate, adaptive immunity also has two major branches of immune responses: the humoral and the cell-

mediated immunity, both governed by different types of cells. Humoral immunity involves the secretion of antibody molecules in response to an antigen and is mediated by B-lymphocytes. Cell-mediated immunity involves the production of cytotoxic T-lymphocytes and activation of certain phagocytes, and it is mediated by T-lymphocytes (Abbas and Lichtman, 2003).
Both classes of cells arise from stem cells in the bone marrow, though they mature in different locations. Maturation of B-cells occurs in the bone marrow whereas T-cells develop in the thymus (Janeway et al., 2002). They differ in phenotype and function but can be distinguished by surface molecules they express. These are proteins and can be detected by monoclonal antibodies. The nomenclature for such markers uses the "CD" (cluster of differentiation) number designation. This system allows us to identify and isolate different lymphocyte populations (Abbas and Lichtman, 2003).

The major function of B-cells is to recognize extracellular antigens and accordingly evolve and proliferate into antibody-secreting plasma cells. Antibodies are determined as soluble glycoprotein molecules that specifically recognize and bind to specific antigens that caused their production by B-cells. This act of binding initiates the process of activation of other cells with the mission to kill or remove the antigenic substance from the body (Roitt et al., 1989).

T-cells are divided into two subsets that are functionally different. Firstly there are T-helper (Th) cells, also called CD4+ T-cells, and these again are classified into Th-1 and Th-2 cells. Th-1 cells collaborate with mononuclear phagocytes and help them kill ingested pathogens whereas Th-2 cells stimulate B-lymphocytes to divide and produce antibodies.
The second subset of T-cells implicates the cytotoxic, CD8+ T-lymphocytes (CTLs). These are involved in directly killing of virus-infected host cells or certain tumor cells (Roitt et al., 1989).

When matured, B- and T-lymphocytes migrate to peripheral lymphoid organs and either stay there or return to blood-circuit via lymphatic vessels (Janeway et al., 2002).

The initiation of adaptive immune response requires that antigen get captured and presented to specific lymphocytes. The cells that carry out this function are called antigen-presenting cells (APCs). The most important APCs are DC, which take up the antigen at the site of infection and transport it to lymph nodes where naïve lymphocytes, cells that have not encountered any antigen before, recognize the pathogen. T cells, however, can detect antigens only when these are bound to host molecules expressed on surface of other cells called major histocompatibility complex (MHC). The stimulation of T-lymphocytes leads to activation of macrophages and B-cells and also causes launching of many other mechanisms in order to combat the infection.
For further development of the adaptive immune reaction, activated T-cells but also macrophages and other leukocytes need to proliferate and differentiate into effector cells on one hand whose task is to eliminate the antigen, and into memory cells on the other hand, which are responsible for augmented responses upon repeated encounters with the antigen (Abbas and Lichtman, 2003).

1.2 ALLERGIC ASTHMA

1.2.1 General introduction to allergic asthma

Allergic asthma is a chronic disease of airway inflammation and airway obstruction characterized by symptoms including wheezing, chest tightness, shortness of breath, cough and mucous hypersecretion (Epstein, 2004b). It occurs as a result of exposure to defined allergens initiating a cascade of cellular activation events within the lungs. The consequence is an inflammatory process orchestrated by a complex assortment of cells (inflammatory, immune and resident lung cells) and other mediators, such as cytokines they release (Epstein, 2004b).

Observing for a longer period, airway hyperresponsiveness (AHR) and increased mucus hypersecretion, as well as damaged airway epithelium are some of the effects. These

appearances can seriously influence both, airway structure and function (Epstein, 2004a).

Inherent in the definition of asthma is the possibility of variable extent and manifestations of the disease between the individuals. Depend on the type of allergen exposure, indices can be seasonal, chronic or intermittent. Therefore, many patients have mild and infrequent symptoms, whereas others may have persistent or prolonged symptoms of great severity (Epstein, 2004b).

Statistics provided by studies reveal that there are approximately 300 million people worldwide affected by asthma (Selgrade et al., 2006) and it is the most common chronic pulmonary disease among children (Asher et al., 1995; Epstein, 2004a). The disease is more and more representing a significant economic and social burden (Asher et al., 1995; Epstein, 2004a; Epstein, 2004b; Selgrade et al., 2006). Rising incidence and prevalence are mainly reported in industrialized countries (Asher et al., 1995; Epstein, 2004a; Selgrade et al., 2006). Here, data concerning deaths from asthma suggest a trend toward an increased mortality rate, this despite of improved means of prevention and treatment and greater availability of effective drugs (Epstein, 2004a).

A number of explanations have been discussed, including the effects of certain medications (e.g. aspirin) (Selgrade et al., 2006), increasing exposure to industrial pollutants and/or indoor allergens (Asher et al., 1995; Epstein, 2004a; Selgrade et al., 2006), overuse of β2-agonists (Epstein, 2004a), fewer viral infections during the childhood (Epstein, 2004a; Selgrade et al., 2006), but also limited exposure to microorganisms and their products in early age (Selgrade et al., 2006).

Truly, remarkable progresses have been made in recent decades, however there is more to be done in order to clarify the cause, the mechanisms and the consequences of the disease.

1.2.2 Pathogenesis

Allergic pulmonary disorder such as allergic asthma develops when an encounter with allergen, that normally does not have any infections or pathogenic potential, induces an immune response. However, it is not clear why this immunological phenomenon affects only some of the individuals (Epstein, 2004b). Of course, the susceptibility varies among different age groups and races, but genetic predisposition combined with geographical and cultural factors certainly have a huge impact on the prevalence of the disease (Selgrade et al., 2006).

The occurrence of allergic asthma requires prior sensitization to a specific allergen. This incident results in the recruitment of many cells and other mediators to the location of the reaction, so that they are advanced to provoke a fast and strong immune response upon the following encounter with the antigen (Epstein, 2004b; Pearlman, 1999). However, once the allergen has breached the barriers and invaded the organism, it gets ingested and fragmented by antigen presenting cells (APC), so they can display its pieces on their surface to the lymphocytes (Epstein, 2004b). Provided that antigenic peptide is attached to MHC class II molecule on APC, then naïve T helper cells can react via their T cell receptor (TCR) with the antigen-MHC-II-complex, an interplay essential for their activation and differentiation (Pearlman, 1999). A factor that may influence the differentiation of Th-cells is the cytokine profile evoked by the antigen (Pearlman, 1999). In case of atopic asthma, T cells turn into Th-2 cells and produce particularly Th-2 type cytokines including IL-4, IL-5, IL-9 and IL-13 (Finotto et al., 2000). IL-4 again favours the generation of Th-2 cells (Pearlman, 1999). Further, the cytokines released by Th-2 subset also affect B cells. Once more, IL-4 plays the key role in activating B cells and triggers their production of IgE immunoglobulins, in other words antibodies. B cells, however, can receive this help from T-lymphocytes, provided that B cells themselves have come across the specific antigen and have identified it (Pearlman, 1999). The synthetized immunoglobulins contain in their structure a site for binding an antigen. Hence, once the antigen is attached, IgE fixates to the mast cell by bridging its high-affinity cell surface receptor FcεRI (Epstein, 2004b). This, in turn, signalizes intracellular events initiating release of inflammatory mediators: histamine, leukotriene, platelet-activating-factor (PAF) and bradykinin (Pearlman, 1999). These chemicals act locally, in airway tissue that is, and are responsible for increased vascular

permeability, airway edema and bronchoconstriction, but also increased mucous secretion, all of them events that illustrate the acute-phase response (Pearlman, 1999). Accompanied by coughing, wheezing, and hindered breathing, the immediate response occurs in the first minutes after allergen encounter. Over the next 3-11 hours there is a progressive tissue infiltration of inflammatory and immune cells, involving eosinophils, neutrophils and basophils, as well as lymphocytes, in response to other chemical mediators. Again, particular cytokines appear to be significant for the recruitment of leukocytes to the site of inflammation. Not only that they draw the cells, IL-5 for example attracts eosinophils as well as basophils, moreover they (TNFα and IL-4) also enhance the number of some endothelial adhesion molecules which act as chemoattractants just like cytokines do (Pearlman, 1999).

The contribution of leukocytes, especially eosinophils, to the extension of inflammatory process in the airway tissue is related to their release of cytokines and particularly cytolytic enzymes that very likely appear to be the main causer for tissue damage. The harm affects not only endothelial and epithelial cells but also nerve endings. Thus, these insights confirm the association of eosinophils, having a leading role in this late-phase response, with the clinical features in this stage of the disease, implying enhanced mucous secretion, airway hyperresponsiveness (AHR) (Bousquet et al., 2000; Pearlman, 1999) and bronchial hypersensitivity (Pearlman, 1999). Long-term consequences of such changes and abnormalities may manifest in reversible airway obstruction and possible lung damage (Epstein, 2004b), however, they even can lead to death (Wardlaw et al., 2002).

1.2.3 *In vitro* models of allergic disease

Many studies have focussed on *in vitro* modelling of different diseases, and even though these model experiments are limited in comparison with *in vivo* assays due to the lack of various structures and microenvironment dependent processes, they still contribute to better understanding of cellular activities.

Standardized *in vitro* models of allergic asthma deal with cells that orchestrate immunological events as well as resident cells from affected tissue in the respiratory tract. These can be obtained directly from asthmatic patients either from blood or from sputum, nasal and bronchial lavages and biopsies (Epstein, 2004b; Hohenadel et al., 2001). Bronchoalveolar lavages (BAL) are mostly utilized for identifying and quantification of cells and mediators present. However, for cultivation, after the purifying procedure, cell suspensions undergo incubation under diverse conditions. The analysis provides remarkable insights about their migration and secretion behaviour, cell proliferation, their growth and apoptosis (Epstein, 2004b). What scientists often do is to manipulate the cytokine environment in the cell culture in order to create a specific model for their experiment. An example of this was the attempt to verify that an increased Th-2 cytokine-induced release of certain proinflammatory mediators by bronchial epithelial cells from asthmatics can be amplified by direct implication of allergen (Lordan et al., 2002). Tests detected enhanced secretion of IL-8 and GM-CSF when cultures were treated solely with allergen or Th-2 cytokines (IL-4, IL-13, TNF-α). Yet, the combined exposure to both trigger types at the same time confirmed augmented expression of Th-2 cytokines in bronchial epithelium which are significant for the disease (Lordan et al., 2002). Another study produced a group of monoclonal antibodies determined as anti-CD63 and examined their effect on mast cells (Kraft et al., 2005). The results revealed that these antibodies suppress adhesion of mast cells to fibronectin and vitronectin, two specific extracellular matrix proteins (ECM), and moreover, that in the cells which are bound to ECM proteins anti-CD63 prevents FcεR1-initiated degranulation of mast cells. The project served as a basic approach for further *in vivo* assays, which evidence that anti-CD63 monoclonal antibodies potently inhibit anaphylactic reactions in rats. These findings suggest their potential benefit in therapeutical development (Kraft et al., 2005). When mentioning drug research, there, *in vitro* assays are very common. Such an example was the investigation of loteprednol etabonate, a soft corticosteroid with wide therapeutic index and favourable pharmacokinetic attributes (Szelenyi et al., 2000). In cultures with human cells loteprednol appeared to be capable of suppressing the secretion of various cytokines including TNF-α, GM-CSF, IL-4 and IL-5, demonstrating its anti-inflammatory effect in treating allergic responses (Szelenyi et al., 2000).

However, not only cell suspensions belong to *in vitro* models, but also biopsies can be arranged as explants cultures. These systems contain the required microenvironmental elements and enable the analysis of cellular interactions and even pathological events like increased mucous production or bronchoconstriction for instance (Epstein, 2004b). In a recent investigation, human bronchial tissue explants and human bronchial epithelial cells were exposed to rhinovirus and lipopolysaccharide (LPS) to test the mucin secretion (He et al., 2004). The findings illustrated that rhinovirus stimulated production of mucin in both, human bronchial tissue as well as in cell culture, and that the raise of the release is related to pathogen concentration. The same goes for LPS, this endotoxin also triggered mucin secretion in both types of ex vivo cultures (He et al., 2004).

Therefore, the data achieved by *in vitro* modelling is indispensable for detection and identification of some aspects of allergic asthma, and the insights gained are often fundamental prerequisite for further promising in vivo studies.

1.2.4 *In vivo* models of allergic disease

Developing *in vivo* models of atopic disorders has offered numerous advantages and progress in understanding not only the onset or initiation, the course and pathobiology of the disease, but it also advanced drug discovery. Due to the ethical barriers humans do not or hardly participate in these studies (Epstein, 2004b). Nevertheless, researchers have established successful animal models of the disease that are capable of replicating the complex biological events occurring in human patients. Monkeys, sheep, pigs, dogs, mice and rats are being used mostly. However, laboratories have focussed on latter two species, primarily for the economic reason, and secondary for their potential of genetic modification, and finally, for the disposability of species-specific reagents (Epstein, 2004b; Kips et al., 2003; Torres et al., 2005). Apart from spontaneously developed responses, *in vivo* models of atopic asthma are mainly based on experimentally induced immunological reactions. A classic example involves intraperitoneal immunization of mice with chicken-egg OVA, followed by repeating intranasal challenge with nebulized

OVA, ending in an inflammatory state primarily ruled by T lymphocytes and airway hyperreactivity (AHR) (Wegmann et al., 2005). The continuous challenge induced elevated TGF-β levels which are associated with airway smooth muscle remodelling in OVA mouse objects. Moreover, the investigators also explained that manifested goblet cell hyperplasia and subepithelial fibrosis throughout the airway tree are related to chronic exposure to OVA (Wegmann et al., 2005). Other allergens used in mouse models of bronchial asthma are house dust mite (HDM) (Fattouh et al., 2005), Aspergillus fumigates (Mehlhop et al., 1997) and ragweed (Fan and Mustafa, 2006).

Generally, most mouse models of asthma are featured with a Th-2 type of immune response implicating high levels of IgE and Th-2 cytokines such as IL-4, IL-5 and IL-13, as well as manifested hallmarks of disease including eosinophilic lung inflammation, mucous hypersecretion and AHR (Epstein, 2004a). The monkey model of asthma exhibits similar results (Van Scott et al., 2004). In that experiment, different ages of probands were observed and the final outcome affirmed augmented responsiveness to adenosine in neonatal animals sensitized with dust mite. In addition, discrepancies concerning resistance in airways and dynamic compliance between adult and neonatal monkeys were detected too (Van Scott et al., 2004). Sheep samples seem to be well suited objects for asthma induction (Bischof et al., 2003). House dust mite sensitized sheep developed allergic response with accentuated accumulation of eosinophils (Bischof et al., 2003). Similar conclusions in further sheep studies followed (Snibson et al., 2005). Another large animal model for testing immunological reactions is dog (Out et al., 2002). Apparently, dogs show many asthma-like symptoms like eosinophilia and enhanced IgE, but also changes is respiratory tract leading to AHR (Redman et al., 2001). Finally, guinea pig belongs to potential representatives of asthmatic disease models as well (Hutson et al., 1988). However, since they lack in genetic diversity, they might not be used for studies as often as mice (Isenberg-Feig et al., 2003).

All together, animal models are optimal tools for exploring pulmonary immunological disorders and underlying mechanisms on cellular and molecular level, and to exert the achieved findings in drug development (Zosky and Sly, 2007). Nevertheless, the fact is: animal species do not accurately replicate humans in regard to physiology and

pathobiology, and thus all the processes involved. Therefore, the data should be processed with caution (Epstein, 2004b; Zosky and Sly, 2007).

1.3 MOUSE MODELS OF ALLERGIC ASTHMA

1.3.1 "Mouse trap" or useful models for studying disease?

The first data on mouse models with asthma-like disorder were released in 1994 (Epstein, 2004a), and since then they have become most popular animal subjects for simulation and studying the disease.
Numerous advantages have contributed to this progression, if we think of low costs they generate, the accessibility to mouse-specific reagents and the remarkable technologies allowing manipulation of their genetic background (Torres et al., 2005).

As it is generally known, no laboratory animal can reproduce allergic respiratory disease to 100 percent as it is observed in humans. Like every other model, mice have their pros and cons as well. The question is whether the strengths or their weaknesses prevail, in other words: Are mice valuable models for the disease?

Since animals do not develop allergic asthma naturally, with some exceptions like T-bet transcription factor-deficient mice (Finotto et al., 2002), or flea-allergic dogs (Epstein, 2004b), asthmatic conditions have to be induced in a model. There is a variety in techniques scientists apply, but in principle the standardized OVA protocol is the most commonly used (Torres et al., 2005; Zosky and Sly, 2007). This method includes sensitizing mice systemically, either through intraperitoneal (i.p.) injection with antigen and an adjuvant added, or immunization with allergen via intranasal/intratracheal routes (Epstein, 2004a; Zosky and Sly, 2007). The systemic priming and subsequent challenges with the antigen lead to immune response in the respiratory tract hallmarked with lung inflammation, airway hyperresponsiveness, mucus hyperproduction, edema, abundant eosinophils and enhanced IgE levels, respectively (Epstein, 2004a).

However, the protocols also undergo modifications. As our knowledge about mice and disease grows, consequently, more questions arise and this is being reflected in the disease induction strategies.

Which allergen to choose? Well, as mentioned, the most frequently used is OVA. However, there have been made studies with a number of other allergens including bovine serum albumin, *Aspergillus fumigatus,* cockroach antigens, birch, as well as ragweed extracts and house dust mite (HDM) (Epstein, 2004a). Especially the latter two stimuli are increasing in popularity, due to the fact they are natural allergens dispersed in the air and capable of eliciting hypersensitivity reactions in humans. Naturally, they invade the organism through respiratory passages, whereas OVA is ingested by gastrointestinal tract, suggesting that immune reaction evoked should commence in our intestines (Torres et al., 2005), nevertheless it has not been evidenced yet that priming in individuals takes place in the respiratory tract when inhaling the allergen (Epstein, 2004a).

On the other hand, diverse experimental methods imply diverse application rates. Basically, standardized protocols dictate high concentrations of OVA. Unlike house dust mites, in terms of this allergen low doses are required for techniques targeting mouse models with chronic asthma (Torres et al., 2005).

For the sensitization, antigens are administered not only in a soluble form but often precipitated in aluminium sulfate (alum), an adjuvant, with the intention to provoke augmentation in Th-2 guided bronchial response. Referring to humans, substances like cigarette smoke, ozone or exhaust fumes for instance, function as adjuvants for atopic diseases of respiratory system (Epstein, 2004a).

Divergent reactions have also been observed among certain mouse strains (Kannan and Deshpande, 2003). Although some researchers regard it as a handicap, discrepancies like these provide a basis for investigating the association between genetic make-up and the pathological events (Shapiro, 2006). Primed BALB/c mice appear to suffer from stronger airway hypersensitivity accompanied with higher levels of IgE and cytokines, compared with primed C57BL/6 strain. Contrarily, A/J mice seem to be more adequate

for developing a chronic condition that is believed to be more like the asthma occurring in humans (Torres et al., 2005).
With this in mind, scientists are making further changes in the immunization protocols. Instead of the usual i.p. antigen injection, they wage new trials recommending not systemic but priming via respiratory routes and the same way for the following challenges (Torres et al., 2005).

Similar thoughts apply to the length of the initiation experiment. Here, basically, a distinction is drawn between brief exposure to high dosed allergen and, conversely, protocols with extended period of exposure using low concentrations of antigen. Evidence shows that first technique, i.e. short exposure, ends in an acute model of allergic asthma (Torres et al., 2005).

To study the acute disease in mice, numerous technologies have been developed. Airway inflammation, for example, is assessed with BAL (bronchoalveolar lavage), and it is determined that cells with the greatest abundance are eosinophils, followed by neutrophils and macrophages. In this context it is necessary to note that eosinophils are a contentious issue regarding mice and men (Epstein, 2004a). Firstly, in contrast to humans, eosinophils in mouse models do not degranulate, hence this controversy is sometimes seen as a serious limitation for mouse subjects (Persson, 2002). The second point of contention affects their function. There are views proposing that eosinophils may be implicated primarily in regulatory operations rather than promoting the inflammation process (Torres et al., 2005).

Concerning the antigen-specific IgE and mast cells, here, opinions differ again. Asthmatic patients have demonstrated their essential role in the development of early- and late-phase-response as well. In mice, assays have exhibited that the initiation and gravity of the asthma onset do not depend on IgE or mast cells. On the other hand, in many investigations the opposite has been proved confirming the relevance of these two players for the disease in mouse systems (Epstein, 2004a). Genetic distinction (Epstein, 2004a), just as varying induction protocols (Torres et al., 2005) could substantiate these discrepancies.

Another feature of atopic asthma is the airway hyperreactivity. Noninvasive and invasive methods are being applied to evaluate this disorder, but laboratories prefer the noninvasive proceeding. The most commonly used is the so-called whole body plethysmography (WBP), a technique comprising stimulation of mice with either allergenic or cholinergic bronchoconstrictors and measuring the following reactivity of the airways (Epstein, 2004a; Kumar and Foster, 2001; Torres et al., 2005). During such operations researchers have encountered another phenomenon that argues against mice, unfortunately. The stimulant metacholine evokes hyperreactivity in human individuals even when the symptomatic condition alleviates, whereas mouse models demonstrate only temporary hyperresponsiveness during the immunization and challenge phases, an occurrence based on anatomical and physiological distinctions between the two species (Epstein, 2004a).

One of the main weaknesses of most mouse models is the deficiency of chronicity that is ascertained in asthmatic patients. In order to generate conditions that are closer to those in humans, induction protocols had to be altered in certain aspects. When testing novel pathways of initiation, investigators acquired significant and interesting findings, namely, if mice are exposured to OVA for longer time with no preceded systemic sensitization, they will develop immunologic tolerance. In other words, the immune response will be suppressed (Torres et al., 2005). It is most likely this incident is related to defence mechanisms of the immune system and supplied the foundation for the allergen immunotherapy (Epstein, 2004a). Similar events were illustrated in primed models after extended exposure to OVA, bronchial inflammation was relieved. The data was encouraging, thus in addition to prolonged exposure, decreased allergen concentration established in protocols as well. In a recent study, a mouse model was designed in accordance with those new instructions. House dust mite antigen was used and the outcome of the experiment was a model of chronic disease with manifestation of airway remodelling, a state more typical for allergic asthma in patients (Torres et al., 2005).

Altogether, mouse models of asthma are useful tools for identifying and testing mechanisms underlying allergic pulmonary disorders.

No question, there are limitations in mouse systems that need to be considered when estimating their suitability, adopting and adjusting the data achieved to humans. Differences in physiology, disputed significance of IgE and mast cells for the response development in mice and men, these are only few of arguments against mice. Furthermore, age of animal subjects is often being criticized. Indeed, modelling is mainly performed in adult animals, whose immune system compared to the young or neonates actually is sophisticated and acts more effectively in combat against pathogens and diseases (Kips et al., 2003; Selgrade et al., 2006). Hence, it surely would be interesting to create a model that, as a neonate and juvenile has been set out to early life influences typical for men including viral infections and environmental factors (i.e. air pollution, natural indoor and outdoor allergens, microorganisms and their products etc.), and observe to what extent these elements play a role in asthma development at various time points (from early age to adults).
Another problem with mice when compared to asthmatic patients is the temporary AHR to bronchoconstrictive agents (Epstein, 2004a).

Nevertheless, it is primarily mouse who brought the science to where it is now. These small animals provided needful understanding for pathogenesis and notable findings for the therapeutical field.
Of course, many issues demand to be elaborated before starting an experimental induction, reaching from the choice of allergen to mouse strain, but it mainly depends on the target of modelling or the hypothesis that needs to be verified (Torres et al., 2005). Consequently, it is important to combine the investigations in both, mice and men, so that the insights gained in animals can be tested in humans.

No doubt, mice are and will remain useful and appropriate means for further research of allergic lung disease.

1.3.2 Sensitization to allergen

To induce allergic reaction in the respiratory system in mice, animals primarily need to undergo a systemic immunization. In the most of prevalent models, mice are sensitized by the intraperitoneal injection of OVA allergen either in combination with adjuvant alum, or in adjuvant-free solubilized state (Epstein, 2004a; Zosky and Sly, 2007). Furthermore, intranasal or intratracheal instillation of particular allergens, as well as i.p. or intratracheal transfer of primed DCs and macrophages have been proved as successful methods for sensitization process (Epstein, 2004a). Immunizing the mice systemically causes activation of their immune system including production of antigen-specific IgE (Kips et al., 2003), and actually provides a base for disease to be initiated and developed by following inhalation of allergen (Epstein, 2004a), an insight gained from models who revealed that asthmatic events featured with increased IgE secretion (Holt et al., 1981; Seymour et al., 1998) as well as the predominantly Th-2-ruled response (Seymour et al., 1998) do not occur upon repeated inhalative challenges alone. This was further encouraged by a demonstration in unsensitized mice, who after being loaded with antigen-stimulated Th-2 cells from OVA-primed wild-type mice, became exposed to aerosolized OVA and, as a result, recipient mice developed allergic pulmonary response (Hogan et al., 1998).

Hence, systemic priming creates a generation of activated Th-2 cells, which are the key players in setting a stage for immunological allergic reactions upon subsequent airway challenges in mouse models.

1.3.3 Factors influencing the response to allergen

A number of elements involved in asthma induction in mice are able to manipulate the procedure and lead to discrepant achievements.

Adjuvants are substances that enhance Th-2-ruled immunological reaction. This trait was evidenced with many essays, in which adjuvant was administered systemically with

no antigen added and as a consequence a non-specific Th-2 cytokines generation eventuated (Epstein, 2004a). Analog the allergen effect, applying the allergenic substances alone in the sensitization phase causes a Th-2 phenotype of asthma (Epstein, 2004a). To conclude, initiation of the disease does not need addition of an adjuvant to antigen. Another assay fortified these findings regarding the adjuvants function, when compared the phenotype of infiltrates in inflamed lungs of mice, which previously were sensitized either with antigen precipitated in alum or immunized with OVA alone. Results demonstrated that infiltrates correlated to combination of antigen and adjuvant were bigger and were settled close to large airways with eosinophils in abundance, in opposite to infiltrates related to soluble OVA, those appeared smaller, harboured fewer eosinophils and were found mainly near smaller airways, resembling rather the nature of human cellular infiltrates (Epstein, 2004a).

Apart from the most popular adjuvant aluminium sulfate (alum), there are various others that participate in such experiments. Some of them, however, appear to be absolutely necessary for development of the disease. Since it is verified that bacterial LPS (lipopolysaccharides) are present in OVA protein, researchers also wanted to explore the effect of this endotoxin, therefore they segregated it from OVA and conducted the induction in a mouse model with the purified OVA. The outcome illustrated alleviation of asthmatic state, alluding to the relevance of LPS for priming the immune system (Epstein, 2004a). On the other hand, a controversial study showed that applying LPS to mice during the nebulization with aerosolized antigen leads to degradation of the immune response. These discrepant findings may be due to differing administration routes of endotoxin, nevertheless it is not clarified yet (Epstein, 2004a).

Fortunately, there are factors that suppress asthma induction as well, even though some of them have pathogenic character. Parallel ongoing infections, Mycoplasma pneumoniae for instance, seem to decrease the allergic reaction. With this evidence the corroboration for hygiene hypothesis grows and further affirms that in industrialized countries where advanced hygiene circumstances and minor infectious impacts are ubiquitous, these conditions just increase the susceptibility for allergic diseases including asthma (Epstein, 2004a; Selgrade et al., 2006).

Then again it is the experimental protocol implying other influencing factors beside the adjuvants. Mouse strain can determine whether the response will turn out milder or stronger.
The choice of allergen is also important, currently, the laboratories incline to use natural aeroallergens that are responsible for human asthmatic events, i.e. house dust mite.

Not to forget, the route of exposure as well as duration are *the* central issues in the sensitization process in mouse models by far. Brief exposures with high amount of antigen are appropriate for acute onset of the disease in mice, whereas chronic models are related to extended challenges with low allergen concentrations. Regarding the route of administration, on the one hand, scientists recommend both steps to be executed via respiratory ways, priming just as challenges, with the argument that this is the natural way how humans encounter antigens (Torres et al., 2005). On the other hand, it is not verified whether individuals are truly primed via airways (Epstein, 2004a). This may be supported by mice who demonstrated that asthma induction requires systemic priming and subsequent respiratory challenges. In other words, if mice are only nebulized for a long time and had no prior systemic administration of allergen, after a while they develop immune tolerance. Now, what about the immune tolerance and humans with chronic state? One possible explanation claims that this protective mechanism underlying the defence system in individuals is impaired (Torres et al., 2005). Other option suggests that, if humans successfully generate immune tolerance to inhaled allergens, then susceptible persons among will develop immune response in case of being primed systemically (Epstein, 2004a).

Last but not least, allergenicity plays role in the immunization course (Epstein, 2004a), although the importance in mouse systems differs from the importance for disease in men.
Since the allergenicity is explicated as the innate potential of a protein to elicit hypersensitivity, and since mice effectively respond to antigens of non-allergenic character, it is clear that disease induction in mice does not require allergenicity. Atopic individuals, however, have in common that they generate an immunological response to a group of certain antigens, thus obviously the allergenic nature is requested for sensitization in humans (Epstein, 2004a).

Altogether, we realize that in the priming phase everything is interconnected. Depending on the aim of the study, these influences need to be deliberated in advance, and accordingly either incorporated or eliminated.

1.3.4 Acute and chronic disease in mouse models

Mouse models are necessary to study and understand the mechanisms of allergic disease and for this purpose they enable manipulation of incidents of the disease. Therefore it is possible to compile a chronology of asthmatic pathology and to distinguish between acute inflammation and chronicity of asthmatic disorders.

Considering the lung physiological and immunological aspects, mouse system offers numerous advantages such as valuable techniques for investigating the immuno-pathological correlations in allergic asthma. The model of acute inflammation of airways, initiated through repeated short-time inhalative allergen exposure, has become established many years ago (Zosky and Sly, 2007). This form of immune response is manifested with coughing, wheezing, shortness of breath and dyspnea (Bousquet et al., 2000; Epstein, 2004a). On the cellular and molecular level it is characterized by lung inflammation, mucus hypersecretion, airway hyperresponsiveness and increased IgE production (Bousquet et al., 2000; Epstein, 2004a; Kumar and Foster, 2001; Torres et al., 2005). Mouse models are valuable in so far as they allow measurement and evaluation of these events. Lung inflammation for example can be assessed on the basis of BAL and histopathology (Epstein, 2004a; Torres et al., 2005). The analysis of BAL fluid provides indication on cell recruitment in the airways (Epstein, 2004a; Hohenadel et al., 2001; Tobin, 2001), including eosinophils, lymphocytes, monocytes and neutrophils (Bousquet et al., 2000; Epstein, 2004a). The insights gained from BAL are mainly consistent with the histological examination of lungs proposing that the inflammation is primarily eosinophilic, peaks during 24 to 48 hours after last challenge, and declines after 11 days (Epstein, 2004a; Kung et al., 1994). Furthermore, the analysis proves that inflammation is constricted to perivascular and peribronchial regions in the lung (Epstein, 2004a; Kung et al., 1994). Bronchial hyperresponsiveness can be

measured by invasive and non-invasive methods. The most frequently used is the non-invasive whole body plethysmography technique that measures AHR in mice in response to bronchoconstricting/allergenic agents (Epstein, 2004a; Kumar and Foster, 2001; Torres et al., 2005). The levels of IgE or IgG can be assessed using ELISA (Coyle et al., 1996). Unlike IgG level, the amounts of IgE are proved to diminish in a period of few months (Epstein, 2004a). Mucous cell hyperplasia as a feature of acute inflammatory reaction is accompanied by mucous hyperproduction, and these structural changes can be evidenced by histological staining of lung sections with PAS (periodic acid-Schiff) (Epstein, 2004a).

Designing a mouse model with chronic disease is a movement towards replication of asthmatic conditions in humans. The attempts to create such conditions with OVA antigen in mice failed. Continuous and prolonged respiratory exposure to OVA rather causes immunological tolerance (Johnson et al., 2004; Torres et al., 2005). In contrast, chronic provocation with house dust mite extract induces severe eosinophilic inflammation with raised levels of IgE and IgG as well as enhanced production of Th-2 type cytokines (Johnson et al., 2004). Moreover, airway remodelling, that belongs to major hallmarks of chronic allergic asthma, has been evidenced in such models. Airway remodelling is described as a result of consecutive repair processes of continuously injured structures in lungs (Bousquet et al., 2000; Epstein, 2004a). It is associated with subepithelial fibrosis, hypertrophy of bronchial smooth muscle, hyperplasia of goblet cells and mucous hyperproduction, along with microvascular deformation (Bousquet et al., 2000; Epstein, 2004a; Johnson et al., 2004; Torres et al., 2005). These changes contribute to enhanced airway obstruction and consequently, to growing respiratory resistance (Bousquet et al., 2000). Additionally, increased AHR is also noted in chronic models. Different from acute disease, chronic subjects demonstrate fewer eosinophils in infiltrates (Epstein, 2004a), but greater numbers of macrophages, plasma cells and T lymphocytes (Epstein, 2004a; Johnson et al., 2004). However, the lung inflammation is reported to resolve completely after few weeks of continuous exposure to antigen, whereas AHR and immunoglobulins remain increased (Epstein, 2004a; Johnson et al., 2004). Apart from that, structural changes stay persistent (Johnson et al., 2004).

In summary, both, acute and chronic form of allergic pulmonary disorder can be investigated in mice. Acute disease models are suitable for exploring the inflammation process and underlying mechanisms, whereas mouse systems with chronic conditions can be employed for studying the long-term processes and effects on lung function due to tissue damage and structural abnormalities.

1.4 Th-2 MEMORY

Immunological memory is a relevant attribute of immune responses. After a disease/infection has been abolished and the invading agents eliminated, an increased number of specific immune cells remain in the body and they serve to protect the host against re-infection. A secondary encounter with the identical pathogen stimulates these long-lasting memory cells, which elicit more rapid and enhanced immune reaction than to the first exposure (Goldsby et al., 2001). The exact process by which immunological memory develops is not thoroughly explored. To date, proposals for the advantages which might drive the evolution of memory include: protection from re-infection, control of long-term infections, and the heredity transmission of immunity to the next generation (Welsh et al., 2004; Wodarz, 2003). However, memory does not always and exclusively stand for host protection, but it can promote disease relapse and extension as well. The significance of Th-2 lymphocytes for induction and evolution of allergic asthma has often been highlighted in experiments (Bell et al., 1996). Deactivating Th-2 cells or their cytokines results in reduction of asthmatic symptoms (Bell et al., 1996; Epstein, 2006; Mojtabavi et al., 2002), and adoptive transfer of allergen-specific Th-2 population to naïve animals leads to disease development (Epstein, 2006; Mojtabavi et al., 2002). No doubt, an existent frequency of Th-2 memory cells is required by immunological memory (Bell et al., 1996; Epstein, 2006; Mojtabavi et al., 2002). A valuable study supporting this perception verified the persistence of Th-2 memory cells in a murine model of allergic asthma (Mojtabavi et al., 2002). Their sensitization protocol involved immunization with soluble, nebulized and not precipitated OVA antigen in order to prevent adjuvant-dependent antigen deposits in the body. After the efficiently induced asthmatic inflammation, mice rested for extended periods of time to

be able to entirely recover from clinical symptoms. More than 1 year later, mice were again challenged with aerosolized OVA and as a result they restored inflammatory events in lungs characterized with eosinophilia, inflammatory infiltrates containing large amounts of eosinophils followed by lymphocytes and macrophages, AHR, mucous hyperproduction and generation of IgE. Furthermore, recovered animals proved to react rapidly against OVA re-exposure by producing Th-2 cytokine RNA in lungs only 3 hours after the antigen stimulation, suggesting the longevity and promptness of Th-2 cells in lungs. To compare, recovered control mice, which did not undergo rechallenge process after 1 year, evidenced long lasting lymphocytic infiltrates without eosinophils, and no other asthma-like features such as AHR, eosinophilic inflammation or increased mucous production. Except in lungs, persistent memory Th-2 population was detected in spleen as well. Beside the rechallenged animals, also spleen isolated cells from recuperated mice exhibited in vitro IL-4 and IL-5 secretion upon antigen stimulation. All in all, findings from this study demonstrate the persistence of Th-2 memory cell population in spleen and lungs that initiates rapid immune response upon second antigen encounter leading to evolution of allergic asthma (Mojtabavi et al., 2002). On the one hand, there is evidence that memory is based on long lasting $CD4^+$ T cells in lung infiltrates proposing that lungs participate in maintaining of Th-2 memory by providing an ambience favourable for survival of memory cells (Epstein, 2004a). On the other hand, to date, it is unclear whether antigen deposits allowing the continuous availability and Ag presentation is involved in the process of memory maintenance and/or to what extent. Although in the assay previously described Ag deposits were prevented, there was no guarantee that during the recovery phase inhaled OVA was eliminated to 100% and that some irreducible minimum of Ag does not remain in respiratory system and support the perpetuation of memory (Mojtabavi et al., 2002). Indeed, researchers have documented that T cell memory is antigen dependent (Kündig et al., 1996). However, some experiments reveal that memory T cells do not require persistent antigen for their survival (Murali-Krishna et al., 1999). Though, they proved the competence of these memory cells to respond to antigen re-exposure with generation of cytokine profile needed for asthmatic reaction. Furthermore, Murali-Krishna et al. indicated that memory CD8 T cell subset is capable of lasting and proliferating without presence of MHC class I molecules. Thus, antigen persistence stays a debatable issue that demands deeper investigation. In addition, cytokines have also been targets of projects in order to verify

their contribution to sustain T cell memory. According to certain studies, in particular IL-7 (Schluns and Lefrançois, 2003) and IL-15 (Berard et al., 2003; Schluns and Lefrançois, 2003) appear to play a role in that process.

All together, great amount of inconsistent data has been presented and gives rise to a myriad of questions concerning T cell memory and its maintenance that need to be clarified.

1.5 DC AND ALLERGIC ASTHMA

DC are bone-marrow derived and highly migratory cells of immune system that belong to antigen-presenting cells (Goldsby et al., 2001). Their main function is to capture antigens, metabolize and present it to T cells in lymphoid organs. To date, their central role in initiation and enhancement of pulmonary immune responses to antigens has been revealed by numerous studies. They appear to have the ability to determine the nature of immune reaction to Ag encounter, which can be either inflammatory or tolerogenic. Furthermore, they seem to possess the qualification to direct the immune response either towards Th-1 or Th-2 pattern (Kuipers and Lambrecht, 2004; Lambrecht, 2005; Upham and Stumbles, 2003).

To be able to present the Ag and activate naïve T cells followed by initiation of immune response, DCs need to undergo their maturation process. Immature DCs are spread throughout lungs and many of them are situated beyond and beneath the respiratory epithelium, the front line of host defence (Lambrecht, 2005). By Ag uptake, DCs migrate with their Ag-load to regional lymph nodes (Lambrecht, 2005; Upham and Stumbles, 2003). They reach the lymph nodes within first 12h (Lambrecht, 2005). When arrived, DCs express Major Histocompatibility Complex Class II (MHC II) molecules and secrete co-stimulatory molecules including CD86 and CD40, what alludes to their partially mature state (Kuipers and Lambrecht, 2004). However, DCs sometimes need some support for their activation and migration. This support involves signals from Pattern-Recognition-Receptors (PRRs) after Pathogen-Associated-Molecular-Patterns

(PAMPs) have bound to them (Eisenbarth et al., 2003; Kuipers and Lambrecht, 2004). Additionally, there are certain chemokines, like CCL21 for instance, and many other poorly explored mechanisms that mediate DC activation and migration as well (Upham and Stumbles, 2003). Once they have processed the ingested Ag, DCs transport its particles to the cell surface where they build a complex with MHC II molecules, and in this form the antigenic material is presented to T cells. This presentation leads to T cell activation and differentiation from naïve into effector T cells that yet proliferate and initiate the development of immune response (Epstein, 2004b). Interestingly, spontaneously migrating DCs have also been observed although in healthy individuals (Upham and Stumbles, 2003). By the arrival to lymph nodes they, however, remained immature, suggesting their potential role in immune tolerance (Kuipers and Lambrecht, 2004; Upham and Stumbles, 2003). Apart from that, there is a report about a subset of DCs that store the Ag for a longer period of time in lung tissue and probably, maintain the ability of Ag presentation (Julia et al., 2002).

The density of DCs in the lung depends on the magnitude of antigen provocation and can be elevated due to bacterial and viral infections, cigarette smoke, but also in response to IFN-γ (Upham and Stumbles, 2003). Indeed, investigations have proved increased amounts of pulmonary DCs and bone-marrow derived precursors in rodents with allergic asthma (Lambrecht, 2005). The number of myeloid DCs in mucosal tissue and BALF reaches the 80-fold higher amounts as a consequence to disease induction. These DCs that are recruited to inflamed lungs are often found in infiltrates together with primed T cells, demonstrating the interplay among these two cell types, that potentially leads to local maturation of DCs in the periphery in addition to lymphoid T cell areas (Lambrecht, 2005). This notion of their correlation and interaction is further supported by the fact that airway DCs secrete chemokines such as CCL17 that draw memory Th-2 cells to the site of inflammation (Lambrecht, 2005). Together with results from other experiments including the report on lung DCs that long after antigen inhalation these cells evidence the capability to activate T cells (Julia et al., 2002), these findings underscore the participation of DCs in maintaining the established inflammation of respiratory system (Lambrecht and Hammad, 2003; Kuipers and Lambrecht, 2004; Upham and Stumbles, 2003; van Rijt et al., 2005). Lambrecht and co-workers have further substantiated this perception. Their study revealed that selective depletion of $CD11c^+$ airway DCs in mouse objects completely abolished all major

features of asthma with Th-2 phenotype, including eosinophilic inflammation, goblet cell hyperplasia and BHR (Kuipers and Lambrecht, 2004; Lambrecht and Hammad, 2003; van Rijt et al., 2005). Accordingly, transfer of DCs efficiently restored the pathobiological events of asthma in those animals (Lambrecht and Hammad, 2003; van Rijt et al., 2005). In addition, they also confirmed that removal of these DCs from immunized mice being subsequently challenged with Ag prevents the initiation of allergic inflammatory response (van Rijt et al., 2005).
Hence, it is obvious that DCs play a pivotal and essential role in development of Th-2 sensitization to inhaled allergens as well as in maintenance of ongoing pulmonary allergic inflammation.

Various subsets of DC stand for various functions and locations. Basically, it has to be distinguished between myeloid and plasmacytoid DC, and Langerhans cells (LC) (Upham and Stumbles, 2003). Myeloid DC (mDC) arise from monocyte lineage that is in common with macrophages, circulate in blood and their survival is granulocyte-macrophage colony stimulating factor (GM-CSF) dependent. Plasmacytoid DC (pDC) are found in blood stream as well, although in lower amounts than mDC, and further they have been identified in tissues. For their maintenance, pDC require IL-13 cytokine. pDCs are believed to participate in combat against viral infections (Upham and Stumbles, 2003). Finally, Langerhans cells are a subtype of DC located in epithelial tissue, particularly confined to epidermis. Their development demands TGF-β. Differing subsets are suspected to mediate the immune response in a different way. In this context, however, the results are conflicting. In humans, myeloid DCs seem to initiate a Th-1 response that is IL-12 dependent, whereas plasmacytoid DCs appear capable of eliciting a Th-2 type of response (Upham and Stumbles, 2003). Contrarily, experiments with mice reveal that administration of OVA-pulsed myeloid DCs to the airways of naïve mice causes sensitization to OVA, that after rechallenging mice with aerosolized OVA results in a Th-2 type of immune reaction with all typical hallmarks of allergic asthma, including eosinophilic inflammation, goblet cell hyperplasia and AHR (Lambrecht, 2005). These data suggest on the one hand the possible existence of two different subpopulations of myeloid DC, and on the other hand that these discrepancies demand further exploration. Nevertheless, there are some other factors proposed to influence the polarization of immune response. IL-12 for example, is the crucial cytokine in

promoting Th-1 ruled responses (Eisenbarth et al., 2003; Upham and Stumbles, 2003). Further, IL-10 is able to suppress production of IL-12 and therefore Th-1 differentiation, hence it rather contributes to Th-2 phenotype. In addition, microbial stimuli, such as lipopolysaccharide (LPS) (Upham and Stumbles, 2003), also showed competence to switch between Th-1 and Th-2 profile (Eisenbarth et al., 2003; Upham and Stumbles, 2003). Actually, the dose of LPS was observed to manipulate the Th-1/Th-2 variation (Upham and Stumbles, 2003). This finding was further supported by other studies indicating that low concentration of Ag promotes Th-2 cell differentiation, whereas high Ag doses advantage Th-1 controlled immune responses (Upham and Stumbles, 2003). Taken together, all these findings point at one, that DC can influence the Th-1/Th-2 balance, and that underlying mechanisms are yet not fully understood.

As mentioned before, DCs not only participate in evolution of immunological responses, but they also play a role in the process of immune tolerance. One of the potent drivers that can affect their tolerogenic function is for instance TGF-β. This cytokine is capable of suppressing the maturation of DC, thus DC remain unable to stimulate T cells (Demeure et al., 2000). IL-10 has been described as another important cytokine for tolerance. It has been documented that pulmonary DC expressing IL-10 can induce foundation of regulatory T cells (Tregs) which then secrete IL-10 themselves and are able to suppress evolution of Th-2 responses and inflammation of lungs (Kuipers and Lambrecht, 2004; Lambrecht, 2005; Upham and Stumbles, 2003). Indeed, in opposite to allergic patients scientists have detected enhanced levels of Ag-specific IL-10 secreting cells in healthy individuals (Kuipers and Lambrecht, 2004). Beside the respiratory tract DCs, a particular subset of DCs is thought to induce Tregs as well, namely plasmacytoid DCs. Although ex vivo, lung pDCs have shown the capability to induce Tregs generation and moreover to suppress formation of Th-2 effector cells that is stimulated by myeloid DCs (Kuipers and Lambrecht, 2004; Lambrecht, 2005). Apart from that, researchers have also examined the effect of different maturation states of DC on tolerogenic process and came to disparate outcome. While one group ascertained that intermediate maturation stadium was essential for tolerogenic pathway, the others showed that exclusively immature DC are responsible for launching the tolerance (Kuipers and Lambrecht, 2004). These data provide very confident results in terms of significance of Tregs induced by DCs, however, concerning the DC maturation stages,

more has to be done to clarify different states from immature toward mature and their impact on immune tolerance.

1.6 MACROPHAGES IN IMMUNE RESPONSE

Apart from DC and B lymphocytes, the population of macrophages exerts the function of antigen presenting cells as well. Macrophages stem from circulating monocytes, whose precursors promonocytes origin from bone-marrow (Goldsby et al., 2001). Generally, these immune cells are distributed through alveolar spaces and conducting airways (Peters-Golden, 2004), but in specific, they are divided in three subtypes due to their exact localization including alveolar, interstitial and intravascular macrophages (Lohmann-Matthes et al., 1994). Among these, alveolar macrophages are the only being in contact with air, as they are placed in the air-tissue interface. Their task is to protect the host against invading agents applying the mechanism called phagocytosis (Goldsby et al., 2001). It is described as ingestion and degradation of pathogenic and/or non-pathogenic material of exogenous or endogenous nature (Goldsby et al., 2001). Cell counts in healthy animals reveal the abundance of alveolar macrophages claiming that they make up over 90% of cells from BAL fluid (Lohmann-Matthes et al., 1994). Actually, macrophages represent the largest population among immune cells in respiratory tract (Peters-Golden, 2004). Phagocytic activity as well as production of cytokines are stronger evident in alveolar macrophages, whereas interstitial representatives display higher expression of MHC II molecules (Lohmann-Matthes et al., 1994). In addition, there are further molecules that macrophages express on their cell surface implying CD11a, CD11b, CD11c (Lohmann-Matthes et al., 1994), CD4, CD14 and CD40 (Goldsby et al., 2001), but F4/80 has also been identified as a macrophage marker (Gonzalez-Juarrero et al., 2003; Morris et al., 1991). According to their high/low/intermediate expression of these surface molecules what is correlated with their current differentiation stage and function, distinct subtypes of macrophages can be distinguished, however some of them are expressed by other APCs like DCs as well (Lohmann-Matthes et al., 1994). Alveolar macrophages are involved in both, innate as well as in adaptive immunity (Lohmann-Matthes et al., 1994; Peters-Golden, 2004).

Their support of innate immunity is demonstrated by their anti-microbial effects (Jansen, 1996; Lohmann-Matthes et al., 1994; Peters-Golden, 2004). The role in adaptive immune responses has, however, suppressive character (Jansen, 1996; Lambrecht and Hammad, 2003; Lohmann-Matthes et al., 1994; Peters-Golden, 2004). Despite the production of MHC II, macrophages are rather inadequate antigen presenting cells and only DCs are known to present the antigen to naive T cells (Jansen, 1996). In this context, macrophages can show their immunosuppressive effect. They are capable of inhibiting the APC capacity of DCs, and thus T cell activation declines (Jansen, 1996). This downregulatory behaviour was demonstrated in several studies. One of them proved that removal of alveolar macrophages before Ag stimulation leads to augmented Ag-induced immune response with manifested AHR and eosinophilia as well as decreased amount of IFN-γ, a Th-1 cytokine that is also secreted by macrophages (Tang et al., 2001). These observations indicate their capability of initiating a protective Th1 type of response (Lambrecht and Hammad, 2003; Peters-Golden, 2004; Tang et al., 2001). Not surprisingly, in contrast to DC, macrophages failed to reconstitute asthmatic conditions in mice that after elimination of DCs and macrophages were relieved from main features of asthma (van Rijt et al., 2005). Therefore, macrophages operate in two different ways to downregulate allergic reactions, either directly or indirectly. On the one hand they suppress DCs function and consequently T cell activation, thus immune response is indirectly prevented or limited (Peters-Golden, 2004). On the other hand, they direct immunological response towards Th1 phenotype (Lambrecht and Hammad, 2003; Peters-Golden, 2004; Tang et al., 2001). In this regard, it is believed that Th1/Th2 polarization occurs due to distinct subclasses of macrophages distinguished by their cytokine profile and products, thus M1 is described as promoter of Th1 response, and M2 in turn contributes to Th2 immunity (Peters-Golden, 2004). According to these data, macrophages have antipodial functions, they exhibit pro- as well as anti-inflammatory effects. The immune-regulatory and/or -modulatory cytokines they secrete include IL-1, IL-6, IL-10, IL-12, IL-18, and further they produce TNF-α, IFN-γ, TGF-β as well as CSFs (Lohmann-Matthes et al., 1994). Their production is pathogen-dependent. Produced by Th1 cells, IFN-γ for example is the strongest activator of macrophages and is secreted by themselves as well (Getz, 2005; Lohmann-Matthes et al., 1994). Macrophages need it to boost their MHC II molecules (Getz, 2005; Jansen, 1996), and for further secretion of IL-6, TNF-α and IL-1

which have pro-inflammatory roles (Getz, 2005). In contrast, IL-12 and IL-18 support differentiation of T cells into Th1 profile (Getz, 2005; Peters-Golden, 2004). There is evidence that healthy individuals exhibit higher levels of these two interleukins than humans with allergic asthma (Peters-Golden, 2004). Moreover, IL-12 and IL-18 induce IFN-γ secretion by NK and T cells (Getz, 2005). Finally, TGF-β together with IL-10 show opposing effects. They possess the strongest capacity to deactivate macrophages, despite these cells generate them on their own (Lohmann-Matthes et al., 1994). This mechanism of self-deactivation is still an unanswered question. Furthermore, they are capable of dampening Th1 and Th2 responses, respectively (Peters-Golden, 2004). Their course of action is conditional on the circumstances, hence they are also referred to as "regulatory cytokines" (Peters-Golden, 2004). In particular IL-10 has been reported to play a role in immunologic tolerance, as it is expressed by regulatory T cells (Kuipers and Lambrecht, 2004; Lambrecht, 2005; Peters-Golden, 2004; Upham and Stumbles, 2003). Beside the cytokines, macrophages also produce substances like nitric oxides (NO) (Holt, 2000; Peters-Golden, 2004), oxygen radicals (Lohmann-Matthes et al., 1994) and metabolites of arachidonic acid like PGE_2 (prostaglandin E_2) and PGD_2 (Lohmann-Matthes et al., 1994; Peters-Golden, 2004). NO on the one hand favours Th2 immunity (Peters-Golden, 2004), on the other hand, it is able to inhibit T cell responses (Holt, 2000; Peters-Golden, 2004). Moreover, it has the ability to interrupt the amplification of APC function of lung DCs that is GM-CSF-dependent (Holt, 2000). Similarly, PGE_2 has been attributed a pro-inflammatory activity due to its tendency to cause edema, however, recently, its bronchodilatory effect has been documented (Peters-Golden, 2004).

In summary, the role of macrophages in immune responses can be described as a regulatory and sometimes even protective one. Unfortunately, this cell population does not raise as much curiosity and interest as DC do, and therefore remains poorly investigated issue.

1.7 THE AIM OF THE STUDY

The goal of this project was to specify and describe a particular population of lung cells that, after the intranasal administration, pick up the antigen and store it for uncertain periods of time. In order to visualize the cells, OVA antigen conjugated to red fluorochrome TexasRed (TR-OVA) and AlexaRed, respectively, was used.

Mice with naïve background were healthy animals who had no preliminary contact with OVA, while in rechallenged and recovered mice allergic pulmonary response to this antigen was initiated. For the purpose of eliciting asthmatic immune reaction, animals from latter two groups were twice systemically primed with OVA injections. Subsequently, inhalative challenge with nebulized OVA was conducted. Six months later, naïve as well as rechallenged group representatives were intranasally administered TR-OVA. Consequently, the cells taking up the antigen were tracked in these two groups. Recovered models served as control mice, since they had not received any further treatment with TR-OVA in order to resolve asthmatic condition. Hence, the intention was not only to characterize the morphology and location of those cells, but also to compare these cells in mice encountering the antigen for the first time to those who have been systemically sensitized to the antigen and have developed immune response as consequence.

In order to analyse the cells containing fluorescent material, lungs were obtained and frozen seven days after the i.n. instillation of antigen. Subsequently, techniques of immunohistochemistry were performed on frozen lung tissue sections. The immunofluorescence staining was targeted at cell surface markers characteristic for APCs implicating CD11b, CD11c, B220, F4/80, and CD4, which is specifically expressed by T cells. Following microscopic analysis provided insights and findings needed to establish the phenotype of the cells including morphology, localization, distribution, amount in comparison to other cell populations, and eventually the function.

Transgenic GFP-DTR mice

Concerning the animals used, it is necessary to note that beside the wild type also transgenic mice were implied. These rodents were DTR-GFP mice, what means transgenic mice expressing the diphtheria toxin receptor (DTR) and green fluorescent protein (GFP) driven by the CD11c promoter. The purpose for their use has been reported by Steffen Jung and colleagues (van Rijt et al., 2005). In their study, van Rijt, Jung et al. documented the ability to deplete lung $CD11c^+$ DC and alveolar macrophages from airways of DTR tg mice as a result of administration of diphtheria toxin (DT). Normally, mice are unsusceptible to DT due to the missing DT receptor. CD11c-DTR mice, however, prove the opposite. With the administration of DT to airways of previously OVA-sensitized mice, lung $CD11c^+$ cells were eliminated. Subsequently, mice were again exposed to aerosolized OVA, however they did not respond to the antigen with cytokine secretion by Th2 cells. Moreover, major events of asthmatic inflammation were resolved. In the further course, adoptive transfer of $CD11c^+$ DC caused the recurrence of the disease events. Transfer of $CD11c^+$ macrophages failed to re-establish the disease. In summary, their experiment demonstrated that lung $CD11c^+$ DC are required for the induction of Th2-guided asthma in mice and emphasized their significance for the therapeutical directions.

In this study, CD11c-GFP-DTR mice were used in order to verify if GFP^+ cells, that also express CD11c marker, co-localize with cells that pick up and retain fluorescent TR-OVA antigen.

2. MATERIALS AND METHODS

2.1 MICE STRAINS

The project involved wild type BALB/c and C57Bl/6 strains. Furthermore, BALB/c transgenic mice expressing diphtheria-toxin-receptor (DTR) and green fluorescence protein (GFP) driven by CD11c promotor were used. The rodents were housed under standard conditions at the animal facility of VIRCC in Vienna, Austria, with the permission of the Animal Ethics Committee of Medical University of Vienna.

2.2 MOUSE MODELS

For the experiment three following groups with mice were established:

1. "Naïve mice": Naïve wild-type and naïve transgenic GFP-DTR mice were anaesthetized with 100µl of a composite containing 2ml Rompun® (Bayer) and 10ml Ketanest® (Pfizer) diluted in 18ml distilled water. Afterwards they were i.n. administered 100µg OVA-Alexa Red (OVA conjugated to AlexaFluor 594) or OVA-Texas Red (both from Molecular Probes).

2. "Memory mice": In order to induce allergic asthma in mice, this group (WT and TG-animals) was twice immunized with i.p. injection of 10µg OVA on days 0 and 21. Subsequently they were treated with 1% aerosolized OVA on day 28. Six months later, when mice already had recovered from the acute disease, they were rechallenged with i.n. instillation of 100µg OVA conjugated to Texas Red (TR-OVA) under preceded anaesthetization.

3. "Recovered mice": Like in the previous "memory" group, here again WT-mice and TG-rodents received separately two doses of 10µg OVA i.p., on days 0 and 21. Aerosol challenge with 1%OVA followed on day 28. To be able to recover from

acute allergic response, these mice had no further treatment with OVA-TexasRed allergen and had to rest for at least four months before removing their lungs.

Recovered mice as well as the naïve mice served as a control group in the experiment.

2.3 ISOLATION OF LUNGS

One week after the i.n. instillation, mice were put down by a lethal dose of anaesthetic. Lungs were taken out and were inflated and perfused with 1ml 40% OCT diluted with autoclaved water. Embedded in OCT, lung blocks were frozen on dry ice and kept at -20°C.

2.4 IMMUNOHISTOCHEMISTRY

2.4.1 Antibodies

For immunofluorescence staining methods either directly fluorescence-conjugated or purified primary monoclonal antibodies accompanied by fluorescence-conjugated secondary antibodies were applied.

Primary antibodies:

Directly conjugated to fluorochrome:

- anti-CD11c-FITC BD Pharmingen
- anti-CD11b-FITC BD Pharmingen
- anti-CD4-FITC BD Pharmingen
- anti-GFP AlexaFluor 488 Molecular Probes

Purified:

- anti-CD11c (hamster) BD Pharmingen
- anti-CD11b (rat) BD Pharmingen
- anti-CD4 (rat) BD Pharmingen
- anti-B220 (rat) BD Pharmingen
- anti-F4/80 (rat) Serotec

Secondary antibodies:

- Goat-anti-hamster IgG AlexaFluor 488 Molecular Probes
- Goat-anti-rat IgG AlexaFluor 488 Molecular Probes

2.4.2 Immunofluorescence staining of frozen sections

Frozen lung blocks were cut on a Cryotom (Leica) in 4µm sections, they were placed on a glass slide and stored at -20°C. The frozen sections were then defrosted for 10 min at room temperature before being fixed in acetone for another 10 min at 4°C. After washing them in PBS, the slides were then incubated with 100µl of diluted primary antibody per section for 60 min at 37°C in dark. In case of using purified primary antibodies, sections were first washed with PBS and then incubated with diluted secondary antibodies conjugated to detectable fluorochrome for further 30 min at room temperature in dark. When the incubation processes were all completed, the slides were washed in PBS and afterward in distilled water. As last step they were embedded with Fluorosave (Molecular Probes). The staining was analyzed with a Nikon Optiphot 2-UD fluorescence microscope and digital camera using a Multiband Filtersystem including red, green and triple filter at 40x and 20x magnification.

2.4.3 Haematoxylin staining

Haematoxylin is a dye that is widely-used in histological analysis for staining of basophilic cell components such as cell nuclei. For the microscopic examination of frozen lung sections from mice haematoxylin was necessary to highlight whether the cells being tracked are located in the parenchymal tissue and if they were in the central part of the lung lobe or rather in the periphery. For this purpose, slides were stained for 15 seconds with haematoxylin either before or after incubating them with the antibodies. The latter option proved to be more efficient and was applied further on.

3. RESULTS

3.1 LUNG CELLS ENGULFING INHALED OVA ANTIGEN: TR-OVA$^+$ CELLS

To visualize and study the cells ingesting the inhaled OVA antigen in the lung, mice were given AlexaRed-OVA or TexasRed-OVA i.n. (these cells are from here on referred to as TR-OVA$^+$ cells). After seven days lungs were isolated and frozen for further immunofluorescent analysis.

The figure below shows pictures of frozen lung tissue sections of naïve and rechallenged (memory) animals. The ingested OVA antigen conjugated to the red fluorochrome is represented by red patches in the tissue. The pictures used demonstrate the average impression of lung sections that have been examined under the microscope. In other words, the distribution of TR-OVA$^+$ cells was not equal throughout the entire lung. Regions were found where barely any or even none red spot was sighted. In contrast, some areas were truly "invaded" by the red signals, what will be shown later. Nevertheless, the TR-OVA$^+$ cells seem to be large cells with irregular shape and unclear surface. Their bright red signal appears as punctate within the cell. They appear as single cells. As shown here, no difference concerning TR-OVA$^+$ population could be discovered between naïve and memory mice lungs.

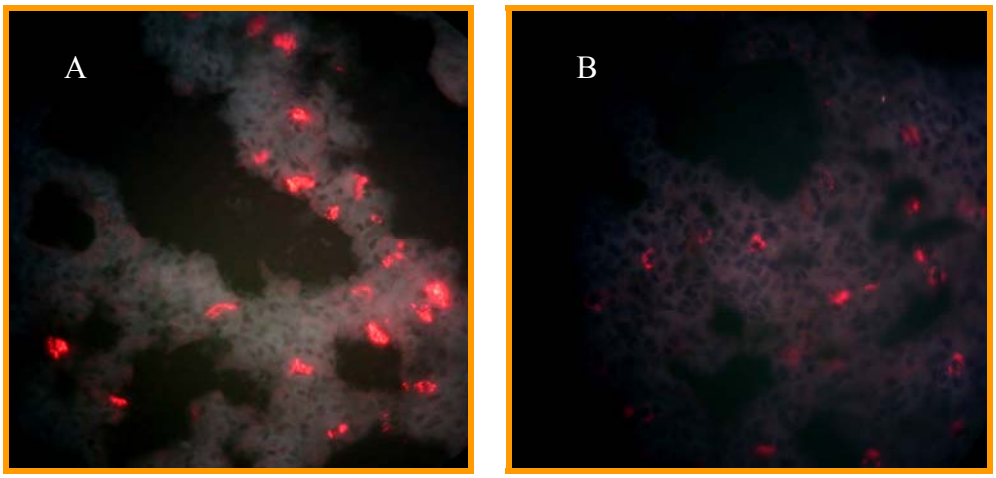

Figure 1. Frozen lung sections of naive (A) and rechallenged (B) mice treated with TR-OVA antigen. Pictures illustrate parenchymal lung compartments with red fluorescent TR-OVA$^+$ cells. They are distributed throughout the tissue with bias for air spaces. Due to the performed haematoxylin staining of rechallenged section (B), red TR-OVA$^+$ signals do not appear as bright as in the naive sample.

3.2 TR-OVA$^+$ CELLS AFTER STAININIG WITH ANTIBODIES

In order to characterize the cells picking up the allergen and to find out something about their appearance and localization in the lung, frozen lung sections were examined by means and methods of immunohistochemistry. Frozen slides with lung tissue of naïve, rechallenged (memory) and recovered (no TR-OVA treatment) mice were stained with fluorescence-labelled antibodies. The staining was directed against cell surface markers specific for APCs involving CD11b, CD11c, F4/80, and B220, but also against marker specific for T lymphocytes like CD4. Moreover, the antibody anti-GFP was applied exclusively for staining sections of transgenic GFP-DTR mice.

3.2.1 TR-OVA$^+$ cells are CD11b$^-$

First immunofluorescent staining was performed with anti-CD11b antibody. The intention was to verify whether TR-OVA$^+$ cells co-express CD11b surface marker which is in first line specific marker for macrophages but it can also be found on some DCs. For that purpose lungs from naïve models as well as from mice with memory disease that have been treated with TR-OVA or AR-OVA, respectively, were harvested and frozen. The slides with tissue were stained either with anti-CD11b-FITC monoclonal antibody or with purified anti-CD11b rat primary antibody followed by secondary fluorescent Ab goat-anti-rat IgG AF488 for detection. Few of the slides were subsequently dyed with haematoxylin. The first impression of this staining revealed that TR-OVA$^+$ cells are negative for CD11b marker, even though the two cell populations (TR-OVA$^+$ and CD11b$^+$) are situated very close to each other. In this regard, the assistance of haematoxylin, which stains the cell nuclei in particular, allowed the observation that TR-OVA$^+$ cells were not placed in proximity of cardinal respiratory bronchi or main pulmonary vessels but they were rather located in the lung periphery. As evidenced in the figure below, both cell subsets reside around the airway and are similar in size, namely extraordinary large. In addition, it seems like the red single

positive TR-OVA cell is positioned in the alveolar space (arrow). Apart from that, $CD11b^+$ cells form clusters.

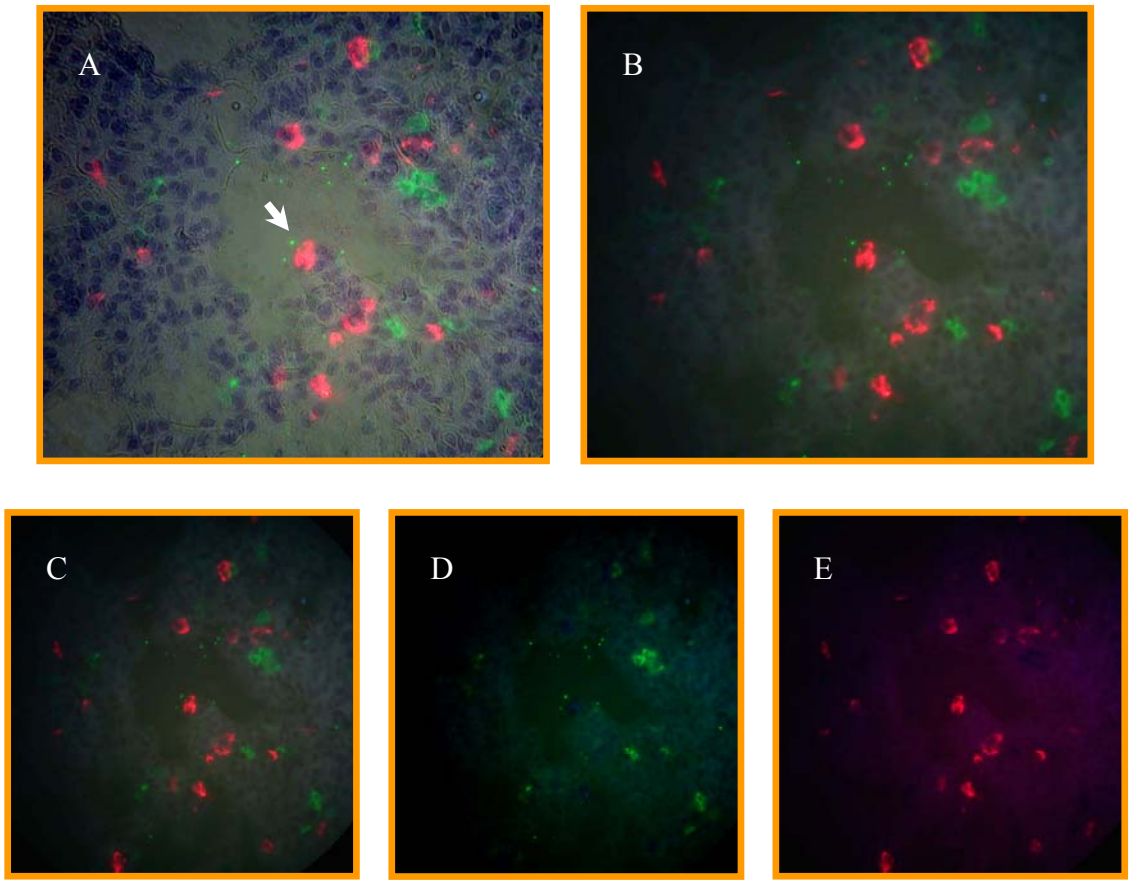

Figure 2. Naive lungs stained with haematoxylin and anti-CD11b Ab (A-E). Slides were first stained with pure anti-CD11b monoclonal Ab followed by secondary green fluorescent goat-anti-rat IgG AF488. In addition, sections were dyed with haematoxylin. Triple filter visualization evidences the close proximity but no co-localization of TR-OVA^+ signals seen in red and $CD11b^+$ cells shown in green (B-E). Without triple filter (A), the vicinity of TR-OVA^+ population to airways is even more apparent.

Comparing the naïve tissues with the memory sections, the TR-OVA^+ profile was virtually the same. At first view it is obvious that the image of naïve mice beneath exhibits a greater number of both, TR-OVA^+ and $CD11b^+$ signals, what would rather have a conflicting notion. However, the image above disproves that claim, since it displays a naïve model bearing fewer cells of both populations. Thus, again it is apparent that spatial dispersion of TR-OVA^+ as well as $CD11b^+$ cells is not constant within distinct sectors of the lung.

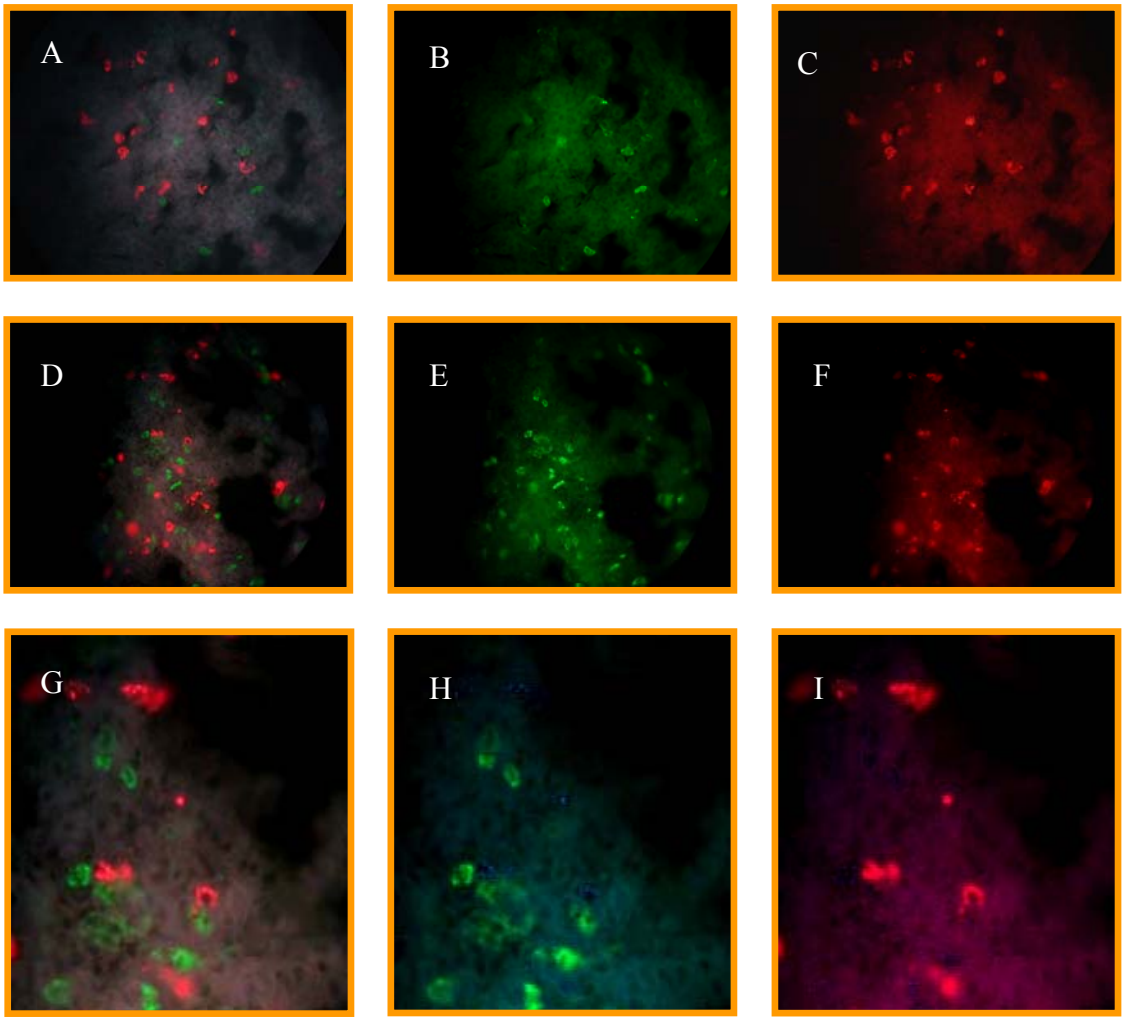

Figure 3. Sections with naive (D-I) and rechallenged lung tissue (A-C) stained for CD11b marker (A-I). Staining was performed with purified anti-CD11b mAb and subsequently with AF488-labelled goat-anti-rat Ab. Cells with ingested red TR-OVA antigen are often positioned next to green single positive CD11b cells as well as to clustered green $CD11b^+$ population, as seen here in a naive slide (G).

3.2.2 TR-OVA^+ cells are $CD11c^+$

Except from macrophages, DCs are also found to express the surface marker CD11c. In order to verify whether TR-OVA^+ cells co-express this marker, lungs of naïve models and mice with memory disease were stained for CD11c. Thereto, anti-CD11c-FITC was used or the other option was the incubation with pure primary hamster anti-CD11c antibody followed by fluorescent secondary goat-anti-hamster IgG AF488. As the

images below prove, TR-OVA$^+$ cells co-localize with the CD11c$^+$ staining. Single TR-OVA$^+$ signal is seen in red and single CD11c$^+$ cells appear green. Double positive (TR-OVA$^+$CD11c$^+$) signals appear yellow-orange (white arrow). All three cell types (single TR-OVA$^+$, single CD11c$^+$ and double positive TR-OVA$^+$CD11c$^+$) are spread through the alveoli tissue with a bias for air spaces and airways. In this regard, there is one distinctive feature noticed in naïve model, and that is single TR-OVA$^+$ red signal within the air space (violet arrow), neither single positive CD11c cells nor double positive signals were sighted within alveoli. However, it has to be noted that TR-OVA$^+$ cells were found within the alveoli in rechallenged mice as well and this will be shown later. CD11c$^+$TR-OVA$^-$ population occurs in clusters, whereas single TR-OVA$^+$CD11c$^-$ and double labelled TR-OVA$^+$CD11c$^+$ cells do not share this manner. When comparing the number of stained cells, it can be stated that single positive TR-OVA red cells and single positive CD11c green cells are balanced, it is however mentionable that the percentage of double labelled population is very low. Concerning these observations, naïve and memory mice are found to be similar. As to the dimension of stained cells, the three subsets resemble each other in size. In terms of the shape, CD11c$^+$ cells do not indicate a particular form neither, the difference to the TR-OVA$^+$ signals however is that CD11c stains the cell surface, whereas TR-OVA stain is kept intracellular.

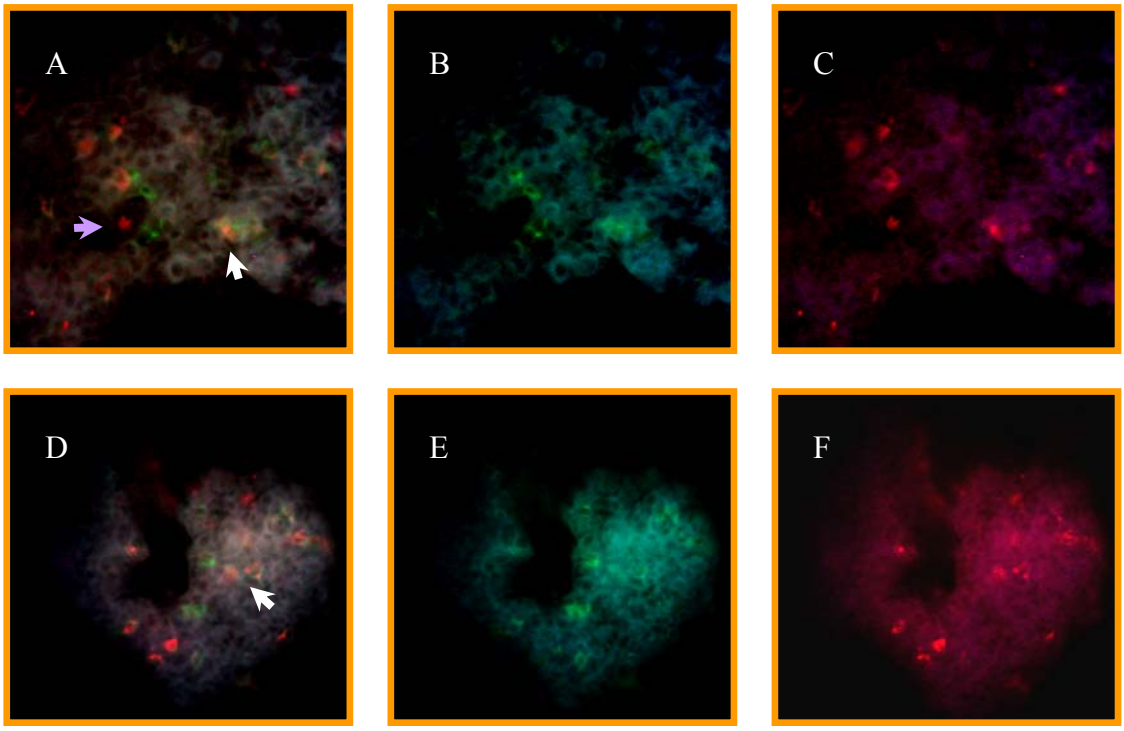

Figure 4. Naive and rechallenged lung compartments stained for CD11c marker (A-F). Mice were administered TR-OVA Ag i.n. and lungs were taken out and frozen. Slides were stained with anti-CD11c-FITC fluorescent Ab. Sections from both mice groups, naive (A-C) as well as rechallenged (D-F), demonstrate co-localization of red TR-OVA stain and green anti-CD11c molecules and these double labelled signals are recognized as yellow-orange cells.

3.2.3 TR-OVA$^+$ cells are F4/80$^+$

The next immunofluorescence staining was targeted at F4/80, which is identified as macrophage cell surface marker. To examine them under the microscope, frozen lung tissues of naïve and rechallenged mice were first stained with anti-F4/80 rat primary antibody and as a detecting secondary antibody goat-anti-rat IgG AF488 was used. The figures below evidence that TR-OVA$^+$ cells co-express the F4/80 marker. Sole TR-OVA$^+$ signal is shown in red colour and single F4/80$^+$ cells are represented in green. Double labelled TR-OVA$^+$F4/80$^+$ cells appear yellow-orange. Comparing the amount of the stained cells, it is obvious that there are more green single positive F4/80 cells than

red cells that have engulfed the TR-OVA antigen. The most slender population is the double positive yellow-orange TR-OVA^{+}F4/80^{+} subset. In this matter naïve and rechallenged models exhibit identical pattern. Single TR-OVA^{+} cells and sole F4/80^{+} cells are similar in size, large that is. The other common morphological feature of these two subsets is the undefined shape, however, not to forget is the fact that F4/80 stains extracellular and TR-OVA is positioned inside the cell. In regard to the localization, all three populations are dispersed through the parenchyma tissue in the lung periphery, particularly focussed on airways. In this context there is some noteworthy similarity affecting localization of TR-OVA^{+} signals in naive and memory mice. Looking at the images below, naïve model shows all three cell types near the airways. In contrast, memory mice indicate double labelled TR-OVA^{+}F4/80^{+} signal not only around but within the air space as well (arrow). This occurrence has been detected in naïve model before, even though it evidenced single positive TR-OVA red cell within the alveoli. Here, during the F4/80 staining, it is probably a coincidence that no single positive TR-OVA or double labelled cells were found within the airway in naïve mice. Apart from that, memory model highlights a kind of cluster containing double positive TR-OVA^{+}F4/80^{+} signals, which does not look cluster-like but rather like a row of cells attached to one another. Unfortunately, this occurrence has been a non-recurring unique observation and does not conform to the average findings from rechallenged mice.

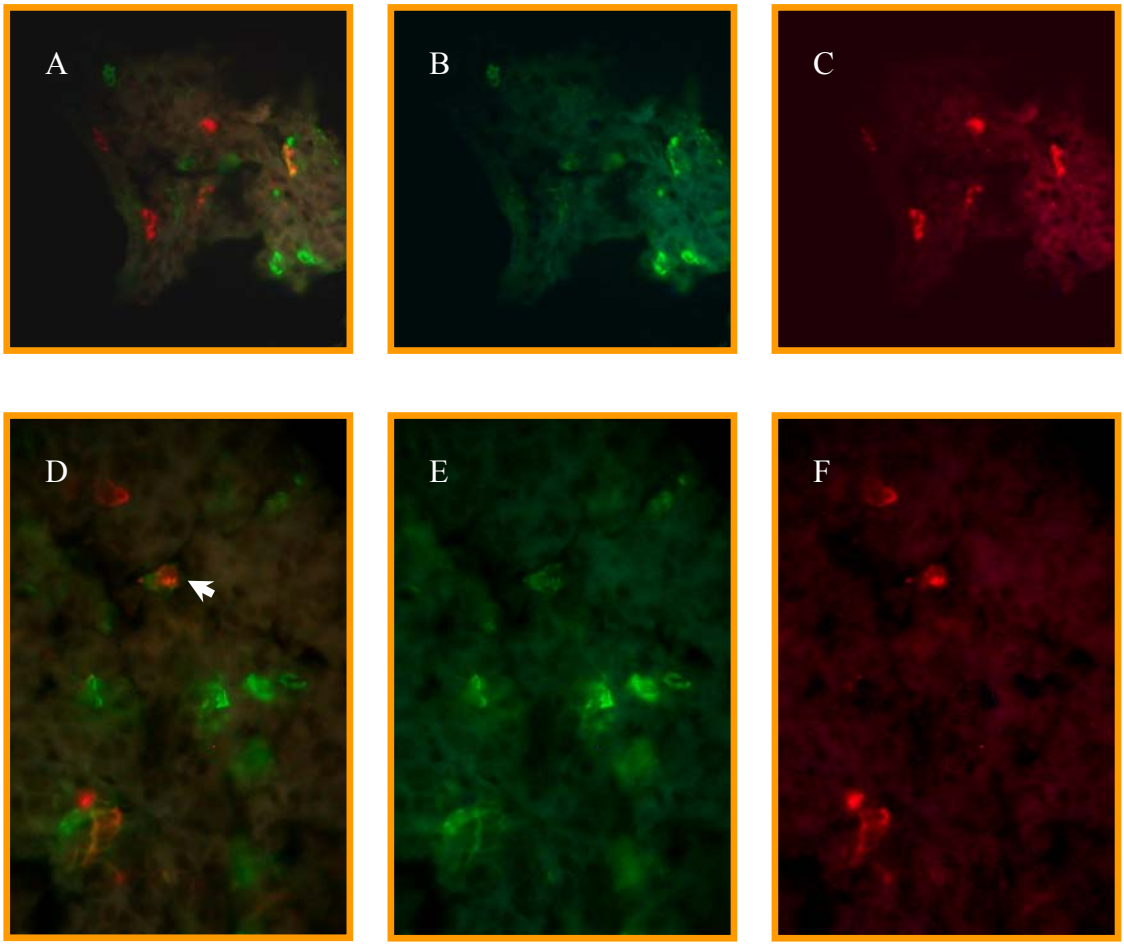

Figure 5. Frozen sections from naive and rechallenged mice lungs stained with anti-F4/80 Ab (A-F). Tissue compartments were incubated with primary anti-F4/80 Ab and for detection, goat-anti-rat attached to green fluorochrome AF488 was applied. Double labelling is evidenced in naive (A-C) as well as rechallenged lungs (D-F), and appears in yellow-orange. Apparently, double positive signal (TR-OVA$^+$F4/80$^+$) is resident except from tissue also in the air space of rechallenged mouse model (D).

3.2.4 TR-OVA$^+$ cells are negative for B220 marker

As B220 is defined as a cell surface marker for B cells, although there are reports claiming that certain subset of DCs also express B220, and since B cells beside DCs and macrophages also belong to APCs, the immunohistological examination with fluorescent antibodies included anti-B220 staining as well. For that purpose frozen slides with sections of naïve and rechallenged animals were incubated with anti-B220 rat primary antibody and subsequently they were stained with fluorescent secondary antibody goat-anti-rat IgG AF488. The pictures demonstrate that B220$^+$ green staining

does not co-localize with TR-OVA$^+$ red signal, no double positive cells were found. Comparing the number of the two cell populations stained, it can be seen that in rechallenged models the TR-OVA$^+$ and the B220$^+$ cells are in balance, naïve models however display more red TR-OVA$^+$ signals than B220$^+$ green cells. Here, it is necessary to allude that in naïve mice the opposite has been noted as well, this means there were areas in naïve mice where B220$^+$ population was in superior number compared to cells that have picked up the TR-OVA particles, in other words both cell types occur in related quantity. The general impression however, was that rechallenged models exhibited a greater amount of B220$^+$ cells but the number of TR-OVA$^+$ signals was similar to naïve sections. As to the site of their disposition, the two subsets are distributed throughout peripheral regions in the lung. Again both cell types demonstrate their close proximity to the airways and to each other (arrow). The cells positive for B220 appear in different sizes, from tiny to midsize, but they are generally smaller than TR-OVA$^+$ cells. Regarding the shape, the majority of B220$^+$ cells are, unlike the TR-OVA$^+$ cells, round or oval.

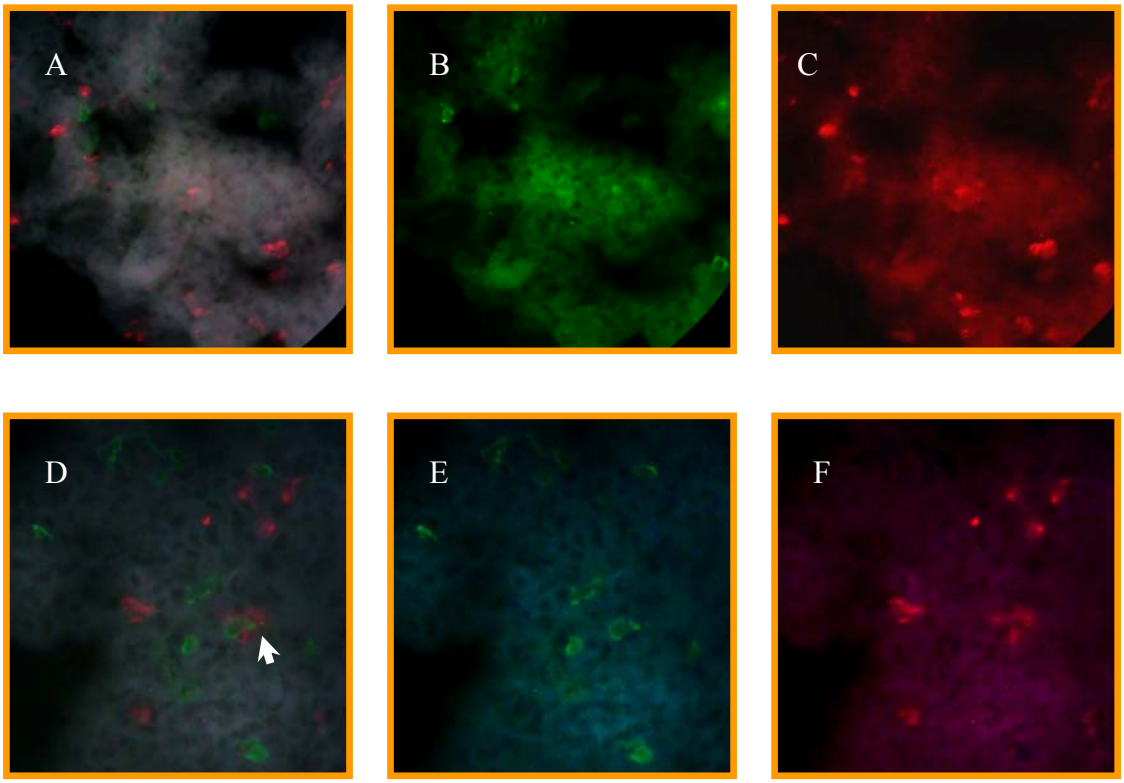

Figure 6. Anti-B220 staining of lung tissue from naive (A-C) and rechallenged mouse models (D-F). Slides were stained with anti-B220 Ab and afterwards with AF488-labelled goat-anti-rat green fluorescent Ab. Both mice groups exhibit no double labelling of green B220$^+$ cells with cells that have taken up OVA antigen attached to red fluorochrome TR.

3.2.5 TR-OVA$^+$ cells are negative for CD4 marker

CD4 is a cell surface marker mainly expressed by T-helper cells, but macrophages also have low expression. The staining was performed with naïve and rechallenged animals. For the microscopic examination, frozen sections were stained with anti-CD4-FITC fluorescent antibody, unfortunately without great success, or purified primary rat antibody anti-CD4 in combination with secondary Ab goat-anti-rat IgG AF488 were applied and achieved respectable results. The analysis revealed that the CD4$^+$ staining did not co-localize with the TR-OVA$^+$ red signal. In terms of localization, both cell types are dispersed through the parenchymal structures preferring the direct vicinity to the airways. Concerning this matter, there was not any discrepancy among naïve and rechallenged mice. Although the two cell populations are sometimes situated relatively proximal to each other, they are not as close as observed in CD11b staining where the vicinity of TR-OVA$^+$ signals to single and accumulated CD11b$^+$ cells was obvious. Further, the figure below shows that cells which have taken up the TR-OVA antigen differ in size and shape from CD4$^+$ cells. Unlike TR-OVA$^+$ profile, the CD4$^+$ cells are small and round or egg-shaped, with a smooth surface. TR-OVA$^+$ cells, in contrast, are large cells without well-defined shape and the nature of their cell surface is not sufficiently visible and remains dubious. Nevertheless, the circumstance that in the images below the naïve models display a bigger amount of CD4$^+$ cells that are beside the single occurrence also aggregated in nuggets, does not represent the totality of the stained sections. Such CD4$^+$ distribution pattern was observed in the rechallenged models as well, however the pictures were selected due to the quality and the phenotype of CD4$^+$ staining was similar in both mice groups.

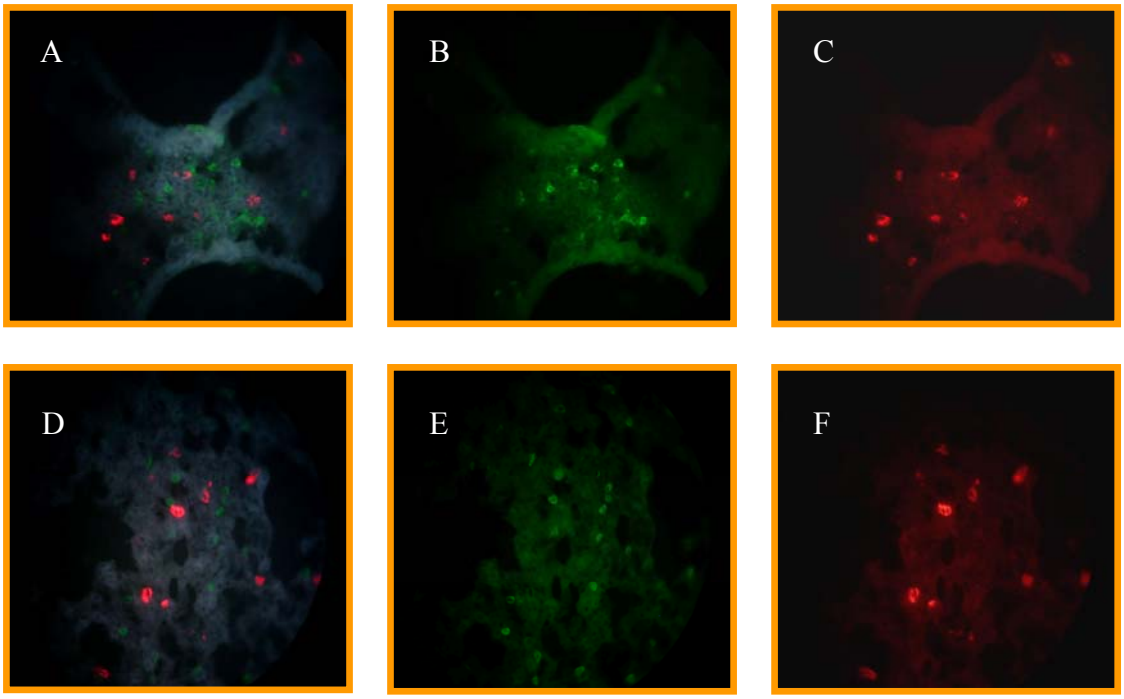

Figure 7. Lung compartments of naive and rechallenged mice stained for CD4 marker (A-F). Frozen sections were incubated with pure anti-CD4 mAb and subsequently were stained with secondary goat-anti-rat AF488 Ab. Neither naive (A-C) nor rechallenged lungs (D-F) reveal co-localization of red fluorescent TR-OVA$^+$ signals with green cells positive for CD4 marker.

3.2.6 TR-OVA$^+$ cells do not co-express GFP-DTR

In the project, beside the wild-type mice, transgenic mice were also used. These express the green fluorescent protein (GFP) and diphtheria toxin receptor (DTR) and this process is regulated by the CD11c promoter. In order to test whether cells with GFP-DTR co-localize with the TR-OVA$^+$ signal, the slides with naïve and rechallenged tissues were stained with anti-GFP AF488 antibody. Unfortunately, the pictures are not in high quality. Due to the weak green fluorescent signal, it was sometimes difficult to find which cells were actually stained. Nevertheless, the pictures shot were still sufficient to provide evidence that TR-OVA$^+$ do not co-localize with GFP$^+$ staining, no double labelled cells were detected. The green GFP$^+$ cells are like TR-OVA$^+$ cells large and with irregular shape. Another common property is the localization, GFP$^+$ subset is also present near the airways in peripheral areas of lungs. In terms of the amount the

impression was that GFP^+ population appeared in bigger numbers than $TR\text{-}OVA^+$ subset. This disparity was observed in both mice groups, naïve as well as memory disease. In addition, the figures illustrate GFP^+ cells clumping together to clusters, a feature which does not match into the $TR\text{-}OVA^+$ cells phenotype.

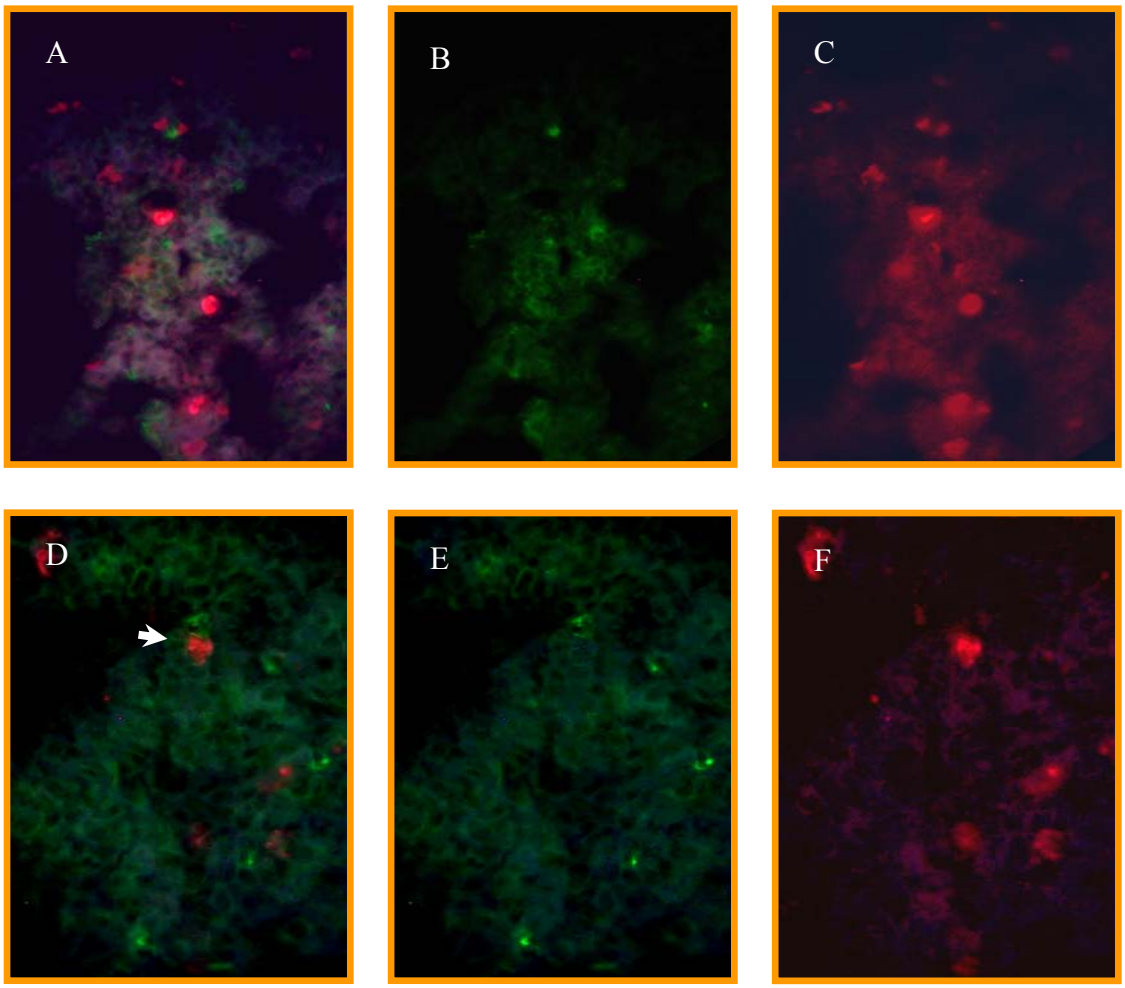

Figure 8. Lung tissue of transgenic GFP-DTR mice stained for anti-GFP (A-F). Staining was performed with anti-GFP AF488 green fluorescent Ab. As evident, no double labelling was observed. Cells with internalized TR-OVA are represented in red and GFP^+ cells appear in bright green fluorescent signals. The two cell types are in close proximity to each other (arrow) like rechallenged mouse sample demonstrates (D). Naive mice show green GFP^+ cells accumulated to cluster (A, B).

4. DISCUSSION

The intent of this project was to ascertain and characterize the pattern of a cell population in the lung of mice that ingest and hold OVA antigen one week after the intranasal administration. To be able to visualize those ingesting cells, the OVA antigen conjugated to a fluorochrome Texas Red was used. Beside the microscopic analysis that provided us an insight into their morphology and localization, we applied methods of immunohistochemistry to upgrade the phenotype and gain indication of their potential function. Accordingly, immunofluorescent staining was targeted against cell surface markers specific for T cells and APCs including: macrophages, DCs and B cells.

Phenotype of TR-OVA$^+$ cells

In our study, we detected the labelled antigen kept in cells in mouse lungs even seven days after the intranasal instillation. These cells, referred to as TR-OVA$^+$ cells, are large in size and do not have an accurately defined shape. Their surface also remained unclear. Further, we assessed that the fluorescent signal appeared spotted throughout the cell. One possible explanation could be that the engulfed fluorescent antigen was fragmented to a certain degree. It would be interesting to track and examine these cells in recovered animals after three to four months for instance. In terms of the localization, TR-OVA$^+$ cells were situated in the lung periphery, particularly in close proximity to airways but red fluorescent signals were observed within the airways and alveolar spaces as well. The distribution however was not constant within the entire parenchymal tissue. Alveolar tissue compartments have been regarded where no red fluorescent signal was sighted. One of the reasons could be the low and insufficient concentration of antigen that was administered, the other cause might be that instilled antigen is not equally transported to all sections of the lung (Vermaelen et al., 2001), or, the distribution of APCs is not consistent throughout the lung tissue due to the complex association to their function (von Garnier et al., 2005). In addition, there was no evidence of aggregating to clusters, TR-OVA$^+$ signals appeared as single cells.

The performed immmunofluorescent staining ought to give us information about cell surface markers TR-OVA$^+$ cells express, according to which we attempted to classify them. The first significant finding was that TR-OVA$^+$ subset is positive for CD11c, even though the double-labelled population appeared in slight amount. All the more, it was surprising that TR-OVA$^+$ cells were negative for GFP, as we expected to see the opposite. The subsequent simultaneous staining of GFP-DTR mice lung sections with anti-CD11c and anti-GFP fluorescent antibodies was not less disputed. It evidenced that GFP$^+$ cells moderately co-expressed CD11c marker, the double positive population of CD11c$^+$GFP$^+$ cells was represented by a short number (data not shown). The reason for this is unknown. Nevertheless, our findings suggest four different populations of CD11c$^+$ cells involving CD11c$^+$TR-OVA$^+$, CD11c$^+$TR-OVA$^-$, CD11c$^+$GFP$^+$ and CD11c$^+$GFP$^-$ population. Hence, these discrepancies require further experiments to clarify the relation among those four subsets. However, for our project the one population of CD11c$^+$TR-OVA$^+$ cells was solely important. The CD11c marker is proved to be variably expressed on lung resident DCs and alveolar and interstitial lung macrophages as well (Cleret et al., 2006; Gonzalez-Juarrero and Orme, 2001; van Rijt et al., 2005). Similarly, the CD11b cell marker is expressed by myeloid DC (Byersdorfer and Chaplin, 2001; von Garnier et al., 2005) and lung macrophages (Strauss-Ayali et al., 2007; von Garnier et al., 2005). However, we could demonstrate that cells picking up fluorescent OVA antigen are negative for CD11b, and according to this evidence we could exclude that they might be conventional DCs. With these results the phenotype of our TR-OVA$^+$ cells was equalized between subsets of DCs and macrophages. After we additionally verified the co-localization of TR-OVA$^+$ with F4/80$^+$ staining, the balance moved towards lung macrophages, as this marker is known to be specific for macrophage lineage cells (Gonzalez-Juarrero et al., 2003; Morris et al., 1991). However, previous studies have reported on DCs that do express F4/80 antigen as well (Belz et al., 2004; Gonzalez-Juarrero and Orme, 2001). In the further run of the project, we ascertained that cells engulfing TR-OVA are B220$^-$, thus they are neither B cells, nor they correspond to plasmacytoid DC phenotype (Martin et al., 2002). Besides that, according to the CD4 staining, we not only detected that our TR-OVA$^+$ do not express CD4 marker, but we also observed that our red fluorescent cells are not placed that near to clusters of CD4$^+$ cells, which we supposed to be T helper cells. Furthermore, no TR-OVA$^+$ signals were found in close vicinity to huge accumulation of T helper cells

around the airways that are probably infiltrates (data not shown), suggesting that there is no interplay among these two cell types.

In this context, we determined no significant differences between naïve models and rechallenged mice with memory disease. We compared diverse patterns of the TR-OVA^+ subset involving distribution throughout distinct sections of the lung with confinement to lung parenchyma, localization in the tissue with preferred proximity to airways, lacking ability of forming clusters, and finally, very similar amount of cells in both mouse systems. Of course, this latter observation is an impression limited by the fact that it is based on individual human sense. Hence, it demands further reliable quantitative analysis provided by FACS. A recent study, for example, reported on a $CD11b^-CD11c^+$ population consistent with our TR-$OVA^+CD11b^-CD11c^+$ subset (Gonzalez-Juarrero et al., 2003). They identified it as a mixture of alveolar macrophages and immature DCs, whose cell numbers decreased during the development of an infection. The reason for this was the change in the expression of these two cell surface markers upon the early and chronic disease, and they suggest that this changing phenotype was due to the differentiation of those cells. Unfortunately, we do not have sufficient data to confirm whether this did or did not happen in our mouse models. Furthermore, concerning the other single stained cell types which did not contain the red OVA antigen, including $CD11b^+$, $CD11c^+$, GFP^+, $F4/80^+$ and $CD4^+$ subsets, we noticed no disparities in cell amounts comparing naïve and memory animals. Only the $B220^+$ staining showed a slight discrepancy among the two animal groups, namely, the rechallenged group exhibited just a bit more $B220^+$ cells than the naïve rodents. Thus, on the one hand, we established that the accumulation of cells addressed, representing APCs and T helper cells, as well as the single stained TR-OVA^+ signals, occurred to closely related extent, reflecting that even naïve animals who had never encountered TR-OVA antigen before, were probably able to develop immune response to this antigen even without prior systemic sensitization. On the other hand, the B220 staining results generated suspicion and doubt on our opinion. Therefore, it would be advisable to verify this assumption with approved and appropriate methods and examine the common hallmarks of asthmatic disorder, such as IgE production and eosinophilic inflammation, analyze the mucus hypersecretion, and test the AHR.

To conclude, although more indications argue for macrophages, it would be premature to define TR-OVA$^+$ as their subset. Indeed, cells taking up red OVA allergen share morphological profile as well as localization pattern with macrophages. Both cell types are large and are situated close to airways but also within alveolar spaces. However, in terms of the function, here, we could not actually determine what role TR-OVA$^+$ cells play in the immunologic processes. What is for sure is that our cells engulf the antigen but do not process it or they process it to a low degree. According to this, we do not believe they present the antigen to T cells. Additionally, this notion was further supported by the fact that we did not observe TR-OVA$^+$ cells in close proximity neither to CD4$^+$ single cells and/or clusters nor to the infiltrates of CD4$^+$ T cells. Unlike this, researchers have described a subset of BAL DCs with CD11c$^+$CD11b$^+$ phenotype that pick up antigen and retain it for up to three weeks after the inhalation, these cells however were proved to highly present the antigen (Julia et al., 2002). They also identified a subset of CD11c$^+$CD11b$^-$ cells, what matches into the phenotype of our TR-OVA$^+$ cells, but these were not capable of capturing the antigen efficiently. Though, at this point it is important to note that this group used much lower concentration of antigen, so this might be the reason for not retaining it. Other group, for instance, characterized cells taking up fluorescent latex beads, either with or without conjugated OVA antigen, as CD11c$^+$CD11b$^-$F4/80$^+$ subset of airway DCs (Byersdorfer and Chaplin, 2001). Their experiment however differs from ours as they exclusively examined BAL cells and we investigated tissue compartments. Another distinction is the timepoint, they tested the antigen uptake 6 h and 48 h after the administration, we analyzed seven days after the instillation. Hence, we realize it is not easy to compare results because there are so many diverse factors that need to be considered and limit the comparison. One last example of a study that achieved results conforming to ours, reported on a long-lived cell population with high endocytic capacity. Von Garnier et al. (2005) identified those cells as lung resident tissue macrophages being positive for CD11c and F4/80 expression and negative for CD11b marker. Moreover, this population appeared incapable of stimulating the T cells in vitro or initiating their proliferation, in contrast, it indicated rather potential suppressive activity. This latter discovery we could not reconcile with our results as we did not performed such attempts. However, the outcome from this study was very close to ours and provides valuable proposals.

Nevertheless, in the end, dealing with so many similar and differing data, controversial findings, it remains impossible to assign our red fluorescent TR-OVA$^+$ population either to DCs or to macrophages. Perhaps they are precursor cells in a late monocytic stage, something like indeterminate cells, which differentiate in the tissue either into macrophages or into DC. It is known that macrophages and DCs may arise from common precursor state (Goldsby et al., 2001; Gonzalez-Juarrero et al., 2003). Therefore, as long as they are immature, the internalized antigen stays non-degraded. Like mentioned before, it would be interesting to pursue these cells for extended periods of time, what would mean to track them in recovered animals. To date, it remains unknown what happens with the deposits of ingested antigen.

Taken together, we investigated a population of lung resident cells that take up OVA antigen conjugated to red fluorochrome after the intranasal administration and keep it for at least seven days. They demonstrate a CD11c$^+$CD11b$^-$F4/80$^+$ phenotype, that is a mixtured phenotype of lung DCs and macrophages. In the experiment, there was no evidence of antigen presentation to T cells. Further studies need to be done in order to understand their development and ascertain their function in allergic reactions. According to our findings, we assume TR-OVA$^+$ cells constitute an intermediate subset with phenotype common for both, DC and macrophages.

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6. APPENDIX

Long-Term Deposition of Inhaled Antigen in Lung Resident $CD11b^{-}CD11c^{+}$ Cells

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Long-Term Deposition of Inhaled Antigen in Lung Resident CD11b$^-$CD11c$^+$ Cells

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In this study we report the characterization of a population of lung resident CD11b$^-$CD11c$^+$ cells that are able to take up inhaled antigen and retain it for extended periods of time. Ovalbumin conjugated to fluorescein-isothiocyanate (FITC-OVA) administered intranasally to mice was taken up by two main populations of cells in the lung, a migratory CD11c$^+$CD11b$^+$ population consisting of dendritic cells (DC), which rapidly transported antigen to the draining lymph node (LN), and a resident CD11b$^-$CD11c$^+$ population that retained engulfed antigen without apparently degrading it for up to 8 wk after administration. The FITC$^+$CD11b$^-$CD11c$^+$ cells did not migrate to draining LN at a detectable rate, and did not up-regulate expression of costimulatory molecules in response to LPS treatment. FITC$^+$CD11b$^-$CD11c$^+$ cells were found in the lung and bronchoalveolar lavage fluid, and their distribution was compatible with macrophages. Although FITC$^+$CD11b$^-$CD11c$^+$ cells expressed the DC marker DEC205 and other molecules associated with antigen-presenting cell function, they did not induce proliferation of antigen-specific CD4$^+$ T cells *in vitro* or acute cytokine production by activated CD4$^+$ T cells *in vivo*. Thus, FITC$^+$CD11b$^-$CD11c$^+$ cells appear to represent an intermediate cell type sharing properties with DC and macrophages. These cells may have a role in modulating the responses of lung resident T cells to inhaled antigens.

Keywords: animal models; antigen presentation/processing; lung inflammation

CLINICAL RELEVANCE

This article reports the characterization of a population of lung cells that take up inhaled antigen and retain it for several weeks. Retention of antigen by these cells had not been reported before, and may have important implications for immune responses in the lung.

The immune response to inhaled allergen involves distinct and well-characterized phases. Pulmonary and airway dendritic cells (DC) are required to transport antigen from tissues to secondary lymphoid organs during the sensitization phase, to drive the activation of naïve CD4$^+$ T cells (1, 2). Antigen-presenting cells (APC) are again required upon antigen re-encounter to present antigen to effector or memory CD4$^+$ T cells in the lung and airways (3). They may include migratory DC populations, that present antigen to T cells in the lung before migrating to the draining lymph node (LN) (4), and other populations of lung-resident APC that migrate to the draining LN at a minimal rate (5). Such resident APC may have an important function in presenting antigen in lung tissue, and perhaps supporting the retention of antigen-specific memory T cells within the lung. In one study, DC isolated from the airway were shown to present inhaled antigen for up to 8 wk after antigen exposure (6). However, it was not established whether those DC are long-lived, or whether they acquire antigen from a separate, long-lived population that cannot directly present antigen to T cells.

Several APC populations are present in lung. Alveolar macrophages phagocytose large amounts of inhaled antigen (7), but also down-regulate the activity of pulmonary DC (8) and suppress T cell activation through the production of NO (9). Murine alveolar macrophages express major histocompatibility complex class II (MHC II) molecules (10) and can present antigen to T cell hybridomas, which have little need for costimulation (11). This suggests that alveolar macrophages could be able to stimulate antigen-specific effector or memory T cells that also have low costimulatory requirements. B cells are also found in the pulmonary tissue of mice, and may also contribute to antigen presentation *in situ* (12).

We wished to track the cells that engulf and present antigen within the lung tissue following intranasal instillation of ovalbumin (OVA) protein conjugated to the fluorescent marker fluorescein isothiocyanate (FITC) (FITC-OVA). We found that several populations of cells take up FITC-OVA within the lung tissue; however, 1 wk after antigen exposure, FITC-OVA becomes restricted to a population of CD11b$^-$CD11c$^+$ cells (hereafter referred to as CD11b$^-$FITC$^+$). These CD11b$^-$FITC$^+$ cells could be isolated from both the lung tissue and bronchoalveolar lavage (BAL) for longer than 8 wk after intranasal antigen administration. Despite expression of MHC II and T cell costimulatory molecules, CD11b$^-$FITC$^+$ cells appeared unable to present their retained antigen to activated Th2 cells *in vivo* or naïve CD4$^+$ T cells *in vitro*. It is possible that these previously unidentified long-lived cells may modulate the response of lung T cells to inhaled antigens, affecting the chronicity of the allergic response that is observed in some disease situations (6, 13).

MATERIALS AND METHODS

Mice

C57Bl/6 mice were from breeding pairs originally obtained from The Jackson Laboratories (Bar Harbor, ME). OT-II mice (14) were a kind gift of Dr. Frank Carbone (Melbourne University, Australia). Experimental procedures were performed with the approval of the Wellington School of Medicine Animal Ethics Committee in accordance with the University of Otago guidelines. C57Bl/6 mice were bred and maintained in the VIRCC animal facility (Vienna, Austria), and used with the approval of the Medical University of Vienna Animal Ethics Committee.

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In Vitro Culture Media and Reagents

All cultures were in complete IMDM (cIMDM), which consisted of IMDM with GlutaMAX and HEPES buffer supplemented with 5% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin (all from Invitrogen Corp., Auckland, NZ) and 50 μM 2-ME (Sigma-Aldrich, Castle Hill, NSW, Australia). Grade V chicken OVA was from Sigma-Aldrich; $OVA_{323-339}$ peptide was from Chiron Mimotopes, Clayton, Australia. Low-endotoxin FITC-OVA and Texas Red–conjugated OVA (TR-OVA) were from Molecular Probes (Eugene, OR).

Intranasal Instillations and Preparation $CD11c^+$ Cells from Lung and LN Digests

Mice were anesthetized and 100 μg of OVA protein, FITC-OVA or TR-OVA in 50 μl of PBS were instilled into one nostril. At different times, lungs were collected, finely sliced, and digested by two consecutive incubations in IMDM containing 1 mg/ml DNase I (Sigma-Aldrich) and 2.4 mg/ml collagenase I (Invitrogen Corp.) at 37°C for 30 min. In some experiments airway cells were removed by BAL before lung collection; this did not affect the number or phenotype of the recovered $FITC^+$ populations. Mononuclear cells were purified by centrifugation over 60% Percoll (Amersham Pharmacia Biotech, Piscataway, NJ). Lung-draining mediastinal LN were harvested and digested for 1 h at 37°C in 2.4 mg/ml collagenase II (Invitrogen Corp.) and 1 mg/ml DNase I in IMDM (15).

Total lung cell preparations were enriched for $CD11c^+$ cells using anti-CD11c MicroBeads (Miltenyi Biotech, Bergisch Gladbach, Germany) and an Auto MACS sorter (Miltenyi Biotech). The enriched population was labeled with fluorescent anti-CD11c and anti-CD11b antibodies, and further purified by electronic sorting using a FACSVantage SE (Becton Dickinson, Mountain View, CA).

Antibodies and Flow Cytometric Analysis

Antibodies were from BD Pharmingen (San Diego, CA), with the exception of F4/80 which was from Serotec Inc. (Raleigh, NC). Anti-FcγRII, anti-CD11c, anti–I-A^b, anti-CD86, anti-CD28, anti-CD3, and anti–DEC-205 were purified from hybridoma supernatants using Hy-Trap protein G columns (Pharmacia Biotech, Uppsala, Sweden) and conjugated to FITC (Sigma-Aldrich) or to allophycocyanin (Prozyme, San Leandro, CA). Cells were pre-incubated in anti-FcγRII mAb and labeled in PBS containing 2% FCS, 2 mM EDTA and 0.01% sodium azide. Analysis was on a FACSort using the Cell Quest software (BD, Mountain View, CA). Live cells were identified by forward scatter/side scatter (FSC/SSC) properties and PI (BD Biosciences) exclusion.

For detection of intracellular Langerin expression, Fc receptors were blocked by incubation in 5% mouse serum. Cells were fixed and permeabilized using a cytofix/cytoperm kit (BD Pharmingen) before incubation with anti-Langerin Ab (16) and anti-rat IgG-PE.

Immunofluorescence and Histologic Analysis

Seven days after intranasal instillation, mice were killed by injection of a lethal dose of anesthetic. The lungs were harvested and frozen in OCT on dry ice and maintained at −20°C. Frozen lung sections (4 μm) were fixed with acetone for 10 min and washed in PBS. Slides were then incubated with anti–CD11c-FITC or anti–CD11b-FITC (Pharmingen) for 60 min at 37°C, washed with PBS and distilled water, and incubated with hematoxylin for 15 s before embedding with Fluorosave (Molecular Probes). Immunofluorescence was visualized with a Nikon Optiphot 2-UD Fluorescence Microscope (Nikon, Tokyo, Japan) with a ×40 objective and a Multiband Filtersystem triple filter (DAPI/FITC/Texas Red; Af-Analysentechnik, Tübingen, Germany).

In Vitro T Cell Proliferation Assays

Decreasing numbers of FACS-sorted $CD11c^+CD11b^+$ and $CD11b^-FITC^+$ lung cells were distributed in triplicate in 96-well plates. OT-II thymocyte suspensions were depleted of APC by 2 h plastic adherence as described (17), and 2×10^5 cells were added to each well. $OVA_{323-339}$ was added to some wells at 10 μg/ml. Bone marrow–derived DC (BM-DC) were generated as described (17) and used at 1.5×10^3 cells/well; rhIL-2 was used at 100 U/ml. Plates were incubated for 3 d at 37°C and proliferation of T cells was evaluated by measuring [^{3}H]-thymidine (Perkin Elmer Life Sciences Inc., Boston, MA) incorporation over the last 8 h of culture. Cells were harvested using a Tomtec automated cell harvester and a liquid scintillation Beta counter (Wallac, Turku, Finland).

Generation of Th2 Cells *In Vitro* and Adoptive Transfer

Th2 cells were generated as previously described (18). Cell suspensions from LN of OT-II mice were depleted of $CD8^+$ T cells and B cells using Dynabeads (Dynal Biotech ASA, Oslo, Norway) and cultured on plates coated with 5 μg/ml anti-CD3 mAb in cIMDM containing 2 ng/ml rhIL-6, 20 U/ml rhIL-2, 1,000 U/ml rmIL-4 and 2 μg/ml anti-CD28 mAb. After 5 d cells were harvested, resuspended in cIMDM containing 100 U/ml rhIL-2 and cultured in fresh plates for a further 2 d. 1×10^7 $V\alpha2^+V\beta5.1, 5.2^+$ cells were injected into the lateral tail vein of recipient mice.

Quantitative and Qualitative Assessment of Leukocytes in BAL Fluid

Airway-infiltrating cells were collected by BAL. Cells were spun on a glass slide, stained using Diff-Quik (Dade International, Dudingen, Switzerland) and counted under a microscope as described (19). A minimum of 200 cells/sample were counted.

RESULTS

DC Transport Antigen from the Lung to the Draining LN

To follow the migration of DC from the lung to the mediastinal LN, mice were given 100 μg of FITC-OVA by i.n. instillation, and the phenotype and number of $FITC\text{-}OVA^+$ cells in the LN was determined by FACS. At 24 h after instillation, 81% of $FITC^+$ cells were $CD11c^+$ $MHC\ II^+$, indicating that antigen was mostly transported by DC (Figures 1A and 1B). In addition, virtually all of the $CD11c^+$ $FITC^+$ cells expressed high levels of MHC II, a phenotype compatible with mature cells emigrating

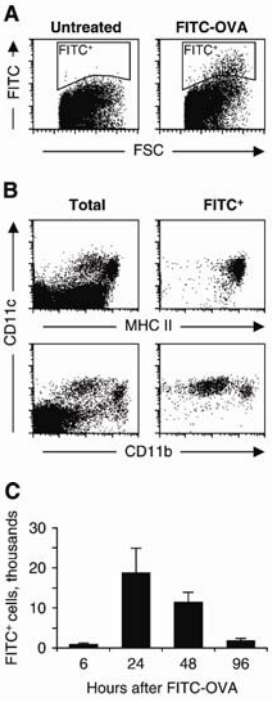

Figure 1. $FITC^+$ cells in the draining LN express CD11c, MHCII, and CD11b. C57BL/6 mice were treated with 100 μg FITC-OVA by intranasal instillation, and 24 h later the draining LN were harvested, digested with collagenase, and analyzed by FACS. Dead cells were identified by FSC/SSC and PI staining and were excluded from analysis. (*A*) FITC staining in total LN suspensions from representative untreated and FITC-OVA treated mice. Gating of $FITC^+$ cells is shown. (*B*) Dot plot analysis of MHCII, CD11b, and CD11c expression on total or $FITC^+$ LN cells. (*C*) Number of $FITC^+$ cells in the draining mediastinal LN at different times after FITC-OVA administration. $FITC^+$ cells were gated as in *A*. *Bars* show the mean ± SEM number of $FITC^+$ cells in the draining LN of four mice.

from tissue. Most of the FITC$^+$ cells expressed intermediate or high levels of CD11b. The number of FITC$^+$ cells in LN was $\sim$ 1,000 at 6 h after instillation and peaked at $\sim$ 20,000 at 24 h (Figure 1C). By 96 h, there were few FITC$^+$ cells remaining in the LN, suggesting that lung deposits of FITC-OVA had been exhausted.

A Population of Lung CD11c$^+$ Cells Retains Antigen for 8 wk after Intranasal Administration of FITC-OVA

The phenotype and number of FITC$^+$ cells in the lungs of FITC-OVA–treated mice was also determined. Surprisingly, the numbers of FITC$^+$ cells in the lung remained relatively constant for up to 28 d, with only a slight decrease by Day 56 (Figure 2A). Profiles in Figures 2B and 2C show that, 1 d after FITC-OVA administration, the FITC signal was present in several different lung cell populations. In contrast, on Day 6 most of the FITC signal was found in a CD11b$^-$CD11c$^+$ population; this population will be referred to as "CD11b$^-$FITC$^+$" from here on. CD11b$^-$CD11c$^+$ cells represented > 90% of the FITC$^+$ cells detected in the BAL fluid of mice treated with FITC-OVA 7 d previously (Figure 2D). However, FITC$^+$ cells in BAL appeared to represent only a proportion of the total CD11b$^-$FITC$^+$ cells in lung, as carrying out BAL before lung digestion failed to completely remove the CD11b$^-$FITC$^+$ population (data not shown).

The phenotype of the CD11b$^-$FITC$^+$ cells was further characterized in lung cell suspensions prepared from mice treated with FITC-OVA 1 wk earlier. Figure 3 shows that CD11b$^-$FITC$^+$ cells did not express the Langerhans cell–specific marker Langerin/CD207, or the lineage markers NK1.1, Gr.1, B220, CD8α, or CD45RB, indicating that they were not Langerhans cells or plasmacytoid DC. CD11b$^-$FITC$^+$ cells expressed the mannose receptor family member DEC-205 and a proportion of cells were also F4/80$^+$. CD11b$^-$FITC$^+$ cells also expressed intermediate levels of MHC II and FcγRII.

Morphology and Localization of CD11b$^-$FITC$^+$ Cells in the Lung Tissue

To examine the morphology of CD11b$^-$FITC$^+$ cells, mice were treated with FITC-OVA or TR-OVA and 1 wk later the CD11b$^-$ fluorescent cells, or the total CD11b$^-$CD11c$^+$ cells, were purified by electronic sorting and spun onto glass slides. CD11b$^-$FITC$^+$ cells were large and round with a smooth cytoplasm and large nuclei (Figure 4A). The fluorescent TR signal appeared as bright, punctate staining throughout the cytoplasm of the cell (Figure 4B) suggesting localization to an endocytic compartment.

To determine the localization of CD11b$^-$FITC$^+$ cells in the lung tissue, mice were treated with TR-OVA and 16 d later lungs were harvested and snap-frozen for preparation of tissue sections and fluorescence analysis. TR$^+$ cells displayed a cytoplasmic bright punctate staining. Simultaneous staining with fluorescent antibodies again showed that the TR$^+$ cells were CD11b$^-$ and CD11c$^+$ (Figures 4E and 4G, respectively), and frequently expressed the F4/80 marker (data not shown). Adjacent lung sections stained with the corresponding isotype control antibodies did not reveal any staining (data not shown).

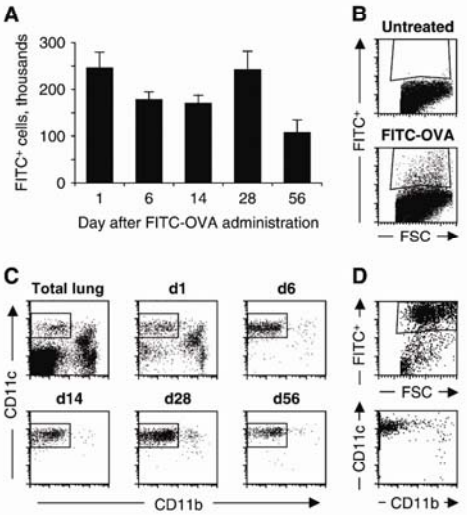

Figure 2. Phenotype of FITC$^+$ cells in the lungs and BAL of FITC-OVA treated mice. C57BL/6 mice were treated with 100 μg FITC-OVA by intranasal instillation, and at the indicated time points lungs were harvested, digested with collagenase, and analyzed by FACS. Dead cells were identified by FSC/SSC and PI staining and were excluded from analysis. (*A*) Number of FITC$^+$ cells in the lung at different times after FITC-OVA administration. FITC$^+$ cells were gated as in *B*. Mean ± SEM for groups of at least four mice are shown. (*B*) FITC staining in total lung suspensions from representative untreated and FITC-OVA–treated mice. Gating of FITC$^+$ cells is shown. (*C*) Dot plot analysis of CD11b and CD11c expression on representative total or FITC$^+$ lung cells at the indicated times after FITC-OVA administration. The CD11b$^-$ CD11c$^+$ population is highlighted. (*D*) Six days after FITC-OVA treatment, airway cells were collected by BAL and analyzed by FACS. The *top panel* shows gating for FITC$^+$ cells on total live BAL cells; the *bottom panel* shows expression of CD11b and CD11c on FITC$^+$ cells. Representative dot plots are shown.

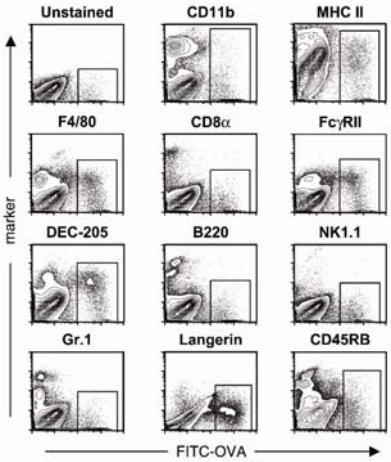

Figure 3. Phenotype of FITC$^+$ cells that remain in the lung after i.n. administration of FITC-OVA. C57BL/6 mice were treated with 100 μg FITC-OVA by intranasal instillation, and 5 d later their lungs were harvested, digested with collagenase, and analyzed by FACS. All samples were incubated with anti-CD11c plus the marker indicated in each panel; dead cells were identified by FSC/SSC and PI staining and were excluded from analysis. For the detection of intracellular markers (e.g., Langerin), cells were fixed after surface staining and permeabilized. Gating of FITC$^+$ cells is shown in each panel; FITC$^+$ cells also expressed CD11c (data not shown).

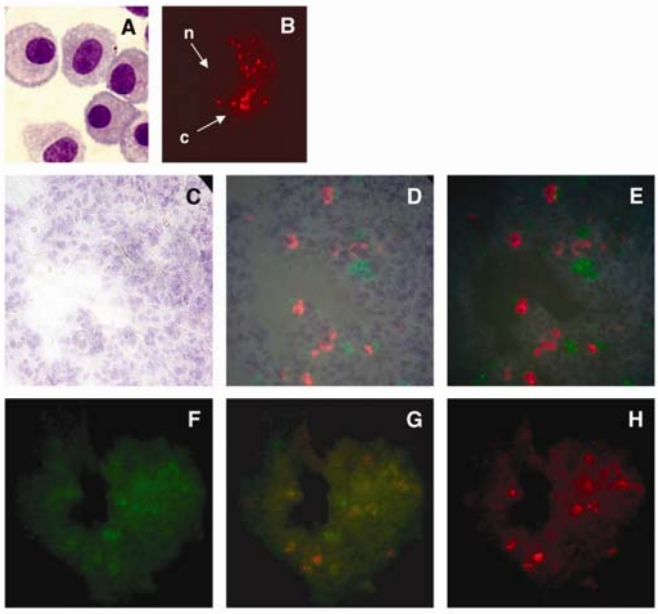

Figure 4. Morphology and localization of CD11b$^-$FITC$^+$ cells in lung tissue. (*A*) C57BL/6 mice were treated with 100 μg FITC-OVA by intranasal instillation, and 7 d later their lungs were harvested and made into single-cell suspensions. The CD11b$^-$CD11c$^+$FITC$^+$ cells were then sorted by FACS, cytospun onto glass slides, stained with Diff-Quik, and examined under a light microscope. Panel illustrates typical morphology of CD11b$^-$CD11c$^+$FITC$^+$ cells. (*B*) As in *A*, with the exception that mice were treated with 100 μg TR-OVA, and sorted cells were observed under a fluorescent microscope. A representative TR$^+$ cell is shown. "n" indicates the nucleus and "c" indicates the cytoplasm of the cells. (*C–E*) C57BL/6 mice were treated with 100 μg TR-OVA by intranasal instillation. Sixteen days later the lungs were harvested, frozen, and stained with Haematoxylin and anti–CD11b-FITC or anti–CD11c-FITC. (*C*) Photomicrograph of frozen section demonstrates an area of lung parenchyma and airway. (*D*) Overlay of hematoxylin and fluorescent image in *E* showing that the majority of the cells are present near the airway. (*E*) Frozen lung section visualized with triple filter (DAPI/FITC/Texas Red) illustrates a cluster of single positive TR-*red* cells and CD11b-FITC single positive *green* cells. Although cells are often in close proximity, no double labeling is observed. (*F*) Anti–CD11c-FITC staining of frozen sections. (*G*) Overlay of fluorescent images in *F* and *H* to show double labeling of TR-red$^+$ CD11c-FITC$^+$ cells, which appear *yellow-orange*. TR-red–CD11c$^+$ cells appear *green*. (*H*) Unstained frozen section to reveal TR$^+$ cells after intranasal administration of TR-OVA.

Microscopic examination of fluorescent frozen lung sections and sections stained with hematoxylin revealed that the fluorescent signal of OVA-TR$^+$ cells was located almost exclusively in the lung periphery. The majority of OVA-TR$^+$ cells were located in the alveoli (Figure 4D), and no large airways were observed in the vicinity of the OVA-TR$^+$ cells. Although the alveolar walls were often compressed, by following the nuclear alignment alveolar lumina could often be appreciated, revealing that the OVA-TR$^+$ cells were located on the luminal side of the basement membrane. OVA-TR$^+$ cells were morphologically and functionally consistent with macrophages, as they were large, were found in large numbers within alveoli, and had phagocytosed OVA-TR. As shown in Figure 4E, the CD11b$^+$ staining did not co-localize with the OVA-TR$^+$ fluorescent signal. CD11b$^+$ cells also appeared large and morphologically consistent with macrophages; however, they were found within the alveoli, as well as between epithelial cells.

CD11b$^-$FITC$^+$ Cells Do Not Up-Regulate Expression of Co-Stimulatory Molecules in Response to LPS Stimulation *In Vivo*

To establish whether CD11b$^-$FITC$^+$ cells might have a role in antigen presentation, the expression of T cell costimulatory molecules was investigated. Mice were treated with FITC-OVA and 10–28 d later the expression of CD80, CD86, and CD40 on FITC$^+$ cells was examined by flow cytometry. At each time point, > 90% of total FITC$^+$ cells were CD11b$^-$CD11c$^+$ (data not shown). These FITC$^+$ cells expressed moderate levels of CD80, low levels of CD86 and undetectable CD40 (Figure 5A).

To determine whether CD11b$^-$FITC$^+$ cells responded to inflammatory stimuli, mice were given FITC-OVA and were treated with 50 μg of LPS intraperitoneally on Day 5. The expression of costimulatory molecules on different populations of lung cells was compared 7 h later. Figure 5B shows that LPS treatment induced up-regulation of CD86 on CD11c$^+$CD11b$^+$ lung DC, but not on CD11b$^-$FITC$^+$ cells or CD11c$^-$CD11b$^+$ macrophages. Some CD11b$^-$CD11c$^+$FITC$^-$ cells also up-regulated CD86 expression in response to LPS, suggesting that there are at least two populations of CD11b$^-$CD11c$^+$ cells in the lung and that they respond differentially to LPS. These two populations of CD11b$^-$CD11c$^+$ cells also differed in intrinsic autofluorescence

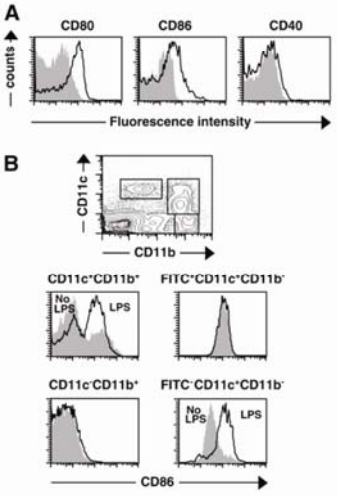

Figure 5. CD11b$^-$FITC$^+$ cells express some T cell costimulatory molecules but do not up-regulate CD86 in response to LPS. C57BL/6 mice were treated with 100 μg FITC-OVA by intranasal instillation, and were killed at the times indicated below. Lung suspensions were prepared and examined by FACS. Dead cells were identified by FSC/SSC and PI staining and were excluded from analysis. (*A*) Representative FACS stainings of lung cell suspensions prepared on Day 14 or 28 after intranasal challenge. *Histograms* show expression of the indicated markers on FITC$^+$ cells, of which > 90% were CD11b$^-$CD11c$^+$. *Open histograms*, cells stained with the indicated antibodies; *solid histograms*, cells stained with an isotype-matched control antibody. (*B*) Representative FACS stainings of lung cell suspensions prepared on Day 6 after intranasal challenge. Some of the mice were injected with 50 μg of LPS given intraperitoneally 7 h before killing. *Histograms* show expression of CD86 in the indicated populations gated as indicated in the *contour plot* at the *top* of the figure. *Solid histograms*, cell populations from untreated mice; *open histograms*, populations from LPS-treated mice.

(data not shown). Similar results were obtained when expression of CD80 and MHC II were examined. $CD11c^+CD11b^+$ lung DC up-regulated CD80 and MHC II expression after *in vivo* LPS stimulation, while $CD11b^-FITC^+$ cells did not (data not shown).

$CD11b^-FITC^+$ Cells Do Not Induce Airway Eosinophilia *In Vivo*

To assess if $CD11b^-FITC^+$ cells were able to induce T cells to secrete cytokines *in vivo*, mice were treated intranasally with FITC-OVA or saline. On Day 7, when the $FITC^+$ population in the lung consisted almost exclusively of $CD11b^-FITC^+$ cells, mice received an adoptive transfer of activated, OVA-specific Th2 cells; intranasal challenge with OVA protein or saline was on Day 8. Figure 6A shows that the BAL fluid of mice challenged with OVA contained large numbers of eosinophils. In contrast, no eosinophilia could be demonstrated in mice that had been treated with FITC-OVA 1 wk before the transfer of Th2 cells. Thus, $CD11b^-FITC^+$ cells were unable to induce IL-5 secretion by activated Th2 cells *in vivo*. In addition, similar numbers of eosinophils were detected in mice pretreated with FITC-OVA or saline, suggesting that $CD11b^-FITC^+$ cells did not suppress the activation of Th2 cells *in vivo*.

We considered the possibility that inflammatory signals might be required to induce the activation of $CD11b^-FITC^+$ cells, or infiltration of activated T cells in the lung tissue. Therefore, mice treated as in Figure 6 were challenged intranasally with 100 ng LPS with or without OVA on Day 7, and lavaged on Day 10. As shown in Figure 6B, no eosinophilia could be demonstrated in mice that did not receive intranasal OVA. It was also possible that $CD11b^-FITC^+$ cells could be located in a compartment of the lung that is not accessible to activated Th2 given intravenously; however, no eosinophils were detected in mice that received activated Th2 cells by intranasal instillation (Figure 6B), despite the presence of some $CD11b^-FITC^+$ cells in the BAL fluid.

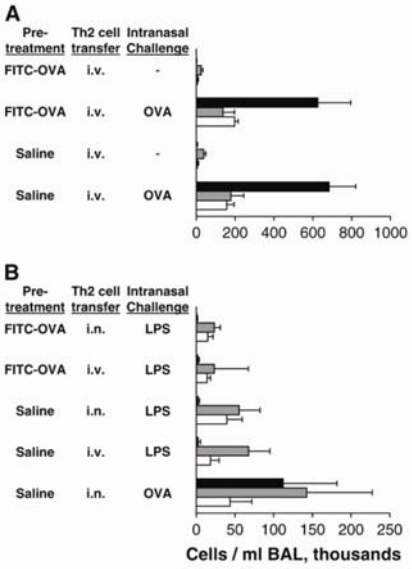

Figure 6. $CD11b^-FITC^+$ cells do not induce or suppress airway eosinophilia *in vivo*. *Solid bars*: eosinophils; *shaded bars*: macrophages; *open bars*: lymphocytes. (*A*) C57BL/6 mice were treated with 100 μg FITC-OVA by intranasal instillation, or saline as a control, and 1 wk later were injected intravenously with 10^7 *in vitro* activated, OVA-specific Th2 cells. One day later, some of the mice received a second intranasal instillation of 100 μg OVA protein. BAL cells were collected 3 d after the second intranasal challenge, counted and spun onto a glass slide. Differential cell counts were performed after Diff-Quik staining. Each *bar* shows the mean ± SEM number of eosinophils, macrophages, and lymphocytes per milliliter of BAL fluid from groups of four mice. (*B*) As in *A*, but Th2 cells were also given intranasally (10^6/mouse), or intranasal challenge was also with 100 ng LPS, as indicated.

$CD11b^-FITC^+$ Suppress Naïve $CD4^+$ T Cell Proliferation *In Vitro*

The ability of $CD11b^-FITC^+$ cells to present antigen was also examined *in vitro*. Mice were treated with FITC-OVA and 1 wk later the $CD11c^+CD11b^+$ and the $CD11b^-FITC^+$ cells were FACS sorted from lung digests, and their ability to present retained FITC-OVA antigen or exogenous $OVA_{323-339}$ peptide to OT-II T cells was tested in a proliferation assay. Figure 7A shows that addition of $OVA_{323-339}$ to cultures containing $CD11c^+CD11b^+$ lung DC resulted in robust T cell proliferation. In contrast, $CD11b^-FITC^+$ cells were completely unable to present their retained antigen or exogenous peptide to specific $CD4^+$ T cells.

To determine whether $CD11b^-FITC^+$ cells were inhibitory to T cells, we tested their ability to suppress the proliferation of OT-II cells to BM-DC and OVA peptide. Mice were treated with FITC-OVA and a week later $CD11b^-FITC^+$ cells were purified from the lungs and titrated into plates containing $OVA_{323-339}$, BM-DC, and T cells from OT-II mice. As shown in Figure 7B, 16 times more $CD11b^-FITC^+$ cells than BM-DC were required to completely inhibit the proliferation of OT-II cells; this inhibition could not be overcome by high doses of IL-2. Inhibition was not antigen-specific since $CD11b^-FITC^+$ cells could also inhibit the proliferation of MOG-specific T cells in response to stimulation with BM-DC and antigen peptide, and T cell proliferation induced by PMA and ionomycin (data not shown).

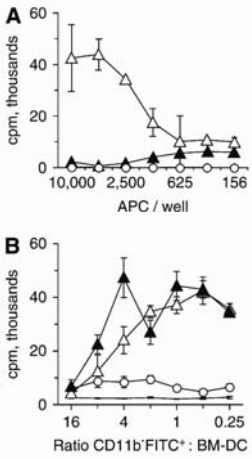

Figure 7. $CD11b^-FITC^+$ cells do not induce the proliferation of OVA-specific OT-II T cells *in vitro*. C57BL/6 mice were treated with 100 μg FITC-OVA by intranasal instillation. One week later, lungs were harvested, made into single-cell suspensions, and $CD11b^-FITC^+$ and $CD11c^+CD11b^+FITC^-$ cells were purified by FACS sorting. (*A*) The indicated numbers of sorted lung cells were incubated with OT-II cells, ± 10 μg/ml $OVA_{323-339}$, for 3 d at 37°C, and thymidine incorporation was measured over the final 8 h of culture. Mean ± SEM cpm for triplicate wells are shown. *Circles*, $CD11b^-FITC^+$ cells with no OVA added, or $CD11c^+CD11b^+$ cells with no OVA added; *solid triangles*, $CD11b^-FITC^+$ cells and $OVA_{323-339}$; *open triangles*, $CD11c^+CD11b^+$ cells and $OVA_{323-339}$. (*B*) Different numbers of sorted lung cells were mixed with 1.5×10^3 BM-DC to obtain the indicated cell ratios. OT-II cells, 10 μg/ml $OVA_{323-339}$, and 100 U/ml IL-2 were added as indicated. Plates were incubated for 3 d at 37°C, and thymidine incorporation was measured over the final 8 h of culture. Mean ± SEM cpm for triplicate wells are shown. *Line at bottom*, OT-II cells only; *circles*, OT-II and $OVA_{323-339}$; *open triangles*, OT-II, BM-DC, $CD11b^-FITC^+$ cells, and $OVA_{323-339}$; *solid triangles*, OT-II, BM-DC, $CD11b^-FITC^+$ cells, $OVA_{323-339}$, and IL-2.

DISCUSSION

Phenotype of CD11b$^-$FITC$^+$ Cells

In this report, we describe the identification and characterization of a population of lung CD11b$^-$CD11c$^+$ cells that retain inhaled FITC-OVA for longer than 8 wk after administration. While it is known that a population of CD11b$^-$CD11c$^+$ cells able to take up antigen resides in the lung (5), the ability of these cells to store antigen for many weeks had not, to our knowledge, been reported.

The identity of CD11b$^-$FITC$^+$ cells was not firmly established. Their morphology was similar to macrophages, and immunohistologic analysis also revealed that the predominant location of CD11b$^-$FITC$^+$ cells near alveoli and on the luminal side of the basement membrane was typical of a subpopulation of macrophages. Intracytoplasmic punctate deposits of fluorescent material were prominent in CD11b$^-$FITC$^+$ cells. Thus, the accumulation of presumably undigested material in these cells would suggest that at least some of their properties differ from those of typical macrophages, which rapidly degrade internalized proteins (20), and are more reminiscent of immature DC (21).

Previous studies have also identified lung CD11c$^+$CD11b$^-$ as macrophages, including alveolar macrophages and a resident population of lung macrophages (12, 22, 23). Alveolar macrophages have previously been described to have a slow turnover (24, 25) and sequester particulate antigen (7), but their ability to retain antigen has not been reported and is not consistent with their proposed function of removing debris. Phenotypic analysis revealed that our CD11b$^-$FITC$^+$ cells also expressed the DEC205 marker, but were CD40$^-$B220$^-$Gr-1$^-$, which is again consistent with the phenotype assigned to alveolar macrophages in two (22, 23), but not one (12) of those earlier studies. The phenotype of CD11b$^-$FITC$^+$ cells was not consistent with conventional DC, which express the CD11b marker, or plasmacytoid DC, which express Gr-1 and B220 (26). However, it is interesting to note that initial studies where the expression of DEC205 by alveolar macrophages was first reported (27) proposed that alveolar macrophages might, in some conditions, give rise to DC. In keeping with this proposal, other authors have used a model of lung infection with *Yersinia pestis* to show that a population of CD11c$^+$CD11b$^-$DEC205$^+$ cells was able to take up bacteria, acquire expression of CCR7, and migrate from the lung tissue to the draining lymph node (28), all properties that are normally associated with DC. Thus, our CD11b$^-$FITC$^+$ cells resemble a population of lung cells whose surface markers and reported properties are intermediate between macrophages and DC.

Antigen-Presenting Function of CD11b$^-$FITC$^+$ Cells

Despite the expression of moderate levels of MHC II and costimulatory molecules, CD11b$^-$FITC$^+$ cells appeared unable to present peptide or protein antigen to naïve CD4$^+$ T cells *in vitro*. We obtained no evidence that CD11b$^-$FITC$^+$ cells present their retained OVA to activated Th2 cells *in vivo,* and attempts to enhance presentation and migration to the airways using LPS were also unsuccessful. This lack of activity might not be surprising given the apparently slow degradation of ingested materials by these cells. Failure to activate Th2 cells *in vivo* in the lung could also reflect the use of limiting amounts of FITC-OVA for the intranasal instillation of antigen. While the dose used (100 μg/mouse) is usually sufficient to elicit a strong response (18), this is in conditions in which the CD11c$^+$CD11b$^-$ and CD11c$^+$CD11b$^+$ populations are both loaded with antigen. It is possible that higher amounts of antigen might be necessary to reveal presentation by CD11c$^+$CD11b$^-$ cells alone.

One previous report by Julia and coworkers documented the ability of a BAL CD11c$^+$CD11b$^+$ DC population to stimulate antigen-specific cells for several weeks after antigen exposure (6). The cell population described in that study differs from the population described here in both the expression of CD11b and in the ability to support cytokine secretion by T cells. In addition, Julia and colleagues identified their cells of interest only on the basis of stimulatory function, and their results are equally consistent with DC being long-lived, or DC taking up antigen from another long-lived population of cells. In our study, cells were directly identified on the basis of retention of fluorescent antigen, allowing us to conclude that the CD11b$^-$CD11c$^+$ cells were loaded with antigen and long-lived. Together, the two studies suggest that a long-lived population of CD11b$^-$CD11c$^+$ lung cells might act as a reservoir of inhaled antigen, which can become stimulatory for T cells if taken up and presented by neighboring lung DC (29, 8, 30), or under conditions that support the differentiation of CD11b$^-$CD11c$^+$ cells into DC.

An alternative possibility is that CD11b$^-$CD11c$^+$ cells may not be stimulatory, but promote tolerance and downregulate local immune responses to protect the fragile lung microenvironment (9). *In vivo*, pre-loading CD11b$^-$FITC$^+$ cells with OVA did not appear to inhibit cytokine production by activated Th2 cells after intranasal OVA challenge. CD11b$^-$FITC$^+$ cells did exhibit some suppressive activity *in vitro*; however, their inhibitory capacity was not particularly potent. Alveolar macrophages can suppress the activity of APC and the proliferation of T cells by producing NO (9, 31), and this inhibition can be reversed by stimulation with PMA or by addition of IL-2 (32, 9). Neither addition of IL-2, nor stimulation with PMA-ionomycin (data not shown) could reverse the suppression induced by CD11b$^-$FITC$^+$ cells, suggesting that a different mechanism was involved. Cytokines such as TGF-β1 and IL-10 have also been reported to inhibit immune responses in the lung (33, 34); however, secretion of these cytokines by CD11b$^-$FITC$^+$ cells was not investigated.

We show in this paper that CD11b$^-$CD11c$^+$ cells remain fluorescent for extended periods after FITC-OVA administration; this was also observed after administration of OVA conjugated to other fluorochromes such as TR or Alexa-Fluor488 (data not shown). Each of these OVA conjugates was rapidly and completely degraded by other cell types, such as for example the CD11c$^+$CD11b$^+$ DC. This suggests that the presence of fluorescence was a marker of incompletely degraded OVA within these cells, as complete protein degradation would be expected to lead to a loss of fluorescent signal.

CD11b$^-$FITC$^+$ Cells in Pulmonary Disease

The apparent limited antigen degradation and processing in CD11b$^-$FITC$^+$ cells could make them attractive targets for intracellular bacteria that reside in endocytic vacuoles, such as mycobacteria. Alveolar macrophages and DC are the favored host cells for mycobacteria, which are able to survive in phagosomes by preventing their acidification and fusion with lysosomes. Interestingly, in this paper we show that CD11b$^-$FITC$^+$ cells do not readily process and present engulfed antigen, suggesting that they could potentially be exploited by infectious agents that do not possess specific immune evasion mechanisms. This may provide an additional explanation for ability of some bacteria to persist inside cells of the immune system.

A second possibility is that CD11b$^-$FITC$^+$ cells might be specialized cells that gradually process stored antigen and present it, directly or indirectly, to resident T cells, modulating their function without inducing full activation. Such low-level stimulation could favor the retention of effector T cells in tissue and the maintenance of protective immunity (35), or possibly prevent T cell activation in conditions of limiting antigen. Memory Th2

cells and lymphocytic infiltrates containing $CD4^+$ T cells have been demonstrated in the lungs of mice more than 400 d after the induction of acute allergic airway disease (13). Experiments are currently in progress to assess whether the lymphocytic infiltrates are in close proximity to $CD11b^-FITC^+$ cells.

In summary, we report a cell population in the airways of mice that is able to retain fluorescent material for extended periods of time. This long-term depot of antigen could be important in modulating immune responses to inhaled antigens. Further work is required to determine the normal function of these cells in different inflammatory or infectious situations.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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