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Functional Dynamics of Resident Alveolar Macrophages in a Mouse Model of Allergic Asthma

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Thomas

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AEC	Alveolar epithelial cells
AHR	Airway hyperreactivity
APC	Antigen-presenting cell
ATP	Adenosine triphosphate
BALT	Bronchoalveolar lymphoid tissue
BMDM	Bone-marrow derived macrophages
CAP	Community-acquired pneumonia
CC	Clara cell
COPD	Chronic obstructive pulmonary disease
CR	Complement receptor
CTL	Cytotoxic T-lymphocyte
DAMP	Danger-associated molecular pattern
DC	Dendritic cell
EB	Elementary body
EBP	Eosinophil basic protein
EMT	Epithelial-mesenchymal transition
FcR	Fc receptor
FEV1	Forced expiratory volume in one second
GC-BP	GC-binding protein
GM-CSF	Granulocyte-macrophage colony stimulating factor
HP	Hypersensitivity pneumonitis
ICAM	Intercellular adhesion molecule 1
ICOS	Inducible co-stimulator
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IFNAR	Interferon α receptor
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible NO-synthase
ip	Intraperitoneal
LDLN	Lung draining lymph nodes
LT	Leukotrien
MARCO	Macrophage receptor with collagenous structure
MBL	Mannose-binding lectin
MCP	Monocyte-chemoattractant protein
M-CSF	Macrophage colony stimulating factor
MGL	Macrophage galactose-type C-type lectin
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinase
MMR	Macrophage mannose receptor

MOMP	Major outer membrane protein
NADPH	Nicotin amide dinucleotide phosphate
NF-κB	Nuclear factor κB
NK	Natural killer
NO	Nitric oxide
NS	Nonstructural
OAS	2',5'-oligoadenylate synthase
OVA	Ovalbumin
PDL	Programmed-death ligand
PEF	Peak expiratory flow
PG	Prostaglandin
PRR	Pattern recognition receptor
PS	Phosphatidyl serine
PSR	Phosphatidyl serine receptor
rAM	Resident alveolar macrophages
RANTES	Regulated upon activation normal T-cell expressed
RB	Reticulate body
RLH	Retinoic acid -inducible gene-like RNA helicase receptor
ROS	Reactive oxygen species
RSV	Resipratory syncytial virus
SP	Surfactant protein
SR	Scavenger receptor
STAT	Signal transducer and activator of transcription
TARC	Thymus and activation regulated chemokine
TCR	T-cell receptor
Th	T helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
WHO	World health organization

PART I

INTRODUCTION

Chapter 1

Pulmonary Inflammation

1.1 Anatomy and histology of the human respiratory system

The respiratory system is the anatomical system of an organism that introduces respiratory gases to the interior and performs gas exchange between the external environment and the organism's circulatory system. In humans and mammals, the respiratory system consists of two major compartments; the upper and the lower respiratory tract. The upper respiratory tract refers to the part of the respiratory system lying outside of the thorax and consists of the oral and nasal cavity, the pharynx and the larynx. The components of the lower respiratory tract, all surrounded by the thorax, include the trachea, the primary bronchi and the lungs. The human lung is a multilobular organ, with the left lung being divided into two lobes and the right into three lobes. The lung connective tissue exhibits high levels of elastin in order to resume its shape after stretching or contracting during inhalation and exhalation respectively. The lungs in turn comprise the secondary and tertiary bronchi, the bronchioles, and the alveoli. The alveolar sacs form the termination point of the respiratory tract. In this alveolar region, molecules of oxygen and carbon dioxide are passively exchanged by diffusion between the external environment and the blood of the organism. A typical pair of human lungs contains 700 million alveoli, providing a total surface of about $100m^2$. The alveolar cavity is surrounded by an epithelial cell layer, embedded in an extracellular matrix and wrapped in a fine mesh of capillaries. The alveolar epithelial barrier consists of two major epithelial cell types. More than 95% of the alveolar surface area is covered by type I (squamous) alveolar epithelial cells (AECs). This cell population forms the structure of the alveolar cell wall and is responsible for the gas exchange function. Type II (cuboidal) AECs are typically found at the alveolar-septal junction and are responsible for the production and secretion of pulmonary surfactant. Pulmonary surfactant represents a mixture of proteins and phospholipids (mostly dipalmitoylphosphatidylcholine) that reduces the alveolar surface tension in order to increase compliance which allows the lung to inflate much more easily, thereby eliminating the work of breathing. The phospholipid components of the pulmonary surfactant are stored in lamellar bodies of the type II AECs and are released into the alveolar lumen from the infant's first breath on. A schematic overview of the anatomy and histology of the human respiratory system is depicted in figure 1.

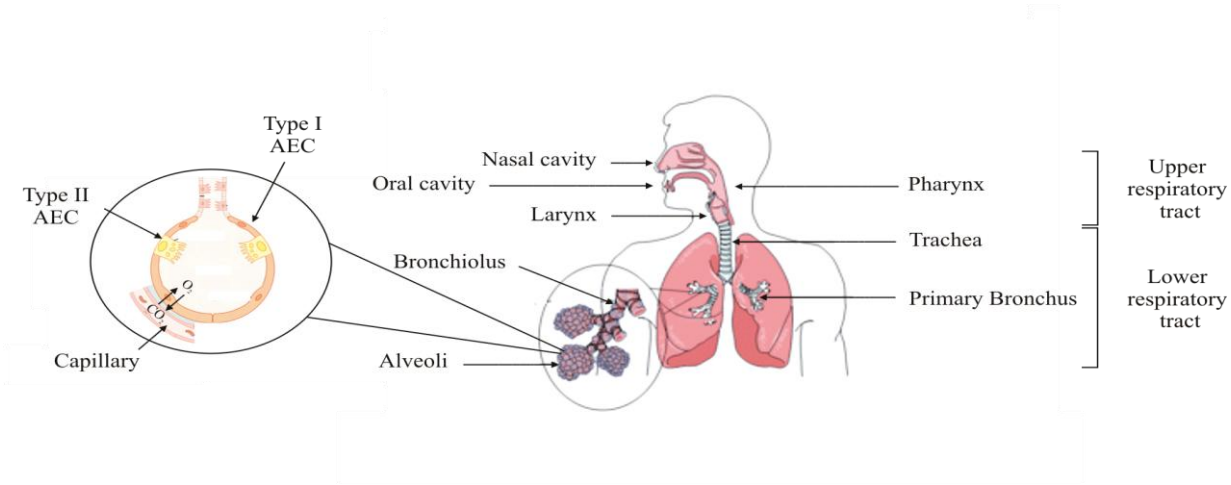

Figure 1: Schematic representation of the human respiratory system (adapted from www.bioedonline.org and [1]). The human respiratory system consists of two major parts: the upper and the lower respiratory tract. Fresh oxygen-rich air enters the body through the oral and/or nasal cavity. Via pharynx, larynx, trachea, and primary bronchi, the inhaled air reaches the lungs. Inside the lungs, the bronchi are further branched into bronchioles. Finally, the bronchioles terminate in alveoli where oxygen from the inhaled air is exchanged for carbon dioxide from the surrounding blood capillaries. The alveolar wall consists of two major airway epithelial cell types. The type I (squamous) AECs, which cover more than 95% of the total alveolar surface, are responsible for the gas exchange. On the other hand, the type II (granular, cuboidal) AECs produce and secrete pulmonary surfactant.

Structural differences between the human and mouse respiratory system

Much of our current understanding of the normal functioning of the lung and mechanisms of lung disease comes from studies utilizing animals. Mice are now widely employed in lung research because of certain advantages this species is thought to provide. However, there are several anatomical differences between the human and mouse respiratory system to consider when using mice as a research platform to study human pathology. Besides the obvious difference in size (the lung capacity of the mouse is approximately 1 ml, which is around 6000 times smaller than that of a human), the anatomy of mouse and human lungs differs profoundly in the number of lung lobes as well. Mice have four right but a single left lung lobe while humans have three on the right and two on the left side. Furthermore, mice have a very rapid but monopodial airway branching into alveolar ducts with relatively few airway generations (13 – 17). In contrast, the human respiratory system displays a dichotomous branching of the airways which extends up to 23 generations before ending in alveolar structures. Compared to the human case, mouse alveoli are relatively small and show a rather thin blood-gas barrier. Together, these differences result in an altered penetration range of inhaled particles, which can influence the outcome of the airway response because of the different cell types that encounter the particles. An important functional difference between

mouse and human lungs is the paucity of submucosal glands and the high numbers of Clara cells. The significance of all the anatomical differences between mouse and human lungs are still unknown although it has been speculated that the mouse lung structure contributes to the high baseline airway resistance observed in these animals. This suggests that inflammatory processes that could compromise lung function in larger animals like humans, might have little effect in mice [2].

1.2 Respiratory diseases: one name, many faces

Respiratory diseases are a common and important cause of illness and death around the world. Common cold is probably the most widespread illness known, with each year more than 62 million reported cases in the U.S.A. (http://www.niaid.nih.gov). Respiratory diseases can be classified in many different ways, such as by the organ or tissue involved, by the type and pattern of associated symptoms, or by the aetiology of the disease. According to the topological classification, respiratory diseases are divided into two major categories: upper and lower respiratory tract diseases. Upper respiratory tract diseases are usually of infectious or allergic nature and often induce rhinitis (inflammation of the nasal mucosa), sinusitis (inflammation of the nares and paranasal sinuses) and laryngitis (inflammation of the larynx). Lower respiratory tract diseases are generally considered as more serious than upper respiratory tract diseases and always affect the lungs. During the acute phase of the disease, pulmonary inflammation is often the main symptom. However, during the chronic phase of the pathology, pulmonary inflammation may be accompanied by lung tissue destruction or remodelling and airway hyperreactivity (AHR), both features leading to respiratory problems which can acquire life-threatening proportions.

Pulmonary inflammatory responses: overview and classification

The air is filled with a variety of (pathogenic) microorganisms, possible allergens, harmful gases and noxious particles. The lungs are exposed to this melting pot of potential danger signals each time we breathe and therefore require a robust and sophisticated immune defence system. A failure to tightly control immune responses to pathogens or foreign particles can result in chronic inflammation and tissue destruction due to an excessive and deleterious response. Depending on the type of inflammatory stimulus, pulmonary diseases and

inflammations are roughly divided into two categories: infectious and non-infectious inflammatory diseases. Pulmonary infectious inflammatory diseases are the leading cause of deaths among all infectious diseases and accounted in 2008 for 3.46 million deaths worldwide (6.1% of all deaths) (www.who.int). Pulmonary inflammatory conditions of infectious origin are generally referred to as pneumonia and depending on the nature of the infectious agents, bacterial, viral, fungal and parasitic pneumonia are distinguished. Bacterial pneumonia is the most common cause of community-acquired pneumonia with *Streptococcus pneumoniae* isolated in nearly 50% of the cases [3]. Other bacterial pathogens, including *Haemophilus influenza* (20%), *Chlamydophila pneumoniae* (13%), and *Mycoplasma pneumoniae* (3%) are frequently isolated as well [4]. Bacteria typically enter the lungs with inhalation. However, bacteria can also reach the lung through the bloodstream if other parts of the body are infected. Often, they live in parts of the upper respiratory tract and are continuously being inhaled into the alveoli. Once inside the alveoli, bacteria trigger an inflammatory immune response via the initial activation of local resident immune cells, including resident alveolar macrophages and AECs. In the acute, innate immune response, neutrophils and monocytes are recruited to the lungs in order to engulf and kill the offending bacterial organism. In the adaptive phase of the immune response, T-helper (Th)1- and Th17-cells are the major local effector T-cells. Especially in the case of intracellular bacterial infections like *Mycobacterium tuberculosis*, an additional supportive cytotoxic $CD8^+$ T-cell response is present. Main cytokines associated with bacterial infections are interleukine (IL)-12 and interferon (IFN)-γ. Humoral B-cell responses lead to the production and systemic presence of immunoglobulin (Ig)G1 and IgG3 isotypes.

In adults, viral pneumonia accounts for approximately one third of the pneumonia cases. Commonly implicated viral agents include rhinoviruses, coronaviruses [5], influenza virus, and respiratory syncytial virus (RSV) [6]. Typically, viruses will reach the lungs by travelling in droplets through the mouth and nose during inhalation. In the alveolar lumen, the virus invades the cells lining the alveoli. This invasion often leads to cell death either through direct killing by the virus or by self-destruction through apoptosis. Pulmonary invasion of viruses leads to the rapid recruitment of natural killer (NK)-cells to the lungs. NK-cells recognize the virus-infected cells and induce apoptosis in these cells in order to eliminate the intracellular virus. A cytotoxic $CD8^+$ T-cell response, often accompanied by a Th1-cell response, is elicited to establish further clearance of the virus from the lungs. The typical key regulator

cytokine found during pulmonary viral infections is IFN-γ. Again mainly IgG1 and IgG3 are found in the serum of patients suffering of viral pneumonia.

Finally, fungal and parasitic pneumonia are two rare types of pneumonia and generally occur in immunecompromised patients like HIV-patients or patients receiving immunosuppressive drug therapy. Pulmonary infection with fungi elicits a monocytic and neutrophilic innate immune response in order to engulf and kill the pathogen. Fungal infections are associated with a predominant Th1- and Th17-cell response, accompanied by IFN-γ production and the systemic presence of IgG1 and IgG3 isotypes in the serum. Pulmonary parasitic infections induce a rapid recruitment of monocytes. Subsequently, a Th2-cell response is mounted which is typically accompanied by the recruitment of eosinophils and mast cells. Both cell populations release toxic molecules through degranulation of intracellular granules in order to kill the parasite. Th2-cell responses are associated with local IL-4 and IL-13 production and systemic IgG2 and IgE humoral B-cell responses.

Pulmonary non-infectious inflammatory diseases arise from pulmonary exposure to environmental agents like chemical gases, ultrafine particulate matter or airborne allergens, and often require some levels of genetic predisposition. While the prevalence of pulmonary infectious diseases is still the highest in developing countries, pulmonary non-infectious diseases are most common in industrialized countries. This category of disease currently affects hundreds of millions of people worldwide and takes a serious bite out of the healthcare budget (www.who.int). The two worldwide leading pulmonary non-infectious inflammatory diseases are chronic obstructive pulmonary disease (COPD) and asthma. In these diseases, the inflammatory component generally fulfils a prominent role in the development of secondary symptoms. COPD is caused by noxious particles or gases, most commonly from tobacco smoke, which trigger inflammatory responses in the lung. The cellular composition of the COPD-associated inflammation consists of a mixture of monocytes, neutrophils, $CD8^+$ T-cells and Th1- and Th17-cells. Hallmark cytokines found in the lungs of COPD patients are IFN-γ and IL-17. Also serum IgG is detected. Due to the persisting inflammatory condition, COPD patients eventually develop structural lung damage and remodelling which in turn result in chronic cough, wheezing and dyspnoea [7].

Asthma actually represents a heterogeneous group of chronic pulmonary diseases with variable aetiologies, phenotypes and symptoms. In 80% of the cases, asthma is allergy-based and is characterized by the presence of a chronic pulmonary inflammation. Generally, two

more or less opposite types of allergic pulmonary inflammation are observed. On the one hand, an eosinophilic Th2-cell response accompanied by the presence of the Th2-cell hallmark cytokines, IL-4 and IL-13 and the systemic presence of IgG2 and IgE isotypes. On the other hand, a neutrophilic Th1- and Th17-cell response can develop which is then accompanied by IFN-γ and IL-17 and the systemic titers of IgG1. In both cases, persistence of the pulmonary inflammation results in structural lung damage and remodelling, eventually leading to recurrent episodes of wheezing, dyspnoea and AHR [8]. A more comprehensive and detailed discussion of the asthmatic pathology is described in chapter 3 of the thesis. A summary of the main features of the discussed respiratory inflammatory diseases is shown in table 1.

	Incidence	Aetiology (trigger)	Leukocyte protagonists	Cytokines/ Antibodies	Chronic symptoms	Treatment
Bacterial pneumonia	10% of hospital admissions in US 3 million deaths annually	Bacteria: *S. pneumoniae* *H. influenza*	Neutrophils Monocytes Th1-cells	IFN-γ IgG1/3	Fever Cough Dyspnoea	Antibiotics: carbapenem cephalosporins
Viral pneumonia	200 million cases annually	Virus: *Influenza* *RSV*	Neutrophils Monocytes $CD8^+$ T-cells	IFN-γ IgG1/3	Fever Cough Dyspnoea	Antiviral drugs: oseltamivir ribavirin
Fungal pneumonia	Opportunistic in immuno-compromised patients	Fungi: *H. capsulatum* *C. Neoformans*	Neutrophils Monocytes Th1-cells	IFN-γ IgG1/3	Fever Chills Fatigue	Antifungal drugs: polyenes imidazoles
Parasitic pneumonia	Opportunistic in immuno-compromised patients	Parasites: *T. gondii* *S. stercoralis*	Eosinophils Mast cells Th2-cells	IL-4/13 IgG2/4 IgE	Fever Chills Fatigue	Antibiotics: pyrimethamine piperazine
COPD	30 million in the US	Noxious particles or gases (mostly tobacco smoke)	Neutrophils Monocytes Th1/17-cells $CD8^+$ T-cells	IFN-γ IL-17 IgG1	Wheezing Cough Dyspnoea	Bronchodilators Corticosteroids
Asthma	300 million affected world-wide	Environmental and genetic factors	Eosinophils Mast cells Neutrophils Th2/1/17-cells	IL-4/13/17 IFN-γ IgG1/2/3/4 IgE	Wheezing Cough Dyspnoea	Bronchodilators Corticosteroids

Table 1: Overview of the main features of the most common infectious (pneumonia) and non-infectious pulmonary inflammatory diseases in humans.

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Chapter 2

Alveolar Macrophages: The Good, The Bad and The Ugly

2.1 Origin and development

Resident alveolar macrophages (rAM) are the most abundant cells in the alveolar spaces and conducting airways of healthy individuals and serve as important sentinels in the recognition of invading pathogens and apoptotic cells. Just like all other resident macrophage phenotypes throughout the body, rAM arise from blood circulating monocytes that populate tissues under steady-state conditions [1]. The macrophage and dendritic cell precursor in the bone marrow gives rise to $GR1^{low}CX_3CR1^{high}CCR2^{-}$ and $GR1^{high}CX_3CR1^{low}CCR2^{+}$ monocyte populations. The proposed model is that cells from the $GR1^{low}CX_3CR1^{high}CCR2^{-}$ monocyte subset are released from the bone marrow into the circulation. This monocyte phenotype responds to pro-inflammatory stimuli and migrates and differentiates into macrophages in the inflamed site. In the absence of inflammation, an unknown regulatory mechanism generates $GR1^{low}CX_3CR1^{high}CCR2^{-}$ monocytes that are postulated to enter the tissues and replenish the tissue-resident macrophage populations [2]. The differentiation of $GR1^{low}CX_3CR1^{high}$ $CCR2^{-}$ monocytes into rAM however, requires an obligate intermediate stage: the development of blood monocytes into parenchymal lung macrophages, which subsequently migrate to the alveolar space [3]. This observation was supported by a comparative genetic profiling study which determined that rAM were phenotypically less related to the monocyte population in comparison to DCs or interstitial macrophages [4]. By abolishing the dependency on the recruitment of blood precursors, the existence of local precursors as reservoir for rAM allows a tight control and prompt adjustment of their numbers. These differences also might correlate with the different functional patterns interstitial macrophages and rAM perform in the lung and reflect the specialization of individual macrophage populations within their microenvironment [5]. Furthermore, Landsmann and Jung provided direct evidence for the ability of both interstitial macrophages and rAM to proliferate. Therefore self-renewal acts also as an important mechanism for replenishing the rAM pool [3]. The primary cytokine governing the development and maturation of rAM during normal homeostasis is granulocyte macrophage-colony stimulating factor (GM-CSF). Although GM-CSF also promotes the monocytic and granulocytic progenitor cell growth, differentiation and activation [6, 7], GM-$CSF^{-/-}$ and GM-CSF $receptor^{-/-}$ mice showed reduced rAM numbers and maturation and exhibited rAM dysfunction while the general hematopoiesis and myelopoiesis was not disturbed [8, 9]. Thus, GM-CSF fulfils a nonredundant and critical role in the rAM development and pulmonary homeostasis.

In steady-state, replenishment of the rAM pool was found to be very slow with turnover rates of only up to 40% over a period of one year [10]. However, upon the induction of a self-limiting lung inflammation in response to LPS or *Streptococcus pneumoniae* infection, rAM turnover was significantly accelerated with at least 50% of the rAM population being replaced by recruited macrophages one week post-challenge [10, 11]. These findings suggest that exudate but not resident AM play an important role in re-establishing alveolar and lung homeostasis.

2.2 Activation of (alveolar) macrophages

Just like other resident macrophage phenotypes, rAM exhibit unique activation patterns upon exposure to cytokines and/or Toll-like receptor (TLR) agonists. At least 2 major functionally distinct activation statuses of macrophages have been extensively studied and these are the classical (M1) and alternatively (M2) activated macrophage activation status. M1 macrophages are induced by pro-inflammatory molecules such as interferon-γ (IFN-γ) and lipopolysaccharide (LPS) and exhibit a crucial role in the elimination of various (intracellular) pathogens in both mice and humans. They possess antimicrobial, anti-proliferative and cytotoxic properties via the production of nitric oxide (NO) and the secretion of pro-inflammatory cytokines such as tumor necrosis factor α (TNF-α) and interleukin (IL)-6 [12]. Prototypical T helper (Th)2-type mediators, such as IL-4 and IL-13, antagonize M1-differentiation and are involved in the development of alternative activation of macrophages [12]. In M2-macrophages, inducible NO synthase (iNOS), catalyzing the production of NO and L-citrulline from L-arginine, is suppressed. Instead, M2-macrophages are characterized by an alternative metabolic pathway of arginine in which arginase converts L-arginine to L-ornithine and urea [13]. M2-macrophages were initially ascribed an anti-inflammatory function because of the secretion of anti-inflammatory cytokine such as transforming growth factor-β (TGF-β) and IL-10 [12], and because of their contribution to wound healing [14] and angiogenesis [15]. At present however, M2-macrophages have also been identified as pro-inflammatory or malignant mediators in the development of a range of pathologies like parasitic infections, hypersensitivity, tumorigenesis and allergy, and their sequelae such as fibrosis [16]. In addition to the classical M1- and M2-macrophage stimuli, also IL-10, TGF-β and immune-complexes are involved in skewing the macrophage activation program [17, 18], thus demonstrating the functional plasticity of these innate cells. rAM are considered alternatively activated, based upon their main biological attributes. These include high basal expression levels of alternative

markers like macrophage mannose receptors (MMR) [19], scavenger receptor A (SR-A) [20], macrophage-galactose type C-type lectins (MGLs)hhhh and the secretory lectin Ym [21] along with low basal phagocytic activity [22].

2.3 The alveolar macrophage: The Good

The function of the lung is to allow the uptake by the body of oxygen and the excretion of carbon dioxide. Gas exchange occurs in the lung alveoli, which are made up of a thin layer of type I alveolar epithelial cells (AECs) interlaced with more cuboidal type II AECs that produce surfactant and have self-renewal and differentiation potential. Lung capillaries are situated in close approximation to the type I cells, separated only by a 0.2 μm thick fused basement membrane, allowing the easy diffusion of gas. With its large surface area, the lung is highly exposed to environmental challenges and is a portal of entry for pathogens and particulate matter present in the air. Yet, the immune defence of this fragile barrier needs not only to be highly effective but also tightly controlled. Clearly, excessive inflammatory reactions damaging the alveolar-capillary wall entail the risk of compromising the gas exchange function of the lung and hence may endanger the survival of the individual. rAM are considered to be a key component in this fine-tuned balance between efficacy and inflammatory tissue damage. In this section, functional aspects of pathogen clearance and immune suppression by the rAM population, being the good cop for the host, are discussed.

2.3.1 Eradication of respiratory bacterial infections

Bacterial infections of the respiratory tract are one of the most common causes of human disease. As the resident phagocyte of the alveolar cavity, rAM are the first effectors of the innate response against bacteria that spread to the distal airways. Once in the alveolar lumen, microbes are readily engulfed by rAM. Being professional phagocytes rAM express a broad spectrum of receptors that participate in the bacterial recognition and internalization. However, since many common respiratory pathogenic bacteria are encapsulated by a polysaccharide layer, opsonisation of the bacteria by immunoglobulin (Ig), complement, and other pulmonary opsonins is important for efficient phagocytosis [23]. Fcγ receptors (FcγRs) and FcαRs, which are abundantly expressed on the surface of AM, bind IgG- and IgA-

opsonised bacteria via the Fc region of the IgG- and IgA-molecule respectively. Complement proteins can opsonise bacteria through antibody-dependent or antibody-independent mechanisms, and complement-opsonised bacteria are recognized and internalized via specific complement receptors (CR). Unlike other macrophages, rAM display CR3 as well as CR4. Both receptors recognize and bind complement protein iC3b. In addition, rAM have high basal expression of SR-A [20]. This pattern recognition receptor (PRR) binds whole bacteria as well as the microbial cell wall components, lipoteichoic acid and LPS [24, 25]. The expression of macrophage receptor with collagenous structure (MARCO), another member of the class A scavenger receptor family, is induced in rAM by exposure to bacterial components, such as LPS and binds to a variety of (non-opsonised) particles including Gram-positive and Gram-negative bacteria [26].

In addition to these generic opsonisation mechanisms, the alveolar cavity contains two compartment-specific opsonin systems; IgA and the pulmonary surfactant. The pulmonary surfactant consists of a complex of lipids and proteins lining the alveolar surface. Next to lowering the surface tension at the air-liquid interface in order to prevent alveolar collapse at the end of expiration, pulmonary surfactant proteins are an integral component of the lung's innate immune system. Both *in vitro* and *in vivo* studies showed that SP-A and SP-D enhance the uptake of particles and bacterial pathogens by direct and indirect mechanisms. Previous research established the binding of SP-A to the lipid A moiety of LPS [27], to desaturated phosphatidyl glycerol [28], to the toxin MPN372 [29, 30] on the surface of *Mycoplasma pneumoniae* and to the Apa and Eap adhesins on *Mycobacterium tuberculosis* [31] and *Staphylococcus aureus* [32]. SP-D interacts with heptose in the inner core oligosaccharide and mannose in O-antigen carbohydrate chains of LPS [33, 34]. Otherwise, SP-A and SP-D may indirectly enhance the phagocytosis of bacteria by upregulating the expression of cell-surface receptors that are involved in microbial recognition. For example, SP-A augments the uptake of *Streptococcus pneumoniae* [20] and *S. aureus* [32] by increasing the cell-surface expression of SR-A on rAM. Several receptors for the opsonising activities of SP-A and SP-D have been identified on the surface of rAM. These include the CD91 complex and the SP receptor 210. A host of studies in humans and animal models indicated that the interaction between SP and these SP receptors on the rAM is critical for the phagocytosis of respiratory bacterial pathogens like *S. aureus* [32, 35-37] and *Mycobacterium sp.* [38, 39].

IgA is the most abundantly produced immunoglobulin isotype in the body [40]. The majority of IgA in the lung is secreted by plasma cells that are densely distributed at the mucosal subepithelium of the airway. These mucosal plasma cells originate mostly from homing IgA-committed B-cells which undergo μ to α class switching at inductive sites of musosal immunity, like bronchoalveolar associated lymphoid tissue (BALT) [41]. IgA-molecules neutralize inhaled bacteria by interfering with their motility or by inhibiting their adherence to the target cells [42]. Subsequently, IgA-coated bacteria interact, through the Fc portion of IgA-molecules, with IgA-specific Fc high affinity receptors, FcαR (CD89). FcαR is expressed on a variety of leukocyte populations, such as rAM, in association with the common FcR γ-chain homodimer [43]. The FcR γ-chain is recognized as a signalling molecule which, upon cross-linking of the FcαR, triggers several biological responses, including phagocytosis and killing of the IgA-opsonised bacterial pathogen [44].

Bacterial internalization is accompanied by the induction of highly efficient antimicrobial mechanisms. As professional phagocytes, rAM produce NO and ROS, like superoxide ions [45]. Oxygen radicals and NO are still considered as major players in the rAM's microbial elimination process. *In vivo* models using mice lacking inducible NO synthase (iNOS), the main source of high-output NO generation in macrophages, demonstrated that rAM-derived NO delivers a crucial contribution to the anti-pneumococcal host defence [46]. Also, *in vivo* treatment of mice with the NO inhibitor, L-NAME, impaired the host defence against *Klebsiella pneumoniae* [47]. However, several studies also highlighted the existence of antimicrobial activity which is not dependent on NO and/or ROS generation. Thus, although NO has been shown to play an important role in the control of microbial proliferation such as in the case of *M. tuberculosis*, Scanga and co-workers found that NO production by itself is not sufficient to control this pathogen [48]. Also in low-dose pneumococcal infection models, decreased expression of NO did not influence bacterial clearance. The same conclusions were true for mice with diminished ROS generation as a result of mutations in the NADPH oxidase complex [49]. These observations indicate that rAM use overlapping antibacterial strategies. Indeed, in addition to these oxidative mechanisms of bactericidal activity, bacterial killing may also result from nutritive antibacterial mechanisms like tryptophan depletion or iron sequestration from the cellular compartment occupied by the bacteria [50]. Recently, Houghton and co-workers provided the first evidence for a direct antimicrobial activity of the rAM-derived matrix metalloproteinase (MMP)-12 in a mouse model of *S. aureus*. Intracellular stores of MMP-12 are mobilized to phagolysosomes after the ingestion of the bacterial

pathogens. Once inside the phagolysosomes, MMP-12 adheres to bacterial cell walls where it disrupts cellular membranes resulting in bacterial death [51]. The presence of multiple redundant recognition and defence mechanisms renders the rAM highly effective in fighting bacterial pathogens simultaneously at different fronts, rendering the cell an important guardian of the lung's immune integrity.

As already mentioned, GM-CSF is a key cytokine in the development and functional maturation of the rAM. The critical role of pulmonary GM-CSF for the rAM's antibacterial activity was extensively confirmed in several *in vitro* and *in vivo* studies. rAMs of GM-CSF$^{-/-}$ mice exhibit a decrease in uptake of both Gram-negative and Gram-positive bacteria due to a diminished capacity to exert complement- and antibody-mediated phagocytosis [52]. Intracellular killing of both Gram-negative and Gram-positive bacteria was also reduced in rAMs of GM-CSF$^{-/-}$ mice. For example, GM-CSF$^{-/-}$ mice showed a significantly increased susceptibility to *Streptococcal* [53] and *Pneumocystis carinii* [54] induced pneumonia due to an impaired superoxide production by rAM. In addition, rAM from GM-CSF$^{-/-}$ mice showed a reduced expression of multiple PRRs, including TLR-4, TLR-2 and CD14, and consistent with that observation a failure to secrete TNF-α following exposure to LPS [52]. These data demonstrate that pulmonary GM-CSF is crucial in establishing the antibacterial response of rAM directly by enhancing phagocytosis and intracellular killing but also indirectly by promoting the expression bacterial-associated PRRs. Overview of the main features of the early immune response of rAM to respiratory bacterial pathogens is depicted in figure 2.1.

2.3.2 Eradication of respiratory viral infections

Viral respiratory tract infections are considered as frequent infectious illnesses afflicting both adults and children. Viral respiratory tract infections result in a surprisingly diverse range of disease severity from the mild common cold to severe and life-threatening conditions. Evidence has emerged in recent years for rAM playing an important role in the first line of defence against respiratory viral pathogens. As is the case with bacterial pathogens, viruses contain conserved structural moieties which are essential for microbial survival and therefore ideal targets for opsonins and PRRs. Although most viral pathogens enter the cell through active, receptor mediated invasion mechanisms, opsonisation of virus particles for immediate uptake by phagocytes plays an important role in the innate immune defence against viruses. Mannose binding lectin (MBL) has been found to bind directly to virions from a number of

viruses, including severe acute respiratory syndrome coronavirus (SARS-CoV). Binding of MBL to the virion blocks the cellular entry of the virus and functions as an opsonin promoting the viral uptake by rAM [55, 56]. Just as in the case with respiratory bacterial pathogens, the pulmonary surfactant system is also able to opsonise virus particles. Both SP-A and SP-D show specific interactions with several viruses. The carbohydrate recognition domain of SP-D binds influenza virus through interactions with viral haemagglutinin (HA) protein and neuraminidase envelope glycoproteins whereas the interaction of SP-A with influenza virus proteins is mediated through a N-linked oligosaccharide on the carbohydrate recognition domain rather than carbohydrate-binding itself. Respiratory syncytial virus (RSV) is recognized by SP-D and SP-A by binding the G-protein and the F-fusion glycoprotein of RSV respectively [57]. During the adaptive phase of the immune response, IgG- and IgA-mediated opsonisation and uptake by rAM constitutes an essential aspect of viral clearance in the airways. For example, binding of neutralizing IgG-molecules to HA-protein of Influenza A is followed by rAM-mediated uptake and delivers a crucial contribution to viral elimination [58]. In addition, protection by the Influenza A virus vaccines based on the conserved ectodomain of matrix protein 2 (M2e) is mediated through an rAM and FcR dependent uptake of virally infected cells that have bound anti-M2e IgG to cell surface expressed M2e [59]. Next to IgG, mucosal production of IgA is also crucial for the neutralization of respiratory viruses. Lung mucosal RSV specific IgA-molecules are found to be effective in the neutralization of RSV via the rAM-mediated uptake of IgA-coated viral particles [60]. Furthermore, in a mouse model of influenza infection, pulmonary levels of neutralizing IgA antibodies were found to be correlated to reduced virus spread during influenza reinfection [61]. Also surface expressed PRRs, like TLR-4, have been shown to interact with different viral envelop glycoproteins, including the F-protein of RSV, while endosomal PRRs watch over the intracellular space by sensing for viral nucleic acids. Double-stranded (ds)RNA, contained in the genome of dsRNA viruses such as Influenza A, or present as a replicative intermediate, are recognized by TLR-3. Single-stranded (ss)RNA from ssRNA viruses like RSV, and DNA from DNA viruses such as human Bocavirus, are recognized by TLR-7 (in mouse) and TLR-8 (in humans), and TLR-9 respectively. A second family of PRRs involved in the recognition of viral RNA by rAM is the RIG-I-like RNA helicase receptor (RLH) family. In contrast to the TLRs, RLH survey the cytoplasm of the cell for the presence of viral RNA [62]. Once activated, these receptors trigger intracellular signalling cascades that culminate in the formation of highly effective antiviral defence mechanisms along with the induction of pulmonary inflammation. Throughout years it has become clear that pulmonary inflammation, in which rAM act as primary mediators,

fulfils a necessary role in the resolution of Influenza A [63] or RSV [64] infection. The initial inflammatory cytokine and chemokine production by rAM recruits different inflammatory leukocytes, including mononuclear phagocytes, to the site of viral infection. The recruited mononuclear phagocytes then contribute to the elimination of the virus through the induction of apoptosis of infected AECs via the release of TNF-related apoptosis-inducing ligand (TRAIL) [65]. This initial antiviral reactivity of rAM is a major determinant for subsequent disease severity. Indeed, depletion of rAM in a mouse model for Influenza A viral infection resulted in increased TNF-α and IL-6 levels and increased inflammation in the lungs. The increased pulmonary inflammatory response was however accompanied by increased morbidity, mortality and uncontrolled viral growth in the lungs [66]. Similar results were observed in mouse models for NDV [67] and RSV [68] infection in which depletion of rAM prior to viral exposure increased the pulmonary inflammation and virus yield. An elegant study performed by Tate and co-workers even provided evidence for rAM being the key modulator for disease severity during Influenza virus infection in mice. They showed that Influenza strain BJx109 (H3N2) infected rAM with high efficiency and was associated with mild disease following intranasal infection of mice. In contrast, Influenza strain PR8 (H1N1) was poor in its ability to infect rAM but was highly virulent for mice. In addition, depletion of rAM prior to infection lead to the development of severe viral pneumonia in BJx109 infected mice but did not modulate disease severity in PR8 infected mice [69]. Thus, the ability or inability of a respiratory virus to infect rAM may be a critical factor contributing to its global virulence in mice. In contrast, Tumpey and co-workers reported decreased levels of inflammatory cytokines in Influenza A infected lungs after depletion of rAM. This decreased inflammatory response was correlated with an increased mortality and pulmonary viral load [70]. These opposing findings may be explained by the use of different virus strains, viral dose, and administration route. Further evidence that the inflammatory reactivity of rAM is strongly influenced by the viral species infecting the airways derives from observations in mouse models of RSV infection. In contrast to Influenza virus, RSV gives rise to alternatively activated rAM, resulting in limited inflammation and lung injury [71]. Because of these conflicting data, the exact role of inflammation and more specifically, the role of rAM as initiators of pulmonary inflammation during respiratory viral infections remains an ongoing matter of debate.

Type I IFNs are crucial mediators of the antiviral immune defence. The prototypic type I IFNs, IFN-α and IFN-β, are pleiotropic cytokines critical for antiviral responses by inducing

cellular resistance to viral infection, apoptosis of virus-infected cells and activation of natural killer (NK) cells and T-cells [72]. In addition, these cytokines are potent activators of rAM's innate antiviral immunity. Autocrine or paracrine IFN-α/β receptor (IFNAR)C engagement results in activation signal transducer and activator of transcription (STAT)-1 dependent production of antiviral proteins, such as 2'-5' oligoadenylate synthetase (OAS)1 which is in charge of viral RNA degradation [73]. Although various cells are reported to have the potential to produce type I IFNs when exposed to viruses *in vitro*, pulmonary production of type I IFNs was originally assigned to plasmacytoid (p)DCs, a rare subset of DCs [74]. Yet, an important study using knock-in mice in which GFP was expressed under the control of the *Ifna6* promoter, demonstrated that during lung infection with Newcastle disease virus (NDV), rAM acted as the primary type I IFNs producers through an RLH-mediated mechanism [67]. It is therefore suggested that type I IFNs produced by rAM activate surrounding cells in a paracrine manner to prepare for any viral encounter. In addition, several respiratory viruses, like RSV, inhibit type I IFN transcriptional activation in rAM by distinct mechanisms in order to impair the rAM-mediated antiviral response [75]. This further highlights the importance of rAM-derived type I IFNs during the innate pulmonary antiviral response. Indeed, infection of rAM from calves with recombinant bovine RSV, lacking the non-structural (NS) proteins which counteract the antiviral effects of type I IFNs, strongly induced type I IFN production in rAM. This increase in type I IFN production by rAM was accompanied by a severely attenuated viral replication in these cells and in calves [76].

Overview of the main features of the early immune response of rAM to respiratory bacterial pathogens is depicted in figure 2.1.

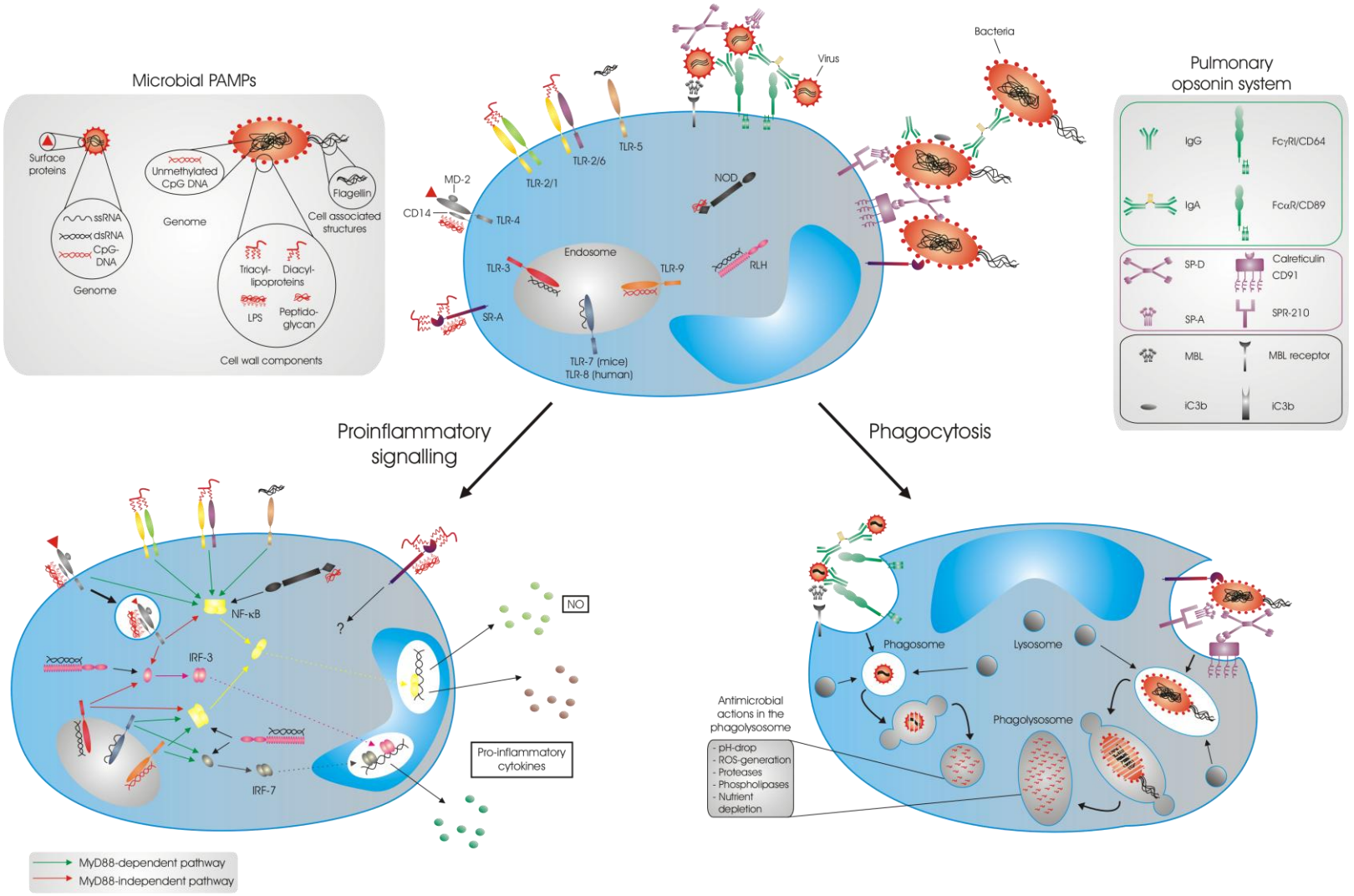

Figure 2.1: Overview of the main features of the innate immune response of rAM to respiratory microbial pathogens.
Microbial pathogens like viruses and bacteria, display a number of PAMPs which are recognized by different families of PRRs including TLRs, RLHs, NODs and SR such as SR-A. In addition, pulmonary proteins and antibodies such as SP-A, SP-D, MBL, iC3b, IgG and IgA are able to opsonise respiratory virsuses and bacteria. Engagement of the different PRR families induces the activation and nuclear translocation of different transcription factors such as NF-κB, IRF-3 and IRF-7 which eventually results in the secretion of proinflammatory mediators like cytokines and NO. Engagement of opsonin receptors on the surface of rAM induces phagocytic uptake and killing of the pathogen through phagolysosomal degradation.

2.3.3 Regulation of immunological homeostasis in the lungs: rAM as the guardian angels of the lower respiratory tract.

The lower respiratory tract is continuously invaded by pathogenic microorganisms and other airborne particulate matter. However, most of these particles are mutely sequestrated by rAM in order to shield the pulmonary environment from the development of exaggerated inflammatory responses. It has been estimated that the pool of rAM can handle up to 10^9 intratracheally injected bacteria before there is 'spillover' of bacteria to DCs and before adaptive immunity is induced [77]. In this context, Dockrell and colleagues demonstrated that depletion of rAM in a low-dose murine pneumococcal infection model shifted the outcome from resolution with complete bacterial clearance and absence of neutrophil recruitment, to one in which neutrophil recruitment is required for bacterial clearance [78]. In contrast, in murine high-dose pneumococcal infection models rAM depletion had no impact on bacterial clearance, presumably because the ability of rAM to clear bacteria is overwhelmed [79].

Several local and unique mechanisms have been identified that allow a specialized modulation of rAM function to meet the need of the tissue for a muted clearance of particulate matter. Under homeostatic conditions, rAM closely adhere to the AECs. This interaction promotes the expression of the TGF-β dependent $\alpha_v\beta_6$-integrin on the surface of AECs. The $\alpha_v\beta_6$-integrin has the potential to bind latent TGF-β and to activate the bound TGF-β in close proximity of the rAM by removing the latency associated peptide, a N-terminal inactivating fragment of TGF-β. Activated TGF-β binds to the TGF-β receptor on the surface of the rAM and induces the phosphorylation of Smad-2 and Smad-3. Smad-signalling eventually will lead to suppression of cytokine production by rAM [80]. The importance of $\alpha_v\beta_6$-integrin for keeping the rAM quiescent under steady-state conditions is illustrated by the fact that $\alpha_v\beta_6$-integrin$^{-/-}$ mice have constitutively activated rAM which are responsible for spontaneously causing MMP-12 dependent emphysema [81]. Besides TGF-β, also IL-10 produced by AECs induces anti-inflammatory signal transduction in the rAM [82]. Yet, another novel homeostatic loop is mediated by the CD200 receptor (CD200R), which is almost exclusively expressed by myeloid cells, including macrophages and DCs [83, 84]. Its ligand, CD200, on the contrary is expressed by a variety of cells including thymocytes, B cells, some peripheral T-cells, neurons in the central nervous systems and endothelium [85, 86]. Binding of CD200 to CD200R imparts a unidirectional negative signal to CD200R bearing cells [83]. The precise molecular mechanisms leading to CD200R mediated suppression is however still unknown. Unlike other tissue macrophages, rAM feature unusually high basal expression levels of CD200R. Under

steady-state conditions, respiratory CD200 ligand expression is limited to the luminal surfaces of the airway epithelium. *In vitro* studies showed that this interaction prevents the activation of rAM in the presence of inflammatory stimuli. Since low levels of CD200R on splenic macrophages have been shown to be increased by IL-10 and TGF-β, the pulmonary pool of these anti-inflammatory cytokines might contribute to the basal high expression of CD200R rAM [87].

Yet, another immune attenuating characteristic of rAM is their markedly reduced IFN-β production in response to viral and bacterial PAMPs. Although rAM adequately produce the immediate/early gene products TNF-α, RANTES, MIP-1α and MIP-1β following TLR-3 or TLR-4 engagement to allow the initiation of inflammation, they fail to autonomously produce IFN-β. As a consequence, autocrine STAT-1 signalling is not induced, impairing the production of potential damaging effector molecules, like NO [88]. Clearly, additional studies are needed to unravel the molecular mechanisms underlying this selective silencing of IFN-β production in rAM.

Besides their role as bacterial opsonins, the surfactant proteins SP-A and SP-D fulfil important immunomodulatory functions in the lung as well. SP-A and SP-D suppress secretion of pro-inflammatory cytokines and reactive oxidant intermediates when rAM are challenged with pathogen-derived cell wall molecules or other immunostimulatory components. It was found that SP-A markedly diminished the pro-inflammatory TLR-2 and TLR-4 response in human rAM resulting in lower phosphorylation levels of Akt and downstream intermediates of the MAPK pathway and absence of nuclear factor (NF)-κB activation [89]. This suppression may at least in part result from a direct binding of the surfactant proteins to the LPS receptor CD14, the TLR-4 adaptor MD-2 and TLR-2 [90, 91]. Also clathrin-dependent endocytosis of SP-A has been reported to block the ability of LPS to induce inflammation in rAM [92]. Surfactant proteins also indirectly contribute to the overall anti-inflammatory environment of the bronchial lumen. SP-A promotes increased expression of the anti-inflammatory IL-10 by AECs and induces secretion of TGF-β [93]. Thus, on the one hand surfactant proteins improve phagocytosis of airborne particulate matter by rAM and on the other hand alter the expression and function of PRRs and inflammatory mediators in order to dampen the rAM's potential for rapid activation so that the antigenic threat is cleared 'silently'.

MUC1, a transmembrane mucin-like glycoprotein ubiquitously expressed on the apical surface of mucosal epithelial cells also contributes to the suppression of rAM inflammatory activity. Indeed, MUC1$^{-/-}$ mice exhibited increased inflammatory responses to *Pseudomonas aeruginosa* compared to their wild-type littermates due to an increased TLR-5 reactivity against the bacterial flagellin [94]. Next to suppression of TLR-5, MUC1 was found to possess anti-inflammatory effects also on other TLR-signalling pathways, including TLR-2, 3, 4, 7 and 9 [95].

Also CD44, a transmembrane adhesion molecule and the major receptor for hyaluronan, limits the responsiveness of murine rAM via the intracellular negative regulators IRAK-M, Tollip and A20 [96].

This plethora of negative regulators maintains rAM in a quiescent state during homeostasis. Once the antigenic challenge exceeds the threshold of immunological homeostasis, inflammatory (TLR-)stimulation of rAM leads to a rapid loss of contact with AECs, which in turn induces a rapid disappearance of $\alpha_v\beta_6$-integrin expression on AECs. Under these conditions, pre-TGF-β is no longer converted to its active form [80] and the IL-10 receptor – IL-10 signal transduction axis is interrupted [82]. Furthermore, the epithelial expression of CD200 is reduced during the onset of an inflammatory response, setting the rAM free from inhibition via CD200R [97]. These processes are responsible for releasing the 'immunological brakes' on rAM. Once activated, rAM generally display a higher oxidative burst and are primed to secrete pro-inflammatory cytokines and chemokines [98].

Strikingly, the switch from an anti- to a pro-inflammatory function of the rAM is not necessarily accompanied by the acquisition of an immunogenic antigen presentation (APC) function by the cell. Although constitutive migration and pathogen transport to lung draining lymphnodes (LDLN) was recently demonstrated [99], rAM are considered as weak APCs due to low expression levels of MHC class II and co-stimulatory molecules. rAM may even exert immunosuppressive functions as suggested by studies using clodronate-filled liposomes to deplete rAM *in vivo*. In these studies, rAM depletion rendered the lungs susceptible to T-cell mediated inflammatory responses to otherwise harmless inhaled antigens [100]. The immune suppressive effects of rAM were initially attributed to a direct suppression of T-cells by NO and the production of anti-inflammatory mediators such as IL-10, TGF-β and prostaglandins. At present, the weak APC function of rAM are mainly ascribed to defective expression of co-stimulatory molecules along with increased expression of co-inhibitory ligands such as

programmed death ligand (PD-L)1 and PD-L2 [101]. In addition to suppression of T-cell activation, rAM actively inhibit the APC function of interdigitating DCs in the airways. Indeed, after rAM depletion, lung DCs exhibited enhanced APC function [102]. rAM depletion also resulted in increased numbers of DCs in the alveolar lumen and augmented uptake of particles by DCs, leading to increased migration of the cells to the LDLN [103]. These studies point to a role for rAM in the steady-state regulation of DC differentiation and migration. An overview of the pulmonary mechanisms pushing and releasing the brakes on rAM-activation is depicted in figure 2.2.

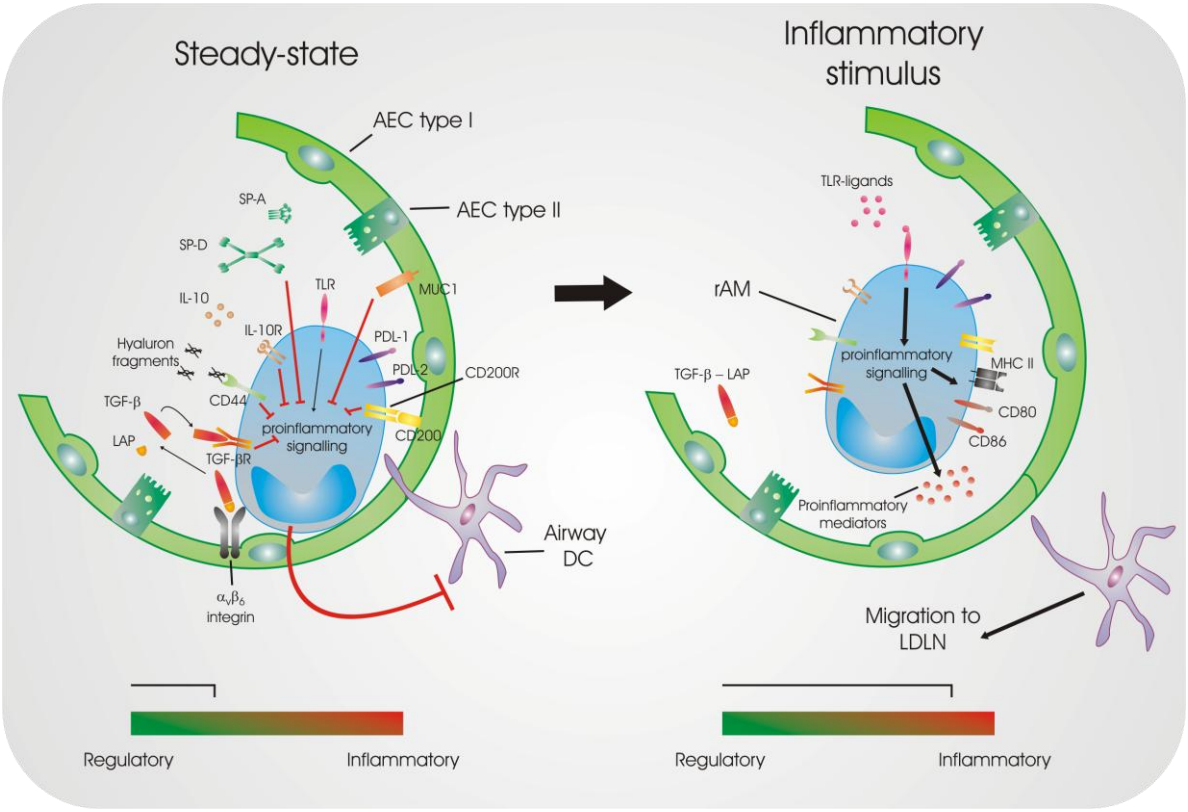

Figure 2.2: Overview of the pulmonary mechanisms pushing and releasing the brakes on rAM-activation. In steady-state conditions (*left panel*) rAM closely adhere to the AECs, thereby inducing the expression of the $\alpha_v\beta_6$-integrin on AECs. This integrin activates TGF-β by removing the latency associated peptide (LAP). Activated TGF-β binds to the TGF-β receptor on the surface of the rAM, leading to suppression of cytokine production. AECs further restrain pro-inflammatory signalling in rAM through the CD200 – CD200R axis and MUC1. Alveolar IL-10, which binds to rAM's IL-10R, hyaluronan fragments, binding to CD44 at the surface of rAM and SP-A and SP-D all inhibit pro-inflammatory signalling in rAM as well. TLR-molecules are present and functional but are overridden by the suppressive mechanisms. Therefore, rAM display a regulatory phenotype in steady-state conditions, suppressing the induction of adaptive immune responses by the expression of inhibitory co-stimulatory molecules like PDL-1 and PDL-2, and inhibiting DC-maturation and trafficking to the LDLN. When inflammatory stimuli (*right panel*), like TLR-ligands, exceed alveolar threshold levels, $\alpha_v\beta_6$-integrin, CD200 and MUC1 expression on AECs is rapidly lost. As a result rAM detach from AECs and escape from TGF-β and CD200 inhibition. Alveolar IL-10 levels drop sharply and hyaluronan fragments are rapidly degraded by inflammatory proteases as well. As a result, rAM display an inflammatory phenotype, characterized by the secretion of pro-inflammatory cytokines and the expression of MHC II and the activator co-stimulatory molecules CD80 and CD86. Furthermore, airway DCs become activated and migrate to the LDLN to induce an adaptive immune response.

2.3.4 Alveolar macrophages in the resolution of inflammation: cleaning up the mess!

Elimination of apoptotic cells is an important step in the resolution of the inflammatory response and represents an evolutionary conserved process from *C. elegans* to man. An inflammatory challenge in the lung, exceeding the local immunological threshold, results in the recruitment of inflammatory leukocytes, which migrate across the endothelial and epithelial barriers into the alveolar airspace [104]. These newly recruited granulocytes have however a limited lifespan, after which apoptosis ensues. Phagocytic removal of apoptotic granulocytes and other inflammatory leukocytes is crucial for preventing the exposure of the surrounding tissue to potentially toxic, immunogenic or inflammatory cellular debris [105], and is mainly carried out by rAM. During apoptosis a series of events culminates in the rearrangement of plasmamembrane components, including phosphatidylserine (PS). The exposed PS is recognized by several rAM receptors, including the PS receptor (PSR), and leads to the uptake of the apoptotic cell [106]. Accordingly, rAM exhibiting an impaired PSR activity are defective in the removal of apoptotic cells from the lung, a feature also observed in the sputum of cystic fibrosis patients [107]. Although the PS – PSR interaction is considered as the main axis for recognition and uptake of apoptotic cells by macrophages, the process of removing dead cells involves multiple other receptors such as scavenger receptors, CD14, CD68, CD36 and vitronectin receptor ($\alpha_v\beta_3$ integrin) [108] which are all expressed on the surface of rAM. Recent studies demonstrated the binding of IgM to oxidized phospholipids, like lysophosphatidylcholine, on late apoptotic cells [109]. Consequently, IgM was identified as a novel opsonin for late apoptotic cells, thereby enhancing their uptake by rAM. This function may be especially significant during pulmonary inflammation when airway levels of IgM increase, thus facilitating the removal of apoptotic inflammatory cells by rAM [110]. Surfactant proteins also contribute to clearance of apoptotic cells. Both SP-A and SP-D enhanced the uptake of apoptotic cells by rAM *in vitro* [111], but only SP-D enhanced apoptotic cell clearance by rAM *in vivo*. Thus, Clark and co-workers reported that SP-D$^{-/-}$ mice have five- to tenfold higher levels of apoptotic rAM in the alveolar spaces. Treatment of these mice with an intratracheally administered 60-kDa fragment of human recombinant SP-D reverted the phenotype of KO mice [112]. Since SP-D and SP-A effectively bind DNA, binding of these collectins to cell-surface DNA represents at least one mechanism by which the surfactant proteins may promote phagocytosis of apoptotic cells by rAM [113].

A consequence of apoptotic body uptake by a phagocyte is the launch of an anti-inflammatory program by the phagocyte. rAM phagocytosis of apoptotic cells indeed results in the release

of anti-inflammatory mediators, such as TGF-β, IL-10 and PGE_2 [114], as demonstrated for apoptotic cells opsonised with surfactant proteins [115] and IgM-coated apoptotic cells [110]. In addition to promoting anti-inflammatory functions, phagocytosis of apoptotic cells actively suppresses pro-inflammatory cytokine production in macrophages in a direct and indirect manner. Clearly, IL-10, TGF-β and PGE_2 inhibit the production of pro-inflammatory cytokines such as TNF-α, GM-CSF, IL-1β, IL-12 and IL-18 [114]. However, ingestion of apoptotic cells also initiates direct inhibition of pro-inflammatory cytokine transcription and translation. Kim and co-workers reported the inhibition of *il12p35* gene transcription by apoptotic cells via a mechanism involving the induction of a novel zinc finger-containing nuclear factor, named GC binding protein (GC-BP), by apoptotic cells in macrophages. Upon contact with apoptotic cells, GC-BP undergoes dephosphorylation and subsequently binds to the promoter region of the *il12p35* gene, thereby actively blocking its transcription [116]. An overview of the main molecular players in the rAM-mediated uptake of apoptotic leukocytes is presented in figure 2.3.

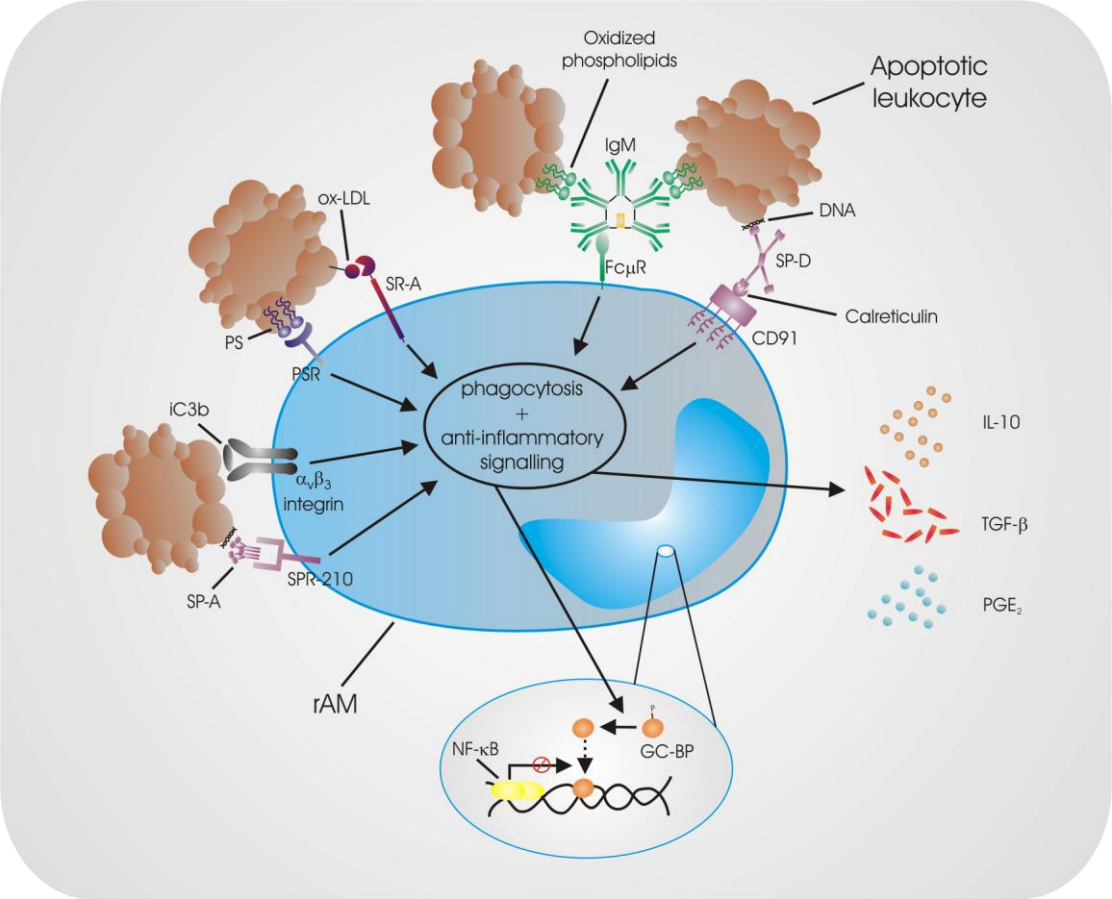

Figure 2.3: Phagocytic uptake of apoptotic leukocytes by rAM.
Apoptotic leukocytes show rearrangements of their plasmamembrane lipid components, resulting in the exposure of PS and ox-LDL to the extracellular space. PS and ox-LDL are recognized and bound by the rAM-expressed PSR and SR-A respectively. In addition, other oxidized phospholipids exposed to the extracellular space by apoptotic leukocytes are bound by IgM. The Fc portion of this antibody isotype in turn binds to the FcμR at the surface of rAM. The pulmonary opsonins SP-A and SP-D (probably) bind to DNA-fragments present at the cell surface of apoptotic leukocytes and are recognized by SPR-210 and CD91/calreticulin of rAM. Finally, iC3b was found to opsonise apoptotic leukocytes and binds to $\alpha_v\beta_3$-integrin expressed by rAM. Stimulation of these receptors results in phagocytosis of the apoptotic leukocyte and the induction of an anti-inflammatory signalling cascade in the rAM. This anti-inflammatory program includes the secretion of anti-inflammatory mediators such as IL-10, TGF-β and PGE_2, and the direct inhibition of transcription of pro-inflammatory genes through activation (by dephosphorylation) of the NF-κB transcriptional repressor GC-BP.

2.4 The Alveolar Macrophage: The Bad and the Ugly

2.4.1 The alveolar macrophage in non-infectious respiratory pathologies: pulmonary criminal at large!

Non-infectious respiratory diseases, like chronic obstructive pulmonary disease (COPD) and asthma, affect millions of people worldwide and are one of the leading causes of death. Harmful environmental factors, including particulate pollutants, gases or allergens often lie at the basis of disease onset. Although fully equipped for the efficient but quiescent elimination

of invading bacterial and viral pathogens, AM display an aberrant pathogenic reactivity during non-infectious pulmonary immune responses.

COPD

A respiratory pathology which deserves special attention in this perspective is COPD, a heterogeneous syndrome associated with abnormal immune responses of the lung to noxious particles and gases leading to lung emphysema and airway obstruction. Cigarette smoke is a common cause of COPD and activates pulmonary phagocytes by triggering PRRs directly or indirectly via the release of damage associated molecular patterns (DAMPs) from stressed or dying cells. Activated DCs induce inappropriate adaptive immune responses encompassing Th1- and Th17-cells, $CD8^+$ cytotoxic T cells and B-cells [117]. Although such inappropriate adaptive immune responses fulfil a critical role in the pathogenesis of COPD with cytotoxic $CD8^+$ T-cell responses in the forefront, different studies in immunodeficient SCID-mice demonstrated that the innate immune system is sufficient to develop cigarette smoke induced inflammation [118] and emphysema [119]. rAM play herein a pivotal role, contributing to several of the clinical features observed in COPD patients. Cigarette smoke exposure leads to engagement of PRRs on the surface of rAM. Different components of cigarette smoke, including endotoxin (LPS), nicotine and free radicals are responsible for triggering rAM PRRs. TLR-4, but also transient receptor potential (TPR)A1 and NOD-like receptor (NLR)P3 are major actors in the inflammatory activation of rAM. DAMPs released by injured airway epithelial cells constitute an important additional source of PRR ligands. High-mobility group box (HMGB)1 [120], uric acid and extracellular ATP [121] are significantly increased in bronchoalveolar lavage fluid of COPD patients compared to smokers without COPD. Activation of PRRs leads to increased expression of pro-inflammatory mediators by rAM, hereby contributing to the inflammatory component of the COPD pathophysiology. The best studied chemokine in COPD patients is IL-8, the major human neutrophil chemoattractant which is found to be significantly more secreted at baseline and after stimulation with IL-1β and cigarette smoke by AM from COPD patients compared to AM from non-COPD smokers and non smokers [122]. This also emphasizes the crucial contribution of IL-1β to the onset and propagation of the pulmonary inflammation associated with COPD. Indeed, IL-1 $receptor^{-/-}$ mice showed attenuated pulmonary inflammation after acute exposure to cigarette smoke and were significantly protected against lung emphysema after chronic cigarette smoke exposure regimens [123]. In addition to their amplifying role in COPD associated pulmonary inflammation, rAM from COPD patients are unresponsive to the anti-inflammatory activity of

corticosteroids. This unresponsiveness is the basis of the poor response of COPD patients to treatment with corticosteroids [122].

rAM also directly contribute to COPD associated lung destruction and lung tissue remodelling. Besides elicited neutrophils, rAM are an important source of oxygen radicals and proteolytic enzymes such as MMP-8, MMP-9, and MMP-12, known to induce lung damage [124]. In addition to their ability to break down the extracellular matrix, MMPs also stimulate mucus production and goblet cell hyperplasia via the proteolytic activation of TGF-α. rAM derived TGF-α further contributes to mucus hypersecretion [125]. Excessive mucus production combined with an impaired mucociliary clearance in turn provoke airway obstruction in patients with COPD [126].

One of the major side effects leading to hospitalization of COPD patients, are secondary respiratory viral and/or bacterial infections. These secondary infections actively contribute to the pathogenesis and course of COPD by causing acute exacerbation of COPD and by amplifying and perpetuating chronic inflammation in stable COPD [127]. Importantly, an impaired phagocytosis of bacteria by rAM in COPD plays an important role in chronic bacterial colonization and acute infectious COPD exacerbation. Indeed, different studies showed that AM from COPD patients exhibited decreased phagocytosis of bacteria such as *Haemophilus influenzae* [128] and *Streptococcus pneumoniae* [129] compared to AM from non-COPD smokers and non-smokers.

Allergic asthma

Allergic asthma is a chronic inflammatory disease characterized by recurrent episodes of airway obstruction and wheezing after exposure to inhaled allergens. In addition to mast cells, eosinophils and allergen specific Th2-cells, also rAM have emerged as important actors in asthma pathogenesis and disease progression. Thus, whereas rAM fulfil a regulatory role at the onset of the asthmatic pulmonary inflammation [100, 102, 130], excessive activation of rAM at later stages of the pulmonary inflammation has an important impact on the progression of the disease. Analysis of AM from asthmatic patients identified these cells as significant sources of IL-13, one of the major key cytokines in asthma [131]. The rAM also showed reduced phagocytosis of apoptotic cells [132]. Both features are predominant characteristics of M2-skewed cells. Moreira and co-workers further showed that rAM from *Aspergillus fumigatus*-induced asthmatic mice expressed high levels of the M2-marker FIZZ1 when compared to

rAM from naïve mice [133]. FIZZ1 has been shown to contribute to asthma and airway remodelling [133, 134]. Thus, even though M2 macrophages are indispensable for tissue repair and the restoration of lung homeostasis, their excessive activation in asthma contributes to increased cell recruitment, mucus hypersecretion and airway hyperresponsiveness. This is further supported by the observation that the lungs of allergen sensitized and challenged mice increased the pulmonary inflammatory response and collagen deposition, a marker for airway remodeling [133]. The enhanced M2-skewing of rAM in allergic asthma intuitively makes sense given the Th2-driven lung environment. However, not all is black and white in the asthmatic lung. Thus, an involvement of endotoxin in the initiation of asthma has been extensively documented as well [135, 136] and both endotoxin and IFN-γ levels were found to be significantly increased in asthmatics with severe forms of the disease. Endotoxin and IFN-γ are prototypic triggers of the differentiation of macrophages into M1 [137]. Furthermore, it has been suggested that the Th2-associated cytokines IL-4 and IL-13 increase the production of the M1-related cytokines IL-6, TNF-α, and IL-12p70 by macrophages after co-stimulation with M1-skewing factors like LPS [22]. Thus determining the precise role of the M1-M2 (im)balance in asthma might determine to what extent rAM exert a regulatory or a pathogenic role in asthma.

Fibrosis

Pulmonary fibrosis evolves from a variety of lung diseases, including COPD, asthma and silicosis, and is characterized by the uncontrolled deposition of collagen and other matrix proteins eventually leading to lung remodelling and irreversible loss of function. Local tissue fibroblasts were believed to be the primary producers of extracellular matrix components. However, the induction and maintenance of a M2-phenotype in rAM is a characteristic feature of pulmonary fibrosis and points towards a role of M2-polarized rAM in the pathogenesis of fibrotic disorders. As already mentioned, induction of the arginase-1 metabolism in M2-skewed rAM by IL-4 and IL-13 promotes collagen deposition and degradation [138]. Next to a disproportionate collagen deposition, IL-13 stimulated rAM also form an abundant source of TGF-β, an important inducer of fibrosis [139]. In a mouse model of bleomycin-induced fibrosis, rAM were found to produce nearly all of the active TGF-β that promotes pulmonary fibrosis [140]. In addition, rAM themselves secrete significant amounts of IL-13, thereby establishing a profibrotic positive feedback loop in the lungs. Finally, rAM are believed to be one of the key sources of a variety of CC-chemokines such as CCL2 [141] or CCL3 [142] which act as crucial profibrotic mediators. Neutralization of these cytokines significantly reduced the development of pulmonary fibrosis.

Hypersensitivity pneumonitis (HP)

HP is an immune complex and cell-mediated immunological disorder disease of the lungs that is caused by the inhalation of antigenic organic particles or fumes. The pulmonary inflammatory response is characterized by the presence of mainly neutrophils, Th1- and Th17-cells, and $CD8^+$ T-cells [143]. rAM display also a preponderant role in the pathophysiology of HP. Following exposure to causal agents, soluble antigens bind to IgG-molecules, triggering the complement cascade. The formation of the C5 fraction activates rAM which in turn release multiple inflammatory cytokines and chemokines like IL-8, RANTES, monocyte chemoattractant protein (MCP)-1 and MIP-1α, thereby contributing to the recruitment of other cells, such as neutrophils, T-cells and monocytes. Furthermore, MIP-1α also promotes the differentiation of Th0-cells to a Th1-cell phenotype [101, 144]. rAM are normally poor APCs, but this function is greatly increased in HP. Israel-Assayag and co-workers demonstrated that, following contact with the antigen, rAM incorporated antigenic particles and presented antigen-derived peptides to T-lymphocytes, resulting in their activation and proliferation. In addition, elevated levels of the intracellular adhesion molecule (ICAM)-1 and the CD80 and CD86 co-stimulatory molecules on the surface of rAM from HP patients and mouse models of HP supports the role of rAM in T-lymphocyte activation in the pathophysiology of HP [101, 145, 146]. HP is also characterized by the formation of granulomas, a firm collection of immune cells which is formed in an attempt to shield off foreign substances when the immune system is unable to eliminate these antigenic threats. Together with recruited monocytes and immature macrophages, rAM can develop into multinucleated giant cells which are grouped to form granulomas [147]. The details of the cell biology of the transformation of macrophages into typical multinucleated giant cells that make up granulomas remain undefined. Next to the pulmonary inflammatory response and granuloma formation, rAM are also inducers of lung injury and remodelling during HP via the secretion of tissue degrading (e.g. MMPs) [101] and remodelling factors (e.g. TGF-β) [148].

2.4.2 Pulmonary innate imprinting: teaching a good child bad manners

Inflammatory responses are characterized as highly dynamic processes. Once local innate immune cells are activated by the inflammatory insult, cytokine and chemokine secretion results in different waves of leukocyte recruitment to the site of inflammation. After the elimination of the antigenic threat, inflammation is cleared and the tissue eventually strives to

regain steady-state conditions. However, research through years has revealed that authentic or initial steady-state conditions are never completely achieved. Therefore, the tissue response to subsequent inflammatory or infectious insults is imprinted and biased by its preceding inflammatory or infectious history even in the absence of cross-reactive immunity. For instance, infection of the lung in the absence of prior infection or inflammation will have a different outcome to same pathogen infecting a lung with resolved infection or inflammation. This alteration is not restricted to latent or concurrent infections or inflammations but can also be influenced by acute inflammatory lung diseases. In addition, clinical and experimental data suggest that influences from prior even unrelated pulmonary inflammatory insults may be long lasting [149, 150]. Although memory functions and long lasting immunological effects were originally ascribed to the adaptive compartment of the immune system, recent research revealed that innate cells, including rAM, are involved in tissue imprinting processes as well.

The outcome of innate imprinting is mostly unfavorable for the host and may depend on the precise sequence of inflammation or infections. This is illustrated by the increased susceptibility to life-threatening bacterial pneumonia in patients infected with seasonal or pandemic influenza infection [151, 152]. The underlying mechanisms responsible for this enhanced susceptibility to secondary bacterial pneumonia after influenza infection have been studied extensively and different independent studies pointed out an important role for the rAM. Didierlaurent and colleagues reported a sustained desensitization of rAM to TLR-ligands including LPS, lipoteichoic acid and flagellin, which lasts for several months after resolution of influenza infection [153]. Although such desensitization might be beneficial in alleviating overall immunopathology, the TLR-hyporesponsiveness of post-influenza rAM was correlated with higher and prolonged respiratory bacterial loads [153]. The first wave of pulmonary inflammation, accompanying the influenza infection is though to increase the rAM's threshold TLR-responsiveness, thereby decreasing its global bacterial recognition. Post-influenza rAM displayed a decreased expression of phagocytosis receptors SR-A and MARCO [154] which is consequently accompanied by an impaired ability to engulf and kill bacteria [155]. In addition, rAM showed an increase in expression of the lung immune homeostasis regulator CD200R after resolution of influenza viral infection which in turn contributes to the observed susceptibility state of the post-infection lung as well [97]. This rAM "paralysis" in the post-infection lung might represent a side-effect of the mechanisms developed in the lung in order to prevent excessive inflammatory responses and bystander tissue damage and alveolar barrier dysfunction. Negative intracellular or intercellular

feedback loops are a common and characteristic feature for dampening inflammatory reactions and are highly pronounced in the lungs. After clearance of influenza viral infection, for instance, excessive pulmonary levels of the anti-inflammatory IL-10 are measured [156].

In contrast to long-lived desensitization of rAM's (antibacterial) functions after influenza infection, the opposite effect of a lasting and persistent (hyper)activation of rAM functions has been demonstrated too. In a mouse model of Sendai virus infection, activated rAM produced high levels of IL-13 and overexpressed the IL-13 receptor. This combination of events established a persistent positive feedback loop, resulting in a chronic lung condition with pathological features resembling asthma and COPD, including chronic mucus cell metaplasia and airway hyperreactivity [157].

It is clear from the described examples that the concept of innate imprinting of rAM has been widely documented in mouse models for infectious respiratory diseases. Recently, persistent activation of dendritic cells was found after the resolution of an allergic airway inflammation. Moreover, this persistent DC activation resulted in an abolished tolerance for new encountered allergens [158]. The extent to which innate imprinting also occurs in rAM following an allergic bronchial inflammation and the nature of its functional outcome remains however unknown.

2.5 Concluding remarks

The air we breathe is filled with a variety of (pathogenic) microorganisms, allergens, harmful gases and noxious particles. AM are the first line of defence of the lungs against this diversity of airborne pathogens and harmful substances. Therefore, AM must be able to mount an appropriate immune response adapted to the specific nature of the antigenic threat. Hereto, AM display an array of sensors allowing the recognition of bacterial and viral microorganisms, and promoting the phagocytic clearance of the microbial threat. In exerting this sterilization function, the lung environment and rAM are finely tuned to avoid an excessive inflammatory response when exposed to minor levels of inhaled particles. This way, bacterial, viral and other particulate matter is eliminated without compromising the alveolar gas exchange function. If levels of inhaled microorganisms or noxious particles do exceed threshold levels, rAM will mount an inflammatory response, enabling the recruitment

of additional phagocytes and immune defences with however an increased risk for inflicting tissue damage. Following the clearance of the antigenic threat, rAM then contribute to the resolution of inflammation by phagocytosis of apoptotic leukocytes. Besides this broad range of functions beneficial for the host, rAM also have a dark side. When the homeostatic capacity of AM is disrupted, the rAM-population becomes hyperreactive, contributing to the pathogenesis of chronic respiratory inflammation associated with diseases like asthma or COPD. Finally, rAM can be the victim of their own phagocytic capacity rendering the host vulnerable to obligate intracellular bacteria like *M. tuberculosis*.

The regulation of rAM-function is therefore of extreme importance for preserving the lung function in an environment highly exposed to microbial and environmental threats. Unravelling the complex cellular and molecular interactions at the basis of this regulatory process is therefore of utmost importance for a full comprehension of pulmonary immunology in health and disease.

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Chapter 3

Allergic Asthma: Taking Your Breath Away

3.1 The asthma syndrome

The 2011 definition of asthma by the WHO stated: *"Asthma is a disease characterized by recurrent attacks of breathlessness and wheezing, which vary in severity and frequency from person to person. In an individual, they may occur from hour to hour and day to day. This condition is due to inflammation of the air passages in the lungs and affects the sensitivity of the nerve endings in the airways so they become easily irritated. In an attack, the lining of the passages swell causing the airways to narrow and reducing the flow of air in and out of the lungs."* This definition adequately illustrates that in the last decades the perception of asthma as an uniform disease gradually evolved to a contemporary view of a heterogeneous disease made up of overlapping, yet separate symptoms and clinical expressions with probably different, but yet ill-defined, causes and natural histories. This heterogeneity is influenced by multiple factors including age, sex, socioeconomic status, ethnicity, and gene by environment interactions [1]. Today, different asthma phenotypes are generally classified according to the frequency of symptoms, forced expiratory volume in one second (FEV1), and peak expiratory flow (PEF) rate, representing a person's maximum speed of expiration. An additional classification of asthma phenotypes is based on whether symptoms are elicited by allergens (atopic) or not (non-atopic or 'intrinsic'). Non-allergic 'intrinsic' asthma generally includes infection- and exercise-induced asthma and occupational asthma.

Respiratory tract infections caused by viruses [2, 3] or *Chlamydophila* species [4] have been epidemiologically associated with asthma and have been implicated in asthma pathogenesis. During childhood, respiratory viruses like respiratory syncytial virus (RSV) and rhinoviruses, are thought to be responsible for the inception of the asthmatic symptoms in high-risk children [2]. Viral and bacterial infection-associated pathology can induce irreversible airway obstruction and airway hyperreactivity (AHR) resulting in the development of asthma symptoms at later stages of childhood. Aberrations in the innate immune response and epithelial barrier function that both facilitate viral replication might be at the origin of inadequate responses to viral or bacterial infections in children predestined to develop asthma. However, because nearly every child has been infected at least once with a respiratory virus or bacteria by the age of two years, additional factors must contribute to the development of 'infection-induced' asthma.

Sustained increased ventilation as a result of frequent heavy-duty training and competition, together with environmental factors like cold air and chlorine in pool water can cause

bronchoalveolar inflammation and AHR and are the probable causes of **exercise-induced asthma**. Although the triggering events of this asthma phenotype are well delineated, the underlying pathogenesis is poorly understood. It has been suggested that cold air induces vasoconstriction of the bronchial circulation and triggers several airway receptors, together leading to pulmonary oedema and airway narrowing. In addition, high respiratory ventilation rates of cold air during exercise lead to considerable loss of water from the lower airways. The resulting changes in osmolarity of the periciliary fluid lining the respiratory mucosal surface is thought to promote bronchoalveolar inflammation [5]. However, it is noteworthy to mention that cold air is not a prerequisite for exercise-induced asthma since breathing hot dry air can result in severe exercise-induced asthma as well [6].

Occupational asthma is a type of asthma associated with a particular work environment and is the most reported occupational respiratory disease. Generally, two types of occupational asthma are distinguished. First, atopic occupational asthma appears after acquiring immune sensitization to the causing agent and is therefore allergy based. Second, non-atopic occupational asthma occurs after exposure to high concentrations of irritants and is characterized by the absence of a preceding sensitization phase [7]. Pulmonary contact with elevated levels of chemicals elicits massive bronchoalveolar inflammation which in turn results in bronchoconstriction and other macroscopic asthma symptoms. Next to genetic predisposition, it is obvious that the working conditions and the company's prevention policy are important factors in the employee's risk for developing occupational asthma.

Finally, **allergic asthma** represents the most common asthma phenotype and is experienced by approximately 80% of the asthmatics. In addition, this type of asthma is often associated with atopy, the predisposition to develop hyperreactivity reactions and produce IgE in response to allergen. Since it constitutes the main focus of this thesis, this asthma phenotype is discussed in more detail in the subsequent paragraphs.

3.2 Allergic asthma: caught by a wolf in sheep's clothing

3.2.1 Incidence and economical impact of allergic asthma

Allergic asthma is one of the most frequent chronic diseases worldwide. It is estimated that 150 million people around the world suffer from allergic asthma. Mortality has reached over

180,000 cases annually and in Western Europe the incidence of asthma has at least doubled during the last ten years. In the United States, there were an estimated 20.3 million asthmatics in 2001; the number of asthmatics has leapt by over 60% since the early 1980s and deaths have doubled to 5,000 a year. Allergic asthma is still the leading cause of hospitalization among young children. Worldwide, the economic costs associated with allergic asthma are estimated to exceed those of TB and HIV/AIDS combined. In the United States, annual allergic asthma care costs (direct and indirect) exceed 6 billion US dollars (www.worldallergy.org).

3.2.2 Pathophysiological features of allergic asthma: the allergic cascade

Because of the ever-increasing prevalence of allergic diseases and more specific of allergic asthma during the last decades, much effort has been put into elucidating the underlying pathophysiological mechanisms. Extensive clinical and experimental research has provided us with valuable knowledge into the allergic cascade and its four temporal phases: allergen sensitization, early-phase effector reactions, late-phase effector reactions, and the chronic persistent phase.

Allergen sensitization

The mucosal surfaces lining the respiratory tract are constantly exposed to a variety of airborne harmless antigens. Depending on the characteristics and concentration of the antigen and the host's genotype, clinical history and environment, immunological sensitization may occur and the otherwise harmless antigen suddenly becomes an allergen [8]. Immature antigen-presenting cells (APC), mostly pulmonary dendritic cells (DCs), sample the allergen in the airway lumen and become activated. Alternatively, allergens can enter the tissue through a disrupted epithelial barrier or, in the case of allergens with protease activity, can gain access to submucosal DCs by cleaving epithelial tight junctions. Activated DCs mature and migrate to the draining mediastinal lymph nodes, or to local bronchoalveolar lymphoid tissue (BALT). Here, the mature DCs present allergen-derived peptides to naïve T-cells via a MHC II dependent mechanism. The presence of early IL-4 skews the differentiation of naïve $CD4^+$ T-cells towards a Th2-cell phenotype. The source of early IL-4 in local or regional lymphoid tissue during the allergic sensitization remained unclear for a long time. Only recently, it was found that IL-4 could be produced by the naïve T-cell itself, upon Notch triggering, as a

consequence of the expression of the Jagged-1 ligand by DCs [9]. Additionally, a variety of other leukocytes, including basophils, mast cells, eosinophils and natural killer (NK)T- cells have been identified as potential IL-4 producers. Th2-cells induce Ig class-switch recombination in B-cells via secretion of IL-4 and IL-13, and the ligation of suitable co-stimulatory molecules. Activated B-cells secrete allergen-specific IgE which enters the blood stream to become distributed systemically. This condition is referred to as atopy. After gaining access to the interstitial fluid, IgE molecules engage the high-affinity IgE receptor (FcεRI) on tissue resident mast cells, thereby sensitizing these cells to respond after re-exposure to the allergen (figure 3.1).

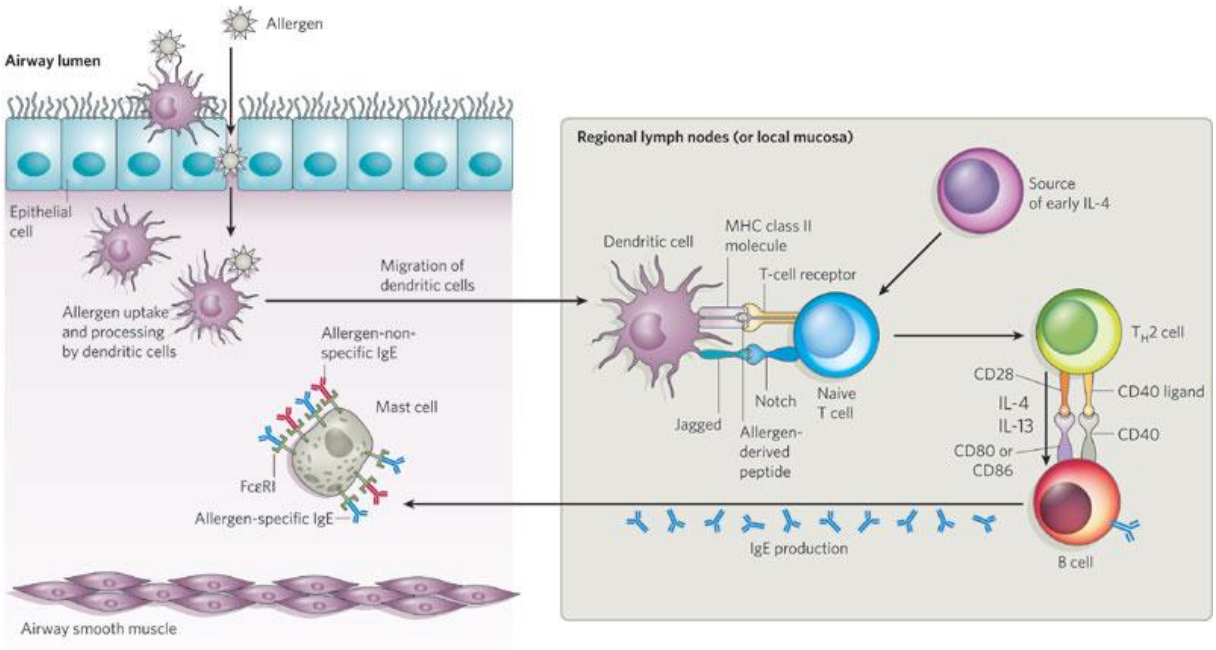

Figure 3.1: Sensitization to allergens in the airways [10]

Early-phase effector reactions

When sensitized individuals are re-exposed to the allergen, a misplaced allergic inflammatory cascade is elicited. Early-phase effector reactions occur within a few minutes after pulmonary allergen re-exposure and mainly involve mediator release by mast cells. As already mentioned, in sensitized individuals, mast cells have allergen-specific IgE molecules bound to their FcεRI. Cross-linking of adjacent FcεRI molecules by bivalent or multivalent allergens results in the rapid engagement of intracellular signalling pathways, eventually leading to the secretion of a variety of biological mediators [11]. Preformed biogenic amines (such as histamine), serglycin proteoglycans (such as heparin), serine proteases (such as tryptases) [12],

and various cytokines (like TNF-α) and growth factors are released in the external environment by the mast cell via degranulation of cytoplasmatic granules. Activated mast cells secrete *de novo* synthesised lipid mediators as well, derived from the catabolism of membrane-associated arachidonic acid into prostaglandins (PG) (particularly PGD_2), leukotriene (LT)B_4, and cysteinyl [13]-LT (especially LTC_4) [14]. Together, these rapidly secreted mediators contribute to the acute symptoms associated with the early-phase effector reactions including bronchoconstriction, vasodilation, increased vascular permeability and mucus hypersecretion [15]. Additionally, cytokine and chemokine production (mainly TNF-α and monocyte chemoattractant protein (MCP)-1 (CCL-2)) by activated mast cells promote the transition to late-phase effector reactions by inducing the recruitment of inflammatory leukocytes to the lungs [10, 16] (figure 3.2).

Late-phase effector reactions

Late-phase effector reactions typically develop two to six hours after initial allergen exposure and involve innate and adaptive leukocytes that have been recruited from the circulation by mast-cell derived cytokines and chemokines [10, 16]. Once activated, recruited immune cells, mainly Th2-cells, become the main producers of inflammatory mediators and hereby elicit a new wave of inflammatory leukocyte recruitment to the lungs [17]. Moreover, macrophages release elastases and matrix metalloproteinases (MMP) which degrade pulmonary matrix proteins like type III collagen. Eosinophil basic protein (EBP), released by degranulation of eosinophils, causes further damage to the epithelial barrier. Th2-cells are central orchestrators in this allergic airway inflammation through the secretion of a variety of key regulatory mediators, including IL-4, IL-5, IL-9, IL-13 and granulocyte-macrophage colony stimulating factor (GM-CSF) and the chemokines thymus and activation regulated chemokine (TARC) (CCL17) [18], eotaxin (CCL-11) and regulated upon activation, normal T-cell expressed, and secreted (RANTES) (CCL-5) [19]. IL-5 and GM-CSF activate eosinophils and prolong eosinophil survival while IL-4, IL-13 and IL-9 cooperate in the induction of mucus hypersecretion and goblet cell hyperplasia [20]. Pulmonary release of TARC recruits Th2-cells to lungs [21] while eotaxin is responsible for the attraction of eosinophils [22]. The secretion of RANTES leads to the recruitment of both Th2-cells and eosinophils to the lungs [10]. The activated status of Th2-cells in the lungs is maintained by local antigen presentation by DCs [23] (figure 3.2).

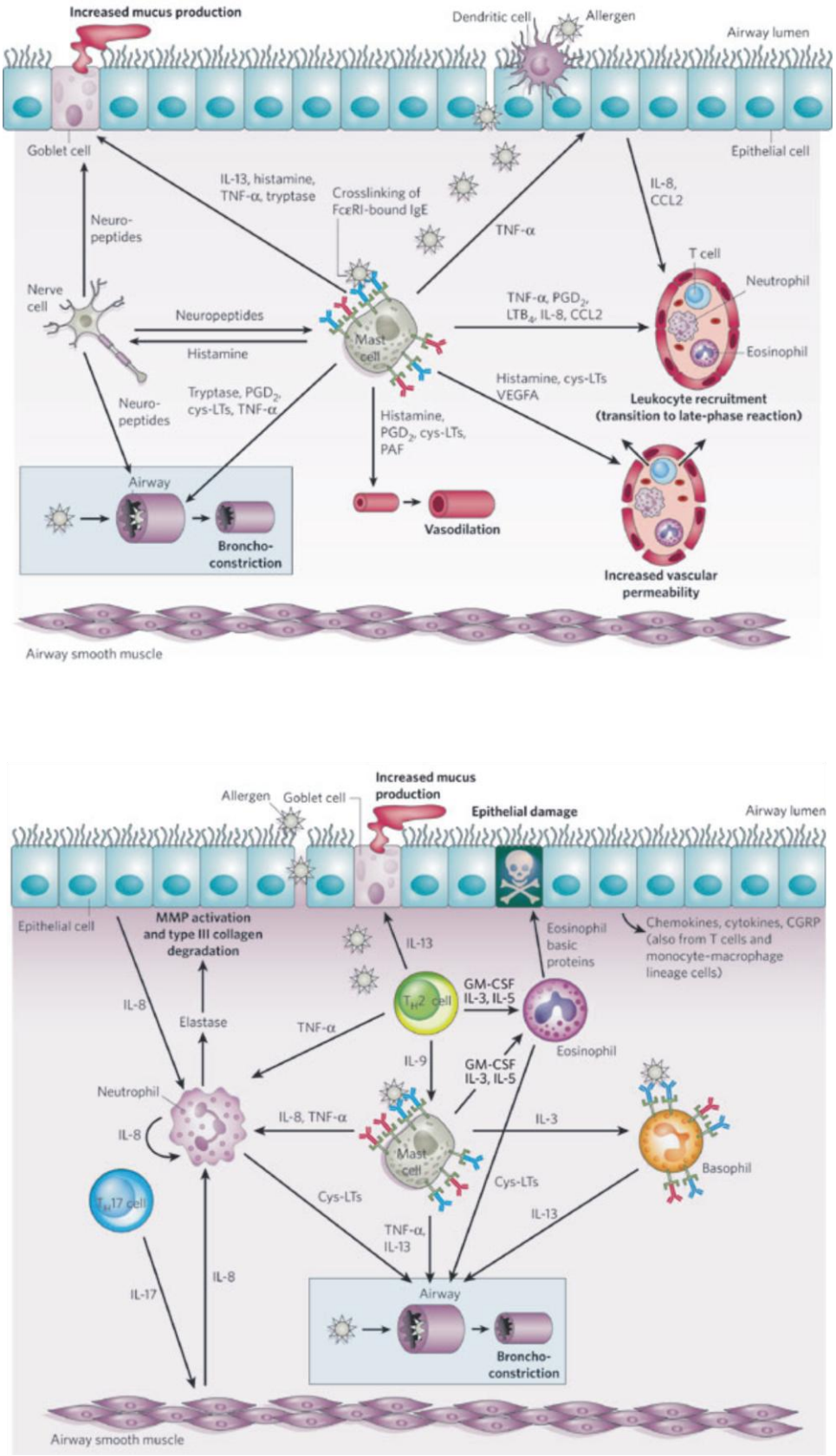

Figure 3.2: Early (upper panel) and late (lower panel) effector phase reactions of allergen induced airway inflammation [10].

Chronic stage of allergic asthma

When allergen exposure is continuous or repetitive, the bronchial inflammation persists and innate and adaptive immune cells are found permanently in the lungs. Complex interactions are initiated between recruited and tissue-resident innate and adaptive immune cells, AECs, structural cells (fibroblasts and airway smooth muscle cells), blood vessels, lymphatic vessels and nerves. This persisted inflammatory reaction is associated with irreversible changes in lung structure and function. Changes in the epithelial barrier, an increased number of mucus producing goblet cells, increased cytokine and chemokine secretion as well as areas of epithelial injury and repair are characteristically observed in patients with chronic allergic asthma. Furthermore, substantial inflammation of the submucosa is present which promotes an increased deposition of extracellular matrix (such as fibronectin and type I, III and V collagen) and a thickening of the airway smooth muscle cell layer [24, 25]. These interactions between the epithelial barrier and the underlying mesenchymal cells are referred to as the 'epithelial-mesenchymal unit' (EMT) and this unit is thought to be the actual regulator of tissue remodelling during the chronic phase of allergic asthma [26]. A continuous mutual positive feedback between the EMT and the bronchial inflammation promotes persistence of the disease (figure 3.3). Airway wall thickening due to fibroblast and goblet cell hyperplasia and excessive extracellular matrix deposition eventually causes a significant reduction of the airway luminal diameter, ranging from 10% to 30% of normal [27].

In individuals with severe narrowing of the airway lumen and persistent bronchial inflammation, AHR and airway obstruction may additionally develop, which in turn cause breathlessness and wheezing. AHR is defined as an increased bronchoconstrictor response to a nonspecific stimulus [28] and is sometimes referred to as 'twitchy' airways. In asthma patients who exhibit AHR, nonspecific stimuli like air pollutants, dust, or cold air produce a marked exacerbation of asthmatic symptoms. Infection with common respiratory viruses such as rhinoviruses, influenza viruses and RSV induce severe asthmatic exacerbations as well [29]. The precise mechanism that controls AHR is poorly understood. However, changes in epithelial cell function [29] and mast cell hyperactivation are thought to be involved [30, 31]. The magnitude of AHR correlates with the level of airway inflammation but other factors including reduced airway diameter, increase in smooth muscle contractility, degree of epithelial injury, dysfunctional neuronal regulation, increase in microvascular permeability, and various leukotriene inflammatory mediators have been associated with AHR as well [20]. In the most

severe cases of airway narrowing by airway remodelling, AHR-induced bronchoconstriction can have a fatal ending.

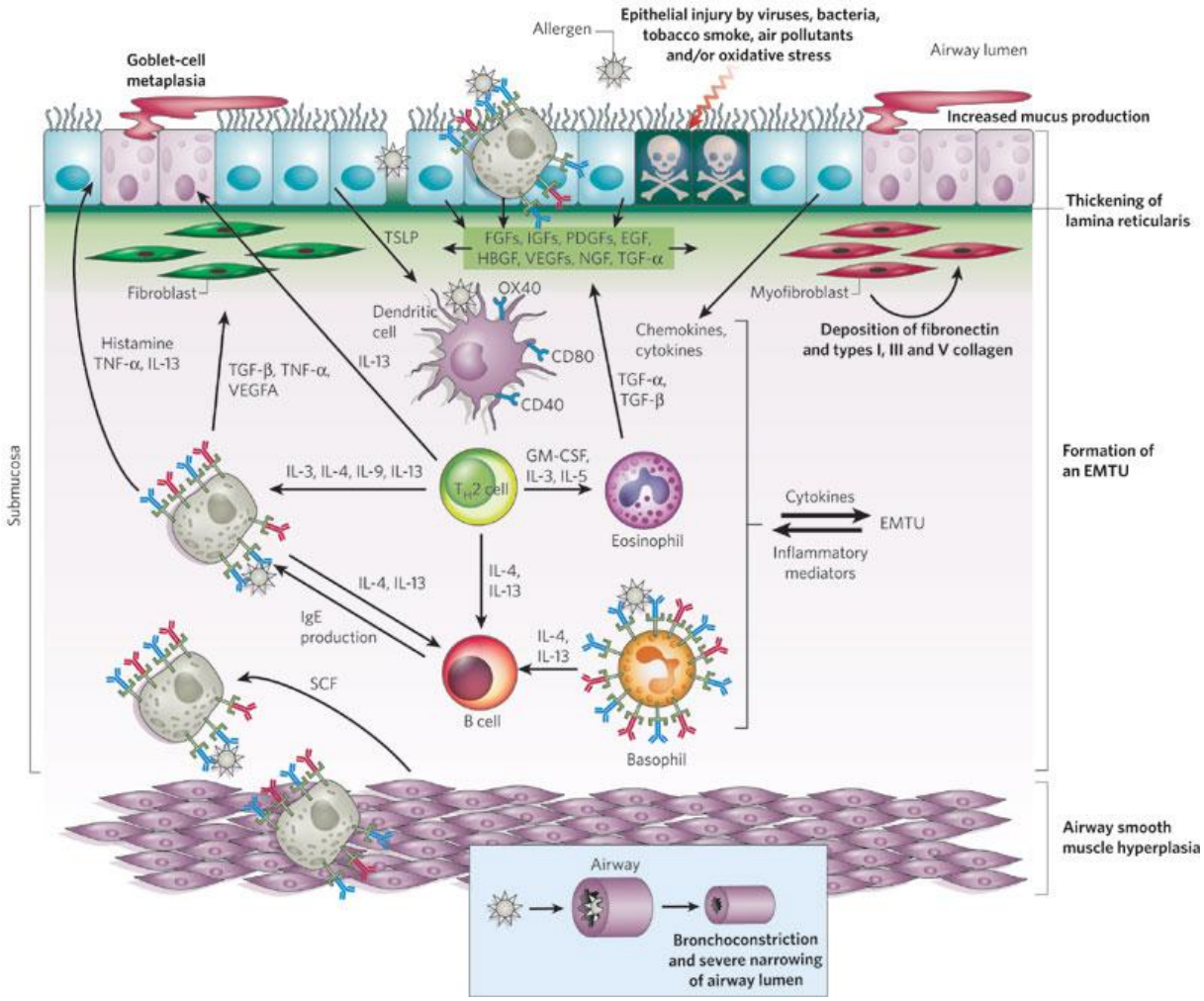

Figure 3.3: Chronic stage of allergic asthma [10].

3.2.3 New players in allergic asthma pathophysiology: asthma beyond Th2-cells

The nomination of Th2-cells and eosinophils as main orchestrators of the pathophysiological features of allergic asthma constituted the central 'allergic asthma dogma' for years. However, asthma is a heterogeneous disease and the emphasis on a Th2-bias and eosinophilic bronchial inflammation fails to explain several clinical observations. During the last decade, a variety of new cell types and cytokines have been identified as critical contributors to the allergic asthma associated pathophysiology. Th1-cells have been observed in the respiratory tract of allergic asthmatics suffering from the 'classical' mild-to-moderate eosinophilic Th2-mediated allergic asthma phenotype. However, mouse models revealed that Th1-cells alone

were not able to induce any of the characteristics of allergic asthma [32]. On the contrary, a number of studies showed that IFN-γ producing Th1-cells inhibited the development of Th2-induced eosinophilia, mucus production and AHR. Shifting the balance towards a Th1-response therefore has long been considered as a potential therapeutic approach for allergic asthma treatment [33-35]. Yet, other studies have shown that Th1-cells enhance pulmonary inflammatory responses and AHR [36, 37] through the recruitment and activation of Th2-cells in the absence of antigen. In addition, Th1-cells are involved in viral infection induced asthma exacerbation through the production of IFN-γ [38]. These conflicting results may indicate that the timing of Th1-cell activation is crucial in determining the net outcome – attenuation versus exacerbation – of the asthmatic response in mild to moderate asthma.

In severe asthma patients a neutrophilic Th1- and Th17-mediated bronchial inflammation is predominant. Although this phenotype of asthma is a less common clinical presentation of the disease, accounting for fewer than 10% of all asthma patients, it contributes to a disproportionate burden of healthcare and economical costs, morbidity and mortality [39]. It has been suggested that as mild or moderate allergic asthma becomes more chronic and severe, the inflammatory phenotype changes from a Th2- towards a Th1- and Th17-type of inflammation [40]. Yet, neutrophilic asthma is observed in a subset of patients with nonatopic, non-IgE dependent asthma as well [41, 42]. In agreement with the Th1-biased neutrophilic nature of the inflammation, Th1-associated cytokines (IFN-γ, IL-12) and a variety of CXC-chemokines are found in a mouse model of neutrophilic asthma [43]. In recent years, Th17-cells have gained increasing attention as potential regulators of this neutrophilic inflammation in patients with severe and uncontrolled asthma. This $CD4^+$ T-cell subset produces IL-17 which causes the release of neutrophil chemoattractant cytokines IL-8 and macrophage inflammatory protein (MIP)-2 from AECs [44]. Increased expression of IL-17A and IL-17F subclasses was detected in the bronchial submucosa of patients with severe asthma [45, 46]. Furthermore, it has been reported that increased AHR in response to methacholine positively correlates with IL-17A levels in the sputum of these patients [47] and that a polymorphism in IL-17F that results in a loss-of-function mutation is inversely related to asthma risk [48]. Moreover, severe asthmatics, exhibiting a neutrophilic Th1- and Th17-mediated pulmonary inflammation, are characterized by unresponsiveness towards corticosteroid treatment. Th17-cells are believed to be involved in the corticosteroid resistance of this subset of asthma patients through the secretion of IL-17 [49].

Besides their potential role in severe neutrophilic asthma, Th17-cells may also play an important role in mild to moderate Th2-biased asthma. IL-17 promotes Th2-responses by synergizing with Th2-derived IL-4 and IL-13 [50-52]. In support of these findings, several studies suggest a pro-inflammatory role for the Th17-axis through the actions of IL-22 and IL-23. Besides IL-17, IL-22 is the other main cytokine produced by Th17-cells and performs similar functions as IL-17 [53]. Increased mRNA transcript and protein levels of IL-22 have been observed in a mouse model of eosinophilic asthma [54]. IL-23, a member of the IL-12 cytokine family, is produced by innate immune cells like DCs and macrophages, and is a potent inducer of Th17-differentiation. Transgenic mice that overexpressed IL-23 in the airways (CCSP-IL-23 mice) showed increased eosinophilia and Th2-cytokine levels whereas IL-23$^{-/-}$ mice showed reduced eosinophilia [55, 56].

Recently, a novel subset of human memory CD4^{+} T-cells that produces both IL-17A and IL-4 has been identified. This novel population of Th17/Th2-lymphocytes was more represented in the blood of patients with allergic asthma than in the blood of healthy donors [57]. The existence of a subset of memory/effector CD4^{+} T-cells that co-expresses the main Th2- and Th17-differentiation transcription factors, GATA-3 and RORγT, was recently reported in mice too. In particular, in a mouse model of allergic asthma, adoptive transfer of allergen-specific IL-17-producing Th2-cells resulted in goblet cell hyperplasia, elevated mucin production and inflammatory leukocyte recruitment to the airways. In contrast, mice that received conventional Th2- or Th17-cells exhibited less airway infiltration of eosinophils or neutrophils respectively, and limited pathophysiological features [58]. It is hypothesized that the Th17/Th2-lymphocyte subset originates from the activity of IL-4 on IL-4 receptor expressing Th17-cells and/or from the activity of IL-1β, IL-6 and IL-21 from innate and tissue cells on Th2-cells [57].

Besides Th2-, Th17-, Th2/Th17- and Th1-cells, a fifth Th-subset, consisting of IL-9 producing Th9-lymphocytes, has been identified as an additional participator in the allergic airway response. Studies in which IL-9 overexpressing transgenic mice [59] and IL-9$^{-/-}$ mice [60] were used, already confirmed the involvement of this cytokine in the development of the allergic eosinophilic airway inflammation, airway remodelling and AHR. Initially, IL-9 production during allergic airway eosinophilia was attributed to Th2-lymphocytes. Comparative analysis of different T-cell subsets for cytokine production identified however a novel Th-cell subset, distinct from the Th1-, Th2-, and Th17-subsets, as an important source

of IL-9 [61, 62]. Yet, the presence of this Th9-subset in the airways during allergic asthma remains to be demonstrated. Nevertheless, the existence of this novel Th-subset opens an exciting new area in the field of allergic asthma due to the important role of IL-9 in the development and maintenance of allergic airway inflammation and airway remodelling.

The role of additional innate cytokines and cells in the generation of allergic pulmonary inflammation is summarized in textbox 1.

- **IL-25**, a member of the IL-17 cytokine family, is secreted by activated eosinophils, mast cells, basophils, and AECs in response to allergens such as ragweed [63]. IL-25 may potentiate Th2-cell airway inflammation and can induce AHR in the absence of Th2-cytokines. Thus, IL-25 is produced during the acute phase of the allergic pulmonary inflammatory response and subsequent IL-25 dependent pathology is generally mediated through downstream Th2-cytokines such as IL-4 and IL-13 [64].
- **IL-33** is a member of the IL-1 cytokine family and many cellular sources during both the innate and adaptive immune components of allergic inflammation are suggested to release IL-33. Murine *in vitro* and *in vivo* studies reviewed in [64] demonstrated that this cytokine acts in the effector phase of the allergic pulmonary eosinophilic inflammation to augment Th2-cytokine production and inflammation via direct effects on both Th2-cells and innate effectors. In addition, IL-33 can generate AHR and goblet cell metaplasia in the absence of adaptive immunity, using pathways that bypass the requirement of Th2-lymphocytes [64].
- **Thymic stromal lymphopoietin (TSLP)** is an IL-7 like cytokine mainly produced by AECs in response to allergens during both the innate and adaptive setting of the immune response. TSLP is thought to activate DCs in order to prime naïve $CD4^+$ T-cells to release Th2-cytokines such as IL-4, IL-5, and IL-13 [65]. TSLP can also directly enhance the Th2-mediated cytokine secretion [66] and can drive the proliferation of activated $CD4^+$ T-cells [67]. Mice with transgenic overexpression of TSLP in AECs displayed a spontaneous pulmonary eosinophilic inflammation with goblet cell metaplasia, perivascular fibrosis, and AHR. Conversely, TSLP $receptor^{-/-}$ mice were protected from antigen-induced pulmonary inflammation [68]. Both studies highlight the role of TSLP as key initiator of the allergic pulmonary Th2-response.
- **Natural killer (NK)T-cells** form a heterogeneous group of cells that share properties of both T-cells (such as the presence of TCR) and NK-cells (such as the expression of the NK1.1 marker). Murine studies have demonstrated that NKT-cells, and more specific the NKT-cell derived cytokines, are elementary for the development of AHR and structural airway changes in allergic asthma since NKT-deficient mice exhibited diminished AHR [69]. Thus, whereas NKT-cells have previously been viewed as cells amplifying established allergic airway inflammation, these innate cells are actually able to initiate pulmonary innate and adaptive host immune responses against allergens as well.
- **γδ T-cells** represent a small subset of T-cells that possess a TCR composed of a γ- and δ-chain. Different studies in humans demonstrated the presence of Th2-type cytokine secreting γδ T-cells in the airways of asthma patients [70, 71]. Also in mice, this T-cell subtype was found to contribute to allergic airway inflammation [72] and the development of AHR [73]. More recently however, it was shown that different subsets of γδ T-cells can have a different influence on the development of airway inflammation, Th2-cytokine secretion and AHR. For instance, $V\gamma1^+$ γδ T-cells enhance AHR while $V\gamma4^+$ γδ T-cells suppress AHR [74, 75]. Therefore, the overall role γδ T-cells in the development of asthma pathogenesis remains uncertain and needs to be further elucidated.

- **Nuocytes or natural helper cells** were recently identified as lineage (lymphocyte, macrophage, DC, basophil, eosinophil, mast cell and NK-cell) negative and ICOS positive innate lymphoid cells which express both the IL-25 receptor (IL-17RB) and the IL-33 receptor (T1/ST2) [76]. It was recently demonstrated that, upon stimulation by IL-25 or IL-33, these cells represent the major innate source of pulmonary IL-13 during lung allergy in mice [77]. Moreover, nuocyte-derived IL-13 plays a crucial role in the development of subsequent airway eosinophilia and AHR [77]. Therefore, nuocytes are considered as important new players in the field of allergic asthma research.
- **Basophils** are mature circulating granulocytes recruited to peripheral tissues in the setting of allergic inflammation and regained interest for their role in allergic eosinophilic asthma. Through recent research, it became clear that basophils are actually critical checkpoints in the development of Th2-immunity. First, basophils deliver a crucial contribution of IL-4 and TSLP during the onset of the allergen-induced airway Th2-response [78]. Second, basophils can also participate in the production of all subgroups of antigen-specific Ig-molecules [79]. Therefore, the biology of this small granulocyte population forms a revisited field of interest for acquiring new insights in the eosinophilic allergic asthma pathology.

Textbox 1: Novel innate cytokines and cells in the generation of allergic pulmonary inflammation.

3.2.4 Endogenous induced tolerance and immune regulation in allergic asthma

Non-asthmatic individuals develop tolerance to allergens that protects against allergic asthma, as manifested by a lack of clinical symptoms in these individuals. Initially, investigators attributed the lack of symptoms in non-allergic individuals to the absence of allergen-specific immune responses. However, this theory is outdated and the precise mechanisms of tolerance are still poorly understood although our understanding has evolved extensively over the past several years.

Hygiene hypothesis and gene-environment interactions

A wide variety of epidemiological observations regarding the effect of environmental factors on the development of allergic asthma have provided important insights into the protective immunity that occurs in non-asthmatic individuals. While the prevalence of allergic asthma dramatically increased over the past two decades, exposure to certain environmental infectious diseases decreased. This observation suggests that certain infections may lower the risk of developing allergic asthma, presumably by enhancing protective tolerogenic immunity against allergens. These observations form the basis for the 'hygiene hypothesis', which suggests that improved hygiene in western societies, together with improved public health

measures and the use of vaccines and antibiotics has reduced the incidence of infections that normally would stimulate the immune system [80]. Additional data that support the hygiene hypothesis include observations that children from large families (having older siblings), children placed in day-care settings in the first year of life (with presumed exposure to infectious agents) or exposure to farm animals in early life all have a reduced risk in developing allergic asthma [81]. Exposure to bacterial endotoxin in childhood may be critical for producing this protective effect. Bacterial and viral infections during childhood increase the 'immunological threshold' of an individual and may expand the Th1-lymphocyte arsenal, which in turn can counterbalance the induction of Th2-responses [82]. More recently, it was demonstrated that early childhood exposure to antibiotics, which may alter the gastrointestinal flora, and exposure to intestinal endotoxin is associated with an increased incidence of allergic asthma [83]. These data point out the crucial character of TLR-signalling by commensal bacteria under normal steady-state conditions in the maintenance of pulmonary epithelial cell homeostasis and the induction of immune tolerance. Moreover, these observations highlight the fundamental relationship between environmental immune stimulation and the development of allergic asthma.

Next to the environment, the host's genetic background delivers a significant contribution to the development of allergic asthma as well. Numerous studies have shown that allergic asthma has a familial nature. For instance, children of asthmatic patients are more likely to develop allergic asthma than those of parents without any history of atopy. Via association studies, positional cloning and genome-wide association experiments several genes or gene loci associated with allergic asthma have now been identified. Allergic asthma susceptibility genes are classified into four main groups [8].

- The first group consists of **innate immunity and immunoregulatory genes**, such as genes encoding PRRs (CD14, NOD1/2, and TLR-2, -4, -6 and -10), regulatory cytokines, TGF-β and IL-10, and transcription factors like STAT-3.
- Asthma susceptibility genes that belong to the second group are associated with **Th2-cell differentiation and effector functions**. These include genes encoding for GATA-3, T-bet, STAT-6, IL-4, and IL-13.
- **Genes implicated in mucosal immunity and AEC biology** are categorized in the third group. Especially genes involved in the structural integrity of the airway epithelial barrier are of major importance, since loss-of-function mutations can lead to a diminished epithelial barrier function which increases the permeability and subsequent sensitization for all kinds of allergens.

- Finally, the fourth group of allergic asthma susceptibility genes consists of **genes linked to lung function and airway remodelling** and comprises genes encoding for metalloproteinases, collagen types, and cytokine receptors in AECs and airway smooth muscle cells.

Environmental factors, like air pollutants, tobacco smoke, and diet, are interacting with these allergic asthma susceptibility genes and such gene-environment interactions influence the net outcome of allergen encounters. Thus, it is increasingly clear that the complex triptych interplay between an individual's personal history of life circumstances, genetic background and environmental factors 'decides' whether a particular individual develops an allergic asthmatic response or not to airborne allergens (figure 3.4).

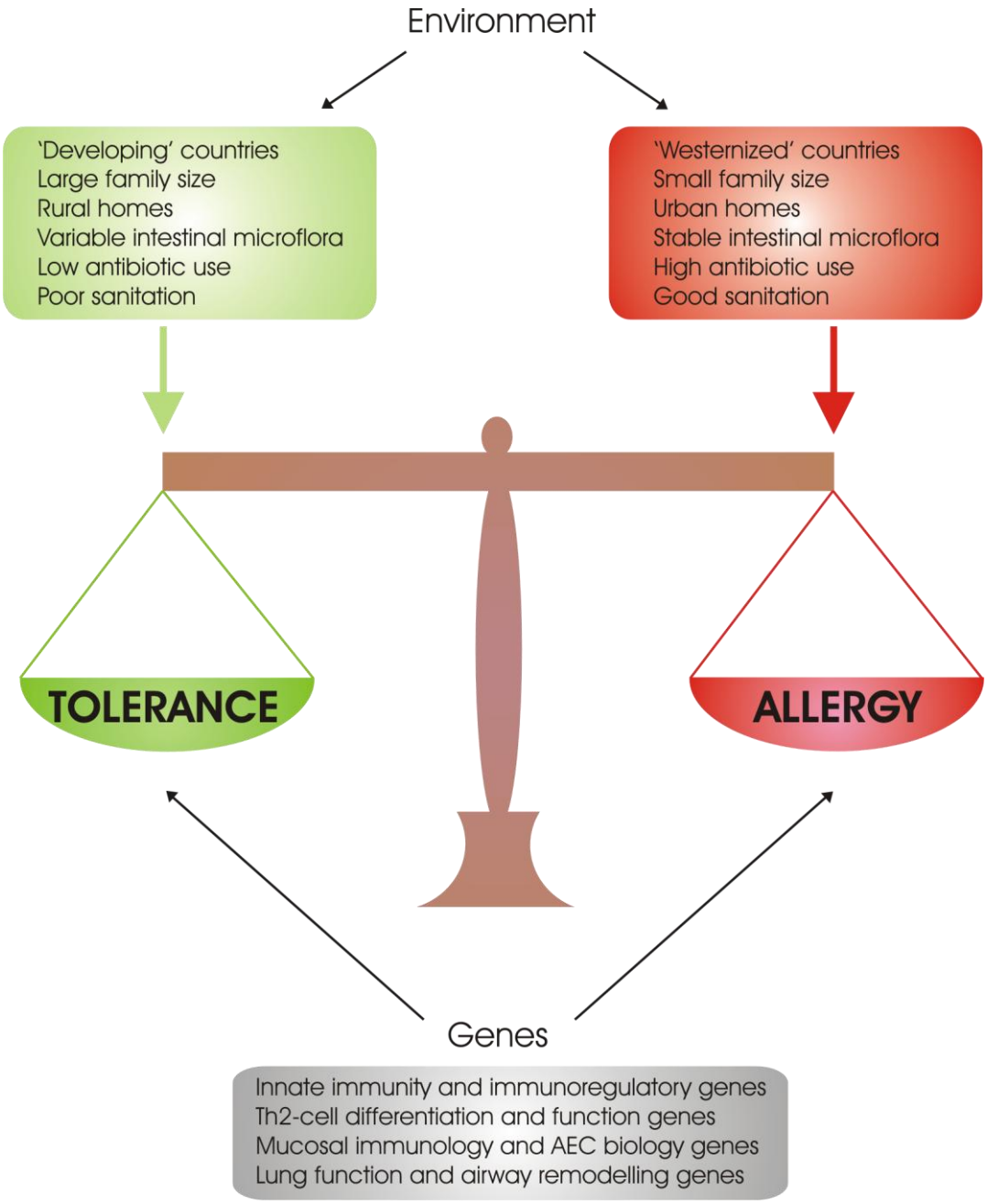

Figure 3.4: Hygiene hypothesis and gene-environment interactions in the development of allergic asthma. The tilting of the balance towards tolerance or allergy is largely influenced by a combination of environmental factors to which the individual is exposed during life and the individuals' genetic background. Adapted from Wills-Karp et al [84].

Cellular and molecular mechanisms of endogenous tolerance to allergens

Immunological studies in non-allergic individuals have demonstrated the presence of allergen-specific serum antibodies of the IgG4-, but not IgE-isotype. These data indicate that non-allergic individuals actually do respond to environmental allergen exposure and are not

ignorant for allergens. Thus, both allergic and non-allergic individuals respond to allergens, but with different forms of immunity (immune deviation) such that non-allergic individuals remain asymptomatic. Allergen tolerance involves multiple mechanisms but a large amount of clinical and experimental research indicated that regulatory T(reg)-cells fulfil a profound role in preventing Th2-responses to allergens. Today, several subtypes of Treg-cells have been described, including central, thymus-generated natural Treg-cells (nTreg) and peripheral, exogenous antigen-specific inducible Treg-cells (iTreg).

In general, Treg-activity is elicited by so-called regulatory DC-subsets, present in mucosal milieus, including the airway mucosa. When airborne antigen is inhaled in the absence of pro-inflammatory signals, it can be phagocytosed by myeloid (m)DC without promoting DC-maturation. Subsequently, antigen-harbouring immature (or partially mature) airway mDCs will migrate to the lung-draining lymph nodes (LDLN) and can elicit a Treg-response. In this case, antigen-specific Treg-cells develop due to the absence of immunogenic co-stimulators, such as CD80, CD86 and CD40, and the secretion of suppressive mediators like IL-10 [85].

A central role in inducing Treg-responses is reserved for airway plasmacytoid (p)DCs. Specific depletion of this regulatory DC-subset in the airways resulted in the loss of inhalation tolerance and the development of features of severe asthma, even in the absence of an adjuvant. Conversely, tolerance was induced after intratracheal injection of cultured pDCs [86, 87]. Therefore a novel concept has been proposed in which the balance between immunogenic mDC- and tolerogenic pDC-mediated antigen uptake plays a pivotal role in regulating the decision between immunity and tolerance against allergens. pDCs elicit central and peripheral Treg-responses through an inducible co-stimulator (ICOS)-ICOS ligand interaction during antigen-presentation [88]. Also, human rAM have been shown to actively induce Treg-cells [89]. These findings suggest that rAM, which express very low levels of co-stimulatory molecules, may induce a form of tolerogenic T-cell responses in the lung similar to that induced by immature mDCs. In addition, it was recently shown that $Foxp3^+$ Treg-cells, expressing membrane-bound TGF-β coupled to latency-associated peptide (LAP), induced Foxp3-expression in naïve $CD4^+$ T-cells which in turn acquired a suppressive phenotype. This alternative process of Treg-induction is referred to as 'infectious tolerance' [90]. Figure 3.5 gives an overview of central and peripheral Treg-cell development.

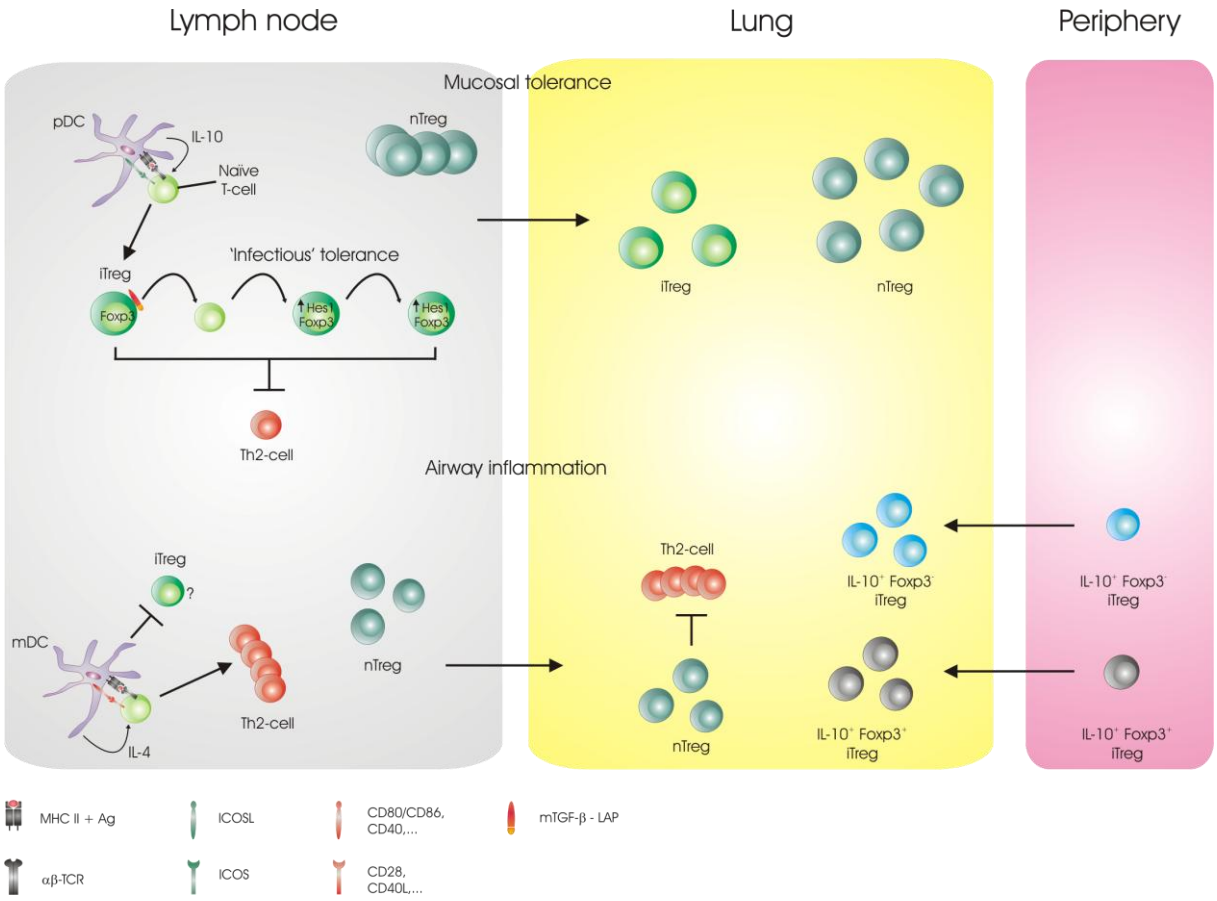

Figure 3.5: Development of Treg-subpopulations in lymph nodes and lungs in allergic and non-allergic individuals.
In non-allergic individuals, antigen-loaded airway pDCs migrate to the LDLN and induce Treg-development through secretion of IL-10 and ICOSL-ICOS co-inhibitory interactions during antigen-presentation. Subsequently, the pool of iTregs is increased via so-called 'infectious tolerance'. In this process, LAP-associated mTGF-β on iTregs induces Hes1 expression in naïve $CD4^+$ T-cells. In turn, Hes1 stabilizes Foxp3-expression in these cells. The iTregs, together with nTregs, efficiently suppress effector Th2-cell development. In allergic individuals, antigen-loaded airway mDCs migrate to the LDLN and induce Th2-development through secretion of Th2-cytokines, such as IL-4, and co-stimulatory interactions during antigen-presentation. Although iTreg-development is suppressed, IL-4 still promotes nTreg proliferation. The Th2-cells and nTregs traffic to the tissue in recall response to inhaled allergen. If priming occurs at a distant site (skin, spleen), IL-10-secreting $Foxp3^-$ and $Foxp3^+$ iTregs are also recruited to the lung in response to allergen challenge to suppress effector T-cell functions.

Two molecules that currently receive the most attention with respect to Treg-mediated suppression of allergic airway inflammation are TGF-β and IL-10. Membrane-bound TGF-β expressed by iTreg-cells can activate Notch-signalling in naïve $CD4^+$ T-cells [91]. Notch-engagement induces the expression of its downstream target gene Hes1 (hairy and enhancer of split 1), a potent repressor of pro-inflammatory gene expression [92]. In addition, Notch-signalling in Tregs stabilizes and promotes Foxp3-expression, the key transcription factor in Treg-differentiation [93]. Although Notch-activation has also been linked with effector Th-cell differentiation, it is likely that the strength of Notch-ligation and the duration of expression of

its target genes, such as Hes1, differs when T-cells are activated or suppressed [94]. Tr1-cells form a subpopulation of iTreg-cells and are an important source of the immunosuppressive IL-10 cytokine. IL-10 induces T-cell anergy, probably via the inhibition of co-stimulation of T-cells [95]. This cytokine has also effects on Ig-isotype switching in B-cells by promoting the secretion of the tolerogenic IgG4 and inhibiting the production of IgE [96]. Recently, research identified IL-35 as a novel inhibitory cytokine that may be specifically produced by nTreg-cells. IL-35 is required for maximal suppressive activity and its inhibitory activity is promoted by IL-10 [97].

With regard to other mechanisms that occur in Treg-mediated suppression, it was shown that activated nTreg-cells from humans and mice contain increased levels of granzyme A [98] and granzyme B [99] respectively. Moreover, it was demonstrated that human and mouse Treg-cells possess cytolytic activity and are capable to elicit granzyme/perforin mediated apoptosis in effector T- and B-cells [98, 100]. Furthermore, several intriguing suppressive mechanisms have been described that collectively mediate 'metabolic disruption' of the effector T-cell target. First, Treg-cells are thought to induce cytokine deprivation-mediated apoptosis [101]. Because of the constitutive expression of CD25, IL-2 is thought to be the key T-cell survival cytokine that is deprived by Treg-cells. However, IL-2 depletion by Treg is not sufficient to suppress effector T-lymphocytes [102]. Second, adenosine nucleoside generation and release via the ectoenzymes CD39 and CD73 by Treg-cells suppresses effector T-cells through the activation of adenosine receptor 2A ($A_{2A}R$) [103]. Interestingly, binding of adenosine to $A_{2A}R$ appears not only to inhibit effector T-cells, but also to enhance the generation of iTreg by inhibiting IL-6 expression and promoting TGF-β secretion [104]. Third, Treg-cells were also shown to suppress effector T-cell function directly by transferring the potent inhibitory secondary messenger cyclic AMP (cAMP) into effector T-cells through membrane gap junctions [105].

In addition to the direct effect of Treg-cells on T-cell function, Treg-cells may also modulate the maturation and/or function of innate cells. A recent study showed that Treg-expressed OX40, via ligation of OX40L, inhibits mast cell degranulation [106]. Furthermore, Treg-expressed cytotoxic T-lymphocyte antigen (CTLA)-4 [107] and lymphocyte activation gene (LAG-3) [108] was found to mediate immune suppression of lung DCs through negative signalling via co-stimulatory molecules, like CD80/CD86, and MHC class II molecules respectively.

A schematic overview of Treg-cell mediated suppression is depicted in figure 3.6.

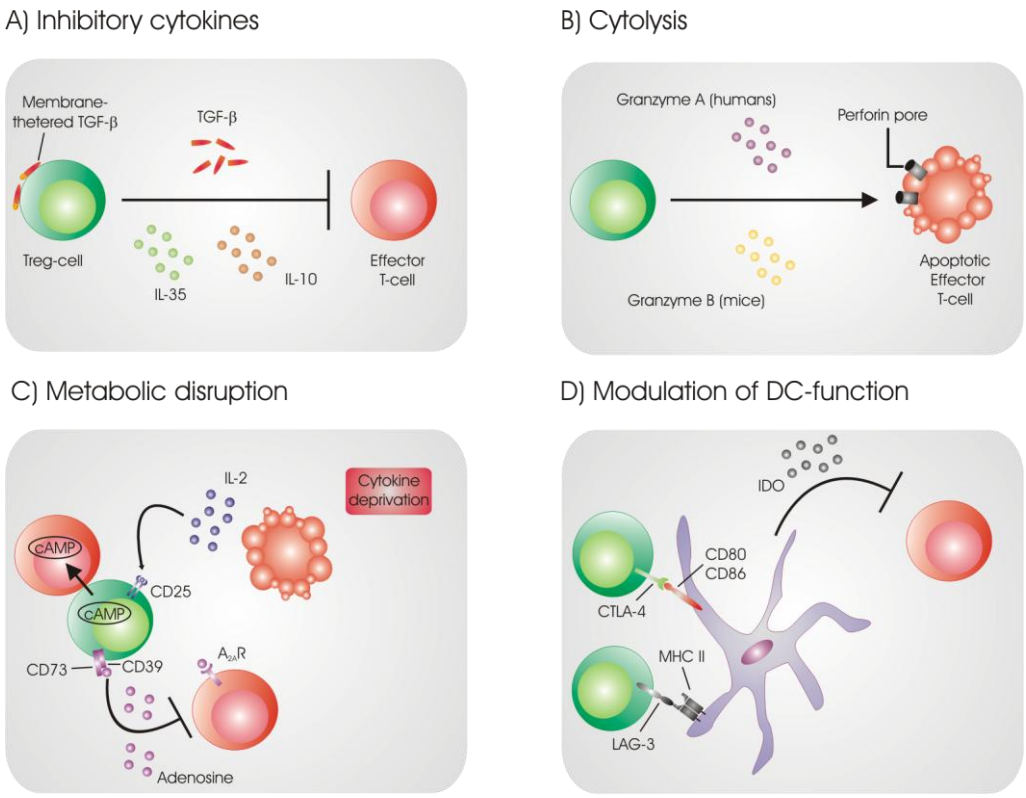

Figure 3.6: Basic mechanisms used by Treg-cells.
a) Inhibitory cytokine (IL-10, IL-35 and TGF-β) mediated suppression. **b)** Treg-induced cytolysis includes granzyme-A (humans) and granzyme-B (mice) dependent and perforin dependent killing mechanisms. **c)** Treg-mediated metabolic deprivation includes high-affinity CD25 (also known as IL-2 receptor ∝)-dependent cytokine deprivation-mediated apoptosis, cAMP-mediated inhibition, and CD39- and/or CD73-generated, $A_{2A}R$-mediated immunosuppression. **d)** Targeting DCs includes mechanisms that modulate DC-maturation and/or -function such as LAG3 (CD223) – MHCII-mediated suppression of DC-maturation, and CTLA-4 – CD80/CD86-mediated induction of IDO-secretion by DCs.

3.2.5 Animal models of allergic asthma: opportunities and pitfalls

Studies in animals have made crucial contributions to our current understanding of the pathophysiology of allergic asthma. Rodents (rats and mice) are the most commonly used species to model allergic asthma because they are relatively cost-effective and a wide range of tools are available to perform detailed mechanistic studies. The use of murine models has the additional advantage that transgenic technology can be employed, since the complete mouse genome sequence has been unravelled [109]. Nevertheless, mice do not naturally develop allergic asthma and the outcome of inhaling inert allergens without prior adjuvant supported systemic sensitization results in immunological tolerance as is the case with non-atopic humans [110]. To circumvent these endogenous tolerance mechanisms, the majority of the models rely on a biphasic protocol. First, mice are systemically sensitized for the allergen

(generally ovalbumin; OVA) via repeated intraperitoneal (ip) administration of the allergen together with a Th2-skewing adjuvant such as aluminium hydroxide ($Al(OH)_3$; alum). Subsequently, the sensitized mice are exposed to allergen through multiple airway challenges for one to ten consecutive days in acute models and up to five weeks in 'chronic' models [111].

Exposure of OVA/alum sensitized mice to nebulised OVA elicits bronchial inflammation characterized by the infiltration of numerous eosinophils and Th2-lymphocytes. The type of inflammatory response found in the airways of these mice together with the presence of antigen-specific serum IgE, resembles the inflammatory phenotype observed in the majority of mild to moderate asthmatic patients. In humans however, the eosinophilic inflammation is localized in the bronchial wall whereas in mice inflammation is peribronchial, perivascular and parenchymal. Another relevant problem that mouse models of allergic asthma encounter is the chronicity of the model. Most mouse models of allergic asthma concern relatively short-term (up to 10 days) exposure to high concentrations of allergen. This is in strong contrast with the recurrent long-term exposure to low concentrations of allergen experienced by asthmatic humans. Prolonged exposure of sensitized mice to high concentrations of allergen eventually leads to downregulation of inflammation and establishment of long-lasting tolerance [112, 113]. In addition to eosinophilic inflammation, airway remodelling and AHR are important chronic clinical features of allergic asthma patients. Therefore, the use of 'standard' short-term models for allergic asthma does not fulfil the demands for studying these chronic asthma symptoms. However, several research groups have developed improved models that better mimic chronic clinical symptoms. Fore instance, exposing sensitized BALB/c mice to prolonged, yet intermittent, exposure to carefully controlled low concentrations of allergen resulted in AHR and significant airway remodelling, whether or not in the presence of an accompanying eosinophilic inflammation. Furthermore, these chronic models of allergic asthma exhibit persistent accumulation of inflammatory leukocytes in the airway epithelial wall and lamina propria and minimize the generation of parenchymal and perivascular inflammation. Conversely, no significant AHR or airway lesions were observed when this protocol was applied to C57BL/6 mice, although this mice strain is often used in the regular short-term models for allergic asthma [114-117]. Clearly, the genetic background of the mouse strain is a determining factor in the establishment of a chronic asthma model as well.

The last decade, other allergens than OVA are employed in allergic asthma research, comprising pollen, house dust mite, ragweed, molds and cockroach proteins. These agents are

airway allergens encountered in real-life. Most of these substances, like house dust mite and cockroach proteins, are auto-sensitizing which means that sensitization can be achieved without the supportive action of an adjuvant. These allergens possess an intrinsic enzymatic activity and therefore often induce limited tissue destruction in the airways, accounting for the necessary (endogenous) danger signals [118]. The use of these compounds allows us to gain more insight in the process of allergen sensitization via the respiratory route which occurs in most human cases of allergen sensitization.

The current insight in the heterogeneous character of allergic asthma has lead to the emerging need for the development of novel animal models representative for the different asthma subtypes. A subtype that gained a great deal of interest in the last several years is severe refractory asthma, featuring a steroid-resistant, neutrophilic, Th1/Th17-driven bronchial inflammation. Recently, a model of this type of allergic bronchial inflammation was proposed, based on the use of the Th1-/Th17-skewing adjuvant Complete Freund's Adjuvant (CFA) instead of the Th2-skewing alum adjuvant. Exposure of OVA/CFA immunized mice to nebulised OVA resulted in a steroid-resistant Th1-/Th17-mediated neutrophilic bronchial inflammation [43]. Again, this model only covers the inflammatory features of the human disease and not its aetiology or more chronic features.

3.2.6 Overview of current and future allergic asthma therapies

Initial approaches to treat allergic asthma emphasised the relief of bronchoconstriction via the use of bronchodilators, particularly β_2-adrenergic agonists. The discovery of airway inflammation as an important pathophysiological component of allergic asthma has lead to the widespread use of corticosteroids as the mainstay of asthma therapy [119]. Today, these drugs are still the most effective anti-inflammatory treatment available for asthma. Treatment with inhaled corticosteroids reverses airflow obstruction and reduces exacerbations which in turn reduces hospitalization and asthma-related deaths and improves quality of life [120]. Despite their efficiency in suppressing inflammation, long-term corticosteroid therapy can however have detrimental side-effects, including cataracts, osteoporosis in elderly patients, and stunting of growth in children [121]. The effectiveness of inhaled corticosteroids, particularly at low to moderate doses, in controlling asthma and reducing exacerbations is improved by combination with long-acting β_2-agonists. This combined treatment protocol has been shown to be more successful than the use of higher doses of inhaled corticosteroid alone [122].

Therefore, this approach is successfully applied in 90% of all asthmatic individuals [123]. In addition, anti-histamines, CysLTR1-antagonists, and mast cell stabilisers can also aid in the treatment. However, all of these therapeutics do not modify the progression of allergic asthma and are not curative [124]. New drugs that alter the course of the disease or that provide a cure with fewer side-effects are therefore urgently needed. Moreover, the presence of a significant population of individuals suffering from severe steroid-resistant asthma strengthens the need for better therapy.

The drive to find new drug targets has lead to the introduction of new classes of anti-mediator agents that are currently in clinical practice. A first class of potential targets for asthma treatment includes inflammatory cytokines and chemokines, transcription factors, enzymes, and immune cell populations. Recruitment of inflammatory cells to the airways by chemokines is a crucial process in the development of asthma. Administration of antisense oligonucleotides that inhibit expression of CCR3 and IL-5, the major mediators of eosinophil recruitment and activation respectively, resulted in decreased sputum eosinophilia, but only a trend for a reduced late response was noted [125]. Cytokines are major targets for asthma therapy because of their key role in (chronic) inflammation and airway remodelling [126]. Throughout years, numerous inhibitory compounds - blocking antibodies, soluble receptors, and protein muteins - against key pro-inflammatory cytokines, including IL-4, IL-5 and IL-13, have been developed and applied in the clinic with varying success. Since adhesion molecules play an important role in several facets of asthma such as leukocyte migration, exocytosis, and respiratory burst, much effort has been made to develop drugs that target these molecules [127]. However, clinical trials of these agents have been disappointing and some of these drugs were placed on hold by the US Food and Drug Administration because of serious side-effects.

Since asthma patients often are atopic, much effort has been directed at the development of a second class of new therapies in which endogenous immunomodulatory mechanisms are exploited. Humanised monoclonal antibodies that bind and thereby block IgE or the low-affinity IgE-receptor (FcεRII or CD23) have been successfully introduced for the treatment of severe refractory asthma [128, 129]. Modulation of DC-function represents a new approach to treat allergic asthma. By selectively antagonizing DC-activation receptors, such as the sphingosine-1 phosphate receptor [130], or agonizing DC-inhibitory receptors, such as the D prostanoid (DP)-1 receptor [131], suppression of DC-function may be achieved, which

eventually may result in decreased airway inflammation and AHR. Subcutaneous allergen-specific immunotherapy increases for instance the production of anti-inflammatory IL-10 by Treg-cells [132, 133]. In addition, oral administration of vitamin D3 restores the ability of Treg-cells to release IL-10 in steroid-resistant asthma patients. This observation suggests that vitamin D3 could potentially increase the therapeutic response to corticosteroids in steroid-resistant asthma patients [134]. Thus, immunomodulatory therapies that reverse the aberrant immune reactivity observed in allergic asthma by for instance enhancing Treg-function or skewing T-cell class switching away from Th2-responses are very promising. The fact that combination therapies are more effective than monotherapy emphasizes the need to evaluate multidrug approaches that are tailored to the genotype and phenotype of the particular asthma patient.

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Chapter 4

Allergic Asthma and Secondary Respiratory Infections

4.1 Respiratory syncytial virus (RSV)

4.1.1 RSV-epidemiology and clinical manifestations

RSV-infection is one of the leading etiological agents responsible for lower respiratory tract infections. Over 70% of one year old children and 100% of children by the age of two have been infected at least once by the RSV-virus [1]. Infection of the nose or eyes occurs by large particle aerosol or direct contact and results in viral replication in the nasopharynx, with an incubation period of four to five days. Eventually, the incubation period can be followed by spread to the lower respiratory tract [2]. Although almost all young children have encountered a RSV viral infection, only a small proportion develops serious lower respiratory tract illness, such as bronchiolitis and pneumoniae, while the majority shows mild respiratory tract symptoms. Nevertheless, RSV-infection is the most frequent viral respiratory cause of hospitalization in infants and young children worldwide [1]. The causal mechanisms are unclear. It is currently believed that environmental factors that affect lung function (e.g. in-house tobacco use) or that increase exposure to infection (e.g. day care, multiple siblings,…) play an important role. In addition, genetic predisposition to severe RSV-infection is indicated by the association of susceptibility with a family history of severe infant lower respiratory tract disease as well as by differences in susceptibility between ethnic, racial and gender groups [3]. More recently, studies have provided evidence that genetic polymorphisms in genes encoding different cytokines and chemokines, and differences in cytokine production at birth are associated with the diversity in the clinical presentation of RSV-disease [4]. In adults, symptoms from RSV-infection usually manifest themselves as rhinitis. However, severe symptoms are commonly observed in the elderly and in immunosuppressed adult patients [5, 6]. Clinical reports have also shown that RSV-infection may cause extra-pulmonary effects at the neurological, endocrine, cardiac and hepatic level [7]. Although the causes leading to these extra-pulmonary symptoms remain elusive, it is possible that both direct organ infection by RSV and damaging inflammatory responses in the lung promoted by the virus contribute to the observed extra-pulmonary effects [8, 9].

To date, only one antiviral drug – ribavirin - is commercially available for treating severe RSV-infection. However, the use of this drug is controversial due to its variable efficacy and questionable cost-effectiveness ratio. Therefore, new pharmacological alternatives for treating RSV are required in order to diminish the adverse inflammatory response elicited by the unbalanced immune response induced by the exposure to the virus. The identification of

different RSV-proteins, including the G- and F-glycoproteins, has allowed developing vaccine candidates. Unfortunately, to date the insufficient efficacy of these vaccines has precluded their introduction into the clinic.

4.1.2. Pathophysiology of RSV-infection

RSV-virion

RSV are spherical or filamentous envelope viruses that belong to the *Paramyxoviridae* family and are characterized by a single stranded negative sense RNA genome (15.2 kb) encoding two non-structural proteins (NS1 and NS2) and nine structural proteins, including the attachment (G) and fusion (F) glycoproteins. The virion of the RSV is enveloped with a lipid bilayer, which is obtained via budding from the host's plasma membrane. A symmetrical helix shaped nucleocapsid is embedded in this lipid bilayer. Five nucleocapsid proteins inside the virion, including the nucleocapsid (N)-protein, the phospho(P)-protein, the antitermination factors M2-1 and M2-2, and the large polymerase (L)-subunit carry out the replication and transcription of the RSV-genome [10]. In addition, the lipid bilayer contains three surface glycoproteins, the G-, the F-, and the small hydrophobic (SH)-protein, which are all separated from each other and can be seen as "spikes" that protrude out of the virion. The F-protein forms trimers and its major function is to direct viral penetration by the fusion between the virion and the host plasma membrane. When expressed on the cell surface, the F-protein also mediates fusion with neighbouring cells, forming syncytia. The G-protein is a type II transmembrane glycoprotein and is a major RSV-attachment protein [11]. It contains a single hydrophobic region which serves as a signal peptide and also as a membrane anchor. The small SH-protein is a short integral membrane protein whose function is unknown. However, it is suggested that the SH-protein enhances the function of the G-protein and/or F-protein. Another RSV-protein is the matrix (M) protein, located in the inner layer of the lipid bilayer. The M-protein is found to play a role in the assembly of virus particles inside the host cells [12]. The remaining two RSV-proteins, NS1 and NS2, are small proteins that appear to be nonessential accessory proteins involved in modulating the host response to infection [13].

RSV-replication cycle

RSV predominantly infects airway epithelium and the first critical step in the infection process is the entry of the virus into the cell. The G-glycoprotein plays a major but not

exclusive role in viral attachment, since recombinant RSV in which the gene encoding for the G-glycoprotein is deleted, is still able to infect mouse and human airway cells, albeit with much lower efficiency [14, 15]. The cellular receptor for the G-glycoprotein has not yet been identified although cell surface glycosaminoglycans, including heparan sulphate and chondroitin sulphate B have been shown to be involved in viral attachment [16]. In contrast to the G-protein, RSV has an absolute requirement for the F-protein for orchestrating the viral penetration by membrane fusion and for mediating the fusion of infected cells with their neighbours through the formation of syncytia. Furthermore, membrane fusion between RSV particles and host epithelial cells via clathrin-mediated endocytosis is considered as a possible mechanism allowing the nucleocapsid to enter the cytoplasm [17]. Viral gene expression and RNA replication occur in the cytoplasm. The nucleocapsid N-proteins tightly encapsulate the viral genomic RNA as well as its positive-sense replicate intermediate, which is referred to as the antigenome. This encapsulation is thought to shield off the viral RNA molecules from immune detection by the host's intracellular TLRs and viral RNA recognition helicases [18]. The large L-protein is the major polymerase subunit and contains the catalytic domains while the P-protein acts as an essential cofactor in viral RNA synthesis [19]. The M2-1 and M2-2 proteins are crucial factors involved in progressive transcription [20] and in regulating the balance between viral RNA transcription and replication [21] respectively. In the absence of M2-1, transcription terminates non-specifically within several hundred nucleotides and results in (reduced) expression of NS1 and NS2 alone [20]. Finally, the M-protein mediates the assembly of the viral RNA-genome, the viral envelope proteins and the nucleocapsid proteins to new RSV viral particles. These eventually leave the cell either through budding to the extracellular space, fusion and syncytia formation with the adjacent cell or following cell rupture.

Host response to RSV-infection

The respiratory epithelium is the first site of encounter between the virus and the host. As a result of this interaction, an early innate immune response is initiated at the site of infection. RSV-attachment to epithelial cells leads to the detection of viral components by several pattern recognition receptors (PRR), like TLR and retinoic acid-inducible gene I-like receptor (RIG-I). TLR-3, expressed by epithelial cells and resident alveolar macrophages (rAM), contributes to the recognition of RSV by binding to viral RNA [18]. The F-protein has been identified as a TLR-4 and CD14 ligand on human monocytes [22]. Furthermore, respiratory

epithelial cells also express TLR-2 and TLR-6 which have been shown to be involved in the control of viral replication [23]. Although TLR-7 expression is upregulated on lung epithelial cells as early as 1h after RSV-infection, the involvement of this receptor in cytokine secretion and modulation of RSV-pathology is only poorly evaluated. It was not until recently that RSV and measles virus were described as the first viruses capable of blocking type I interferon (IFN)-secretion through TLR-7 and TLR-9 [24]. In agreement with this observation, deletion of TLR-7 was found to worsen the RSV-induced pathology [25]. In addition, substantial changes in TLR-expression can be observed which are likely to play an important role in the clinical outcome of the infected individual [26]. For instance, it has been described that expression of TLR-4 is significantly increased in epithelial cells after RSV-challenge and during the inflammatory response induced by the virus [27]. Early RSV-detection by rAM plays an important role in the initial clearance of the virus since rAM-depletion prior to infection results in increased pulmonary virus titers [28].

Activation of PRRs at airway epithelial cells (AECs) or rAM induces widespread changes in the cellular expression of genes encoding for a variety of factors, including surfactants, cytokines, chemokines, and cell surface molecules. Some of these factors exert direct antiviral properties while others stimulate the influx and activation of inflammatory leukocytes. Engagement of TLRs and RIG-I stimulates the NF-κB pathway leading to the production of TNF-α, IL-6, CCL2, RANTES and IL-8. These chemokines and cytokines promote the recruitment and activation of especially neutrophils, natural killer (NK)-cells and monocytes [29]. NK-cells are important effector cells in viral clearance by orchestrating cytotoxic lysis of infected airway cells. Another major function of activated NK-cells is the production of early IFN-γ which primes the subsequent antiviral adaptive Th1-cell and cytotoxic T-lymphocyte (CTL) immune response [30]. Another set of important molecules that are secreted by immune and non-immune cells, mainly plasmacytoid (p)DCs, rAM, and AECs upon RSV-infection, are IFN-α and IFN-β. Expression of type I IFNs is induced by the IFN-regulatory factors (IRF) transcription factors after TLR-ligation. Once secreted, type I IFNs bind in an autocrine or paracrine way to surface receptors belonging to the type I IFNAR-complex, which in turn activate intracellular signalling pathways that promote the activation of antiviral responses [31, 32]. This type I IFN-induced cellular antiviral response is characterized by the expression of IFN-stimulated genes (ISGs) that trigger antiviral effectors, such as Mx GTPase, RNA-dependent protein kinase (PKR), ribonuclease L (RNase L), oligo-adenylate synthetase (OAS). Mx GTPase captures viral nucleocapsid proteins in the cytoplasm and blocks their

movement into the nucleus. Active PKR phosphorylates eukaryotic initiation factor 2α (eIFα) which leads to the inhibition of viral transcript translation, thereby blocking viral protein synthesis. Viral RNA-induced activation of OAS results in the conversion of ATP into 2',5'-linked oligomers of adenosine (2-5A), which binds to RNase L. The binding of 2-5A to RNase L enables the latter to cleave viral RNA. On the other hand, RNase L cleaves some self mRNAs in order to produce small RNA fragments which again act as ligands for RIG-I. These events further amplify the type I IFN production and the overall innate antiviral host response [33]. In addition to type I IFNs, surfactant protein (SP-)A and D also appear to play a role in the antiviral defence by binding to the viral surface glycoproteins F and G [34]. Once bound, SP-A and SP-D have been reported to neutralize the virus and to increase viral uptake by rAM and neutrophils. Recent studies linking surfactant gene polymorphisms to RSV-susceptibility in children further emphasize the importance of this innate defence mechanism [35, 36].

CTLs deliver a major contribution to the clearance of the RSV primary infection, and there is a robust expansion of RSV-specific $CD8^+$ T-cells in the lung. Human CTLs, recognizing the N-, SH-, F-, M-, M2- and NS2-proteins are already identified [37]. Upon activation, CTLs carry out cytolysis of RSV-infected host cells through the release of perforin and granzyme. However, perforin and granzyme-mediated killing of infected target cells is not compulsory for the clearance of the virus. Alternative killing mechanisms, such as FasL-mediated induction of target cell apoptosis, have been found to be involved in the clearance of the virus [38]. By being a significant source of IFN-γ, Th1-cells contribute to the activation of CTLs and NK-cells, and to the amplification of the host's protective adaptive immune response. The importance of the cellular adaptive immune response against RSV has been demonstrated by adoptive transfer of sensitized $CD4^+$ or $CD8^+$ T-lymphocytes in RSV-infected mice. Both cell types reduced shedding of the virus in the lungs [39]. The protective role of cellular adaptive immunity during RSV-infection is however still a matter of debate due to its potential association with pulmonary tissue damage. For instance, in mice lacking both $CD4^+$ and $CD8^+$ T-lymphocyte subsets, RSV was cleared very slowly but did not induce any significant disease [40]. Furthermore, adoptive transfer of sensitized $CD4^+$ or $CD8^+$ T-lymphocytes to RSV-infected mice induced increased pulmonary tissue damage [39]. Thus, next to their role in RSV-immunoprotection, both $CD4^+$ T-cells and CTLs are also potent inducers of RSV-associated immunopathology.

Although the humoral immune response is not likely to influence the course of the primary RSV-infection, there is good evidence that protection against subsequent RSV-infections is mediated through neutralizing antibodies. Serum antibody titers increase following RSV-reinfection, and there is a gradual acquisition of protective antibodies in the serum [41, 42]. Patients of any age with low titers of serum neutralizing antibodies are at greater risk for developing severe, lower respiratory tract RSV-disease than those with high antibody titers [43]. Additionally, breast fed infants with high titers of transplacentally acquired antibodies are less likely to succumb to severe RSV-induced bronchiolitis [44] and passive immunization with RSV-specific IgG has been shown to be effective in protecting against hospital admission [45]. Neutralizing antibodies include secretory IgA and serum-derived IgG. Secretory IgA is particularly important in protecting the upper respiratory tract, which is accessed only very inefficiently by serum IgG [46]. The IgA-response is short-lived following primary infection but can increase in duration following reinfection. Serum IgG-antibodies are more efficient in accessing lower respiratory tract and can provide substantial protection in that compartment. Upon RSV-reinfection, IgA- and IgG-molecules neutralize the invading particles by opsonising them for subsequent rAM-mediated phagocytosis [10]. Because neutralizing serum antibodies provide substantial protection, eliciting a strong and potent humoral response is a promising and intensively investigated vaccination strategy.

4.1.3 RSV-infection and asthma: the chicken or the egg?

Wheezing illnesses, such as allergic asthma, have been associated with viral respiratory infections since many years. The relation between respiratory viral infections and the development of asthma has been best characterized for respiratory infections due to RSV. Clinical studies highlighted the fact that hospitalization due to RSV-infection in early childhood promotes the development of asthma during later life [47-50]. This possible causative correlation was also suggested in an elegant epidemiological study in which it was shown that timing of birth in relationship to the annual winter RSV-peak predicts an infant's likelihood of developing childhood asthma. Infant birth approximately four months before the seasonal RSV winter peak resulted in a 29% increase in odds of developing asthma in later life compared to birth 12 months before the peak [51]. Furthermore, it was shown that ameliorating or preventing RSV lower respiratory tract infections during infancy was associated with a decrease in recurrent wheezing at later age. For instance, prophylactic use of palivizumab

(Synagis), a humanized monoclonal antibody against the RSV F-protein, substantially reduced hospitalization for severe RSV-infections in infants [52]. The prevention of hospitalization for severe RSV-infections in palivizumab-treated children was associated with a decrease in the development of wheezing at later age [53]. However, palivizumab prophylaxis only decreased later recurrent wheezing in nonatopic children whereas infants with an atopic family history were not 'protected' [54]. These results highlight the crucial role of the individual's genetic background in the delicate interplay between early RSV-infections and the development of recurrent wheezing or asthma at later age. Indeed, by comparing the correlation between severe RSV-infections during infancy and the development of asthma during later life in monozygotic and dizygotic twins Thomsen and co-workers identified a common genetic source for both disorders. Moreover, they stated that severe RSV-infections during infancy are not a direct cause of asthma but rather an indicator for the underlying genetic predisposition to asthma [55]. A variety of experimental data obtained from animal models supported this premise. For instance, guinea pigs infected with RSV and then exposed to aerosolized allergen developed higher titers of allergen specific IgG1 compared to non-infected allergen challenged animals [56]. Furthermore, prior RSV-infection of BALB/c mice resulted in increases in AHR after subsequent pulmonary exposure to nebulised allergen [57]. Different properties and characteristics of the RSV-infection cycle and pathology can contribute to these observations. The first contact of RSV with the host's target AECs may already act as a potential instigator of allergic asthma by promoting allergen sensitization. Initial interactions between RSV-particles and the airway epithelium through TLR-4 increase the expression of this PRR on AECs. RSV-induced TLR-4 expression may then hypersensitize AECs to LPS, augmenting their ability to initiate inflammatory responses to low amounts of LPS in allergen particles [27]. Additionally, during the propagation of the RSV-infection, viral induced damage to the pulmonary epithelial barrier provides endogenous danger signals which can function as adjuvant during host sensitization for allergens. In order to avoid the antiviral effects of CTL- and Th1-responses, RSV features a number of properties that skew the host's immune response from a protective CTL-/Th1-response to non-protective Th2-immunity. After the initiation of RSV-infection, the typical Th2-cytokines IL-4, IL-5, IL-10 and IL-13 are produced in the lungs by different hematopoietic and non-hematopoietic cells [58]. These cytokines have been associated with delayed viral clearance and are important in promoting AHR [59] and inducing B-cell proliferation and IgE production [60]. In addition, these cytokines upregulate the expression of MHC II molecules on the surface of macrophages which in turn increases the APC-activity of these cells during pulmonary

allergen encounter [61]. Persistence of these inflammatory Th2-cytokines together with other RSV-induced pro-inflammatory cytokines, including IL-6, IL-1Rα, IL-1β, and GCF, in fully recovered patients with RSV can provide a substratum for the development of subsequent asthma [62]. Indeed, blocking IL-13 during primary RSV-infection in mice that were subsequently sensitized and challenged with allergen reduced the levels of AHR [63]. Furthermore, T-cells from children hospitalized for RSV in their infancy secreted more IL-4 in response to aeroallergens than control subjects [64]. Chemokines produced during RSV viral infection may contribute to the enhanced development of consequent allergic asthma as well. For instance, RSV-infection induces the secretion of eotaxin (CCL11) which is responsible for the chemoattraction and activation of eosinophils [65]. Studies in which mice were infected with RSV virus and then sensitized to allergen suggest that RANTES (CCL5) might fulfil an important role in the predisposition of virally infected mice to allergic asthma too [66]. Moreover, RSV-infection of conventional myeloid DCs significantly decreased the ability of this leukocyte population to induce Th1-responses while the expression of IL-10 was increased [67-69]. Thus, in an ongoing RSV-infection, allergens might induce a skewed Th2-response due to the alteration of DC-subset activation. Finally, RSV-pathology can provoke structural lung remodelling, including smooth muscle cell hypertrophy and mucus hypersecretion, resulting in airway luminal narrowing which in turn may increase AHR during subsequent allergen exposure.

Several epidemiological studies have implicated a number of respiratory viruses in the induction of asthma exacerbations, including infection with rhinoviruses, influenza, parainfluenza, and adenovirus. However, more recent research demonstrated that severe asthmatic exacerbations in children [70] and adults [71] were often associated with RSV-infection (27% and 37% respectively). Infection of AECs with RSV induces the secretion of a wide range of cytokines and chemokines which are able to initiate a pulmonary inflammatory response [29]. Leukocyte populations, including neutrophils, eosinophils and $CD8^+$ T-lymphocytes accumulate in the lungs during RSV-infection. The combination and interplay of inflammatory mediators released by these different leukocytes can increase AHR and airway remodelling, eventually leading to the exacerbation of asthmatic reactions. For instance, elastase released through neutrophil degranulation may cause airway obstruction by promoting goblet cell mucus secretion [72]. Eosinophil granule-derived proteins have also been retrieved from nasal secretions of asthmatic children with wheezing illness caused by RSV [73]. In these children, significant increases in the level of CCL5 were detected as well [74]. In line

with this observation, different studies suggest a critical participation of CCR1, the main CCL3/CCL5 receptor expressed on both Th1- and Th2-lymphocytes, in RSV-associated allergic asthma exacerbations. In allergic $CCR1^{-/-}$ mice, subsequent RSV-infection resulted in a reduction in AHR and mucus production, accompanied by a reduced amount of IL-13 in the lungs as well as reduced numbers of T-lymphocytes and eosinophils as compared to wild-type mice [75]. CCR1-expression on T-cells during acute RSV-infection is therefore thought to exacerbate allergic airway disease through increased recruitment of both allergen specific and virus specific T-lymphocytes, possibly sharing a similar chemokine receptor profile, to the lung and lymph nodes [76].

Also exaggerated mucus production is a hallmark of asthma exacerbation and significantly contributes to morbidity and mortality in asthma [77]. The mucus that occludes the airways during an asthma exacerbation is quite a complex biological material, comprising a mixture of mucin proteins, plasma proteins, and products of dead cells [78]. In asthmatic subjects, the major mucin components of airway mucus secretions are MUC5AC and MUC5B, both contributing to the viscoelastic properties of the mucus [79]. Lukacs and colleagues have shown that RSV-infection induces secretion of MUC5AC and GOB5 by AECs in an IL-17 dependent manner [25]. Therefore, by acting as an additional inducer of pulmonary MUC5AC production, RSV can increase total mucin protein levels in the lungs of asthmatic individuals, thereby increasing the risk to develop asthma exacerbation. Interestingly, in animal models, $CD8^+$ T-lymphocytes are involved in the response to both allergens and viruses [80]. Thus, the role of $CD8^+$ T-lymphocytes for virus-induced asthma exacerbation has become an area of great interest due to the realization that these cells might influence Th2-type cytokines during the response of asthmatic individuals to viral infection.

Research through the past years has highlighted the fact that individuals suffering from allergic asthma are likely to be more prone to developing secondary respiratory viral infections, such as RSV. Corne and co-workers demonstrated the occurrence of more severe and prolonged virus-induced symptoms in asthmatic patients compared to non-asthmatic control subjects, suggesting for the first time that there might be inherent differences in the way asthmatics respond to respiratory tract viral infections [81]. Wark and co-workers subsequently provided mechanistic insight into this clinical observation. Compared to AECs from non-asthmatic subjects, rhinoviral infection of AECs from asthmatic subjects showed a diminished secretion of IFN-β accompanied by higher levels of viral replication [82]. As IFN-β

plays a pivotal role in the innate antiviral defence against RSV as well, impaired airway epithelial IFN-β responses might underlie the enhanced susceptibility of asthmatic individuals for RSV viral infections. At present, the precise mechanism of deficient IFN-β production in asthmatics remains unknown. Genetic polymorphisms in genes encoding transcription factors or signalling molecules required for the expression of type I IFNs is one plausible explanation [83]. Interestingly, recent experimental work highlighted the possibility that excess TGF-β, present in the lungs of asthmatic patients, can be responsible for the observed enhanced viral replication through its suppressive actions on IRF-3 which is responsible for *ifn-β* gene transcription [84]. As already mentioned, the balance between Th1- and Th2-cell cytokine production is crucial to viral clearance. There are data supporting the notion that within a pre-existing type II cytokine asthmatic microenvironment, the normally effective type I antiviral immune response might be inhibited because of the presence of Th2-cytokines. Children suffering from asthma and exhibiting an increased Th2-cytokine profile during RSV-induced bronchiolitis, were at greater risk of developing wheezing during follow-up compared to non-asthmatic control children [85]. Recently, it was also demonstrated that TLR-7 function is impaired in blood mononuclear cells from adolescents with mild-to-moderate asthma [86]. Together with the observation that the pulmonary response to RSV-infection was more pathogenic in TLR-$7^{-/-}$ mice, as assessed by significant increases in inflammation and mucus production [25], impairment of TLR-7 function in asthmatics may increase the susceptibility to RSV-infection. In recent years, a large number of genetic polymorphisms relevant for both viral infection and asthma have been identified too. Polymorphisms in the gene encoding for IRF-1, a pivotal regulator of IFN-γ production, have been associated with the development of allergic disease or atopy [87]. In addition, a significant association between genetic polymorphisms in the promoter region of the gene encoding for suppressor of cytokine signalling (SOCS)1 and adult asthma has been observed. This polymorphism resulted in the increased production of SOCS1 protein, which in turn inhibited phosphorylation of STAT-1 in response to IFN-β stimulation [88]. It is likely that as technology continues to improve, future studies will reveal other polymorphisms playing a key role in the immune response of asthmatic individuals to (RSV) viral infection.

Understanding the mechanisms provoking RSV-induced exacerbations in asthmatic subjects may offer significant opportunities for improved disease management. Prevention of infection through the use of vaccines or monoclonal antibodies would be by far the most effective therapeutic approach. In addition, the use of antiviral agents, such as viral attachment

inhibitors or viral protease inhibitors should be considered when treating hospitalized subjects suffering from severe asthmatic exacerbations. Furthermore, as allergic asthmatic individuals are likely to exhibit deficient antiviral IFN-β responses, exogenous IFN-β supplementation would boost the host's antiviral reactivity. Nevertheless, real therapeutic success has been hampered because of problems of virus specificity, side effects, delivery problems, and the need for early administration after diagnosis of infection. Therefore, in-depth studies of the molecular pathways underlying virus-induced inflammation and pathology are still required to identify new targets for controlling virus induced asthma exacerbations.

A schematic overview of the correlation between RSV-infections and allergic asthma is depicted in figure 1.

4.2 *Chlamydophila pneumoniae* (*C. pneumoniae*)

4.2.1 *C. pneumoniae* epidemiology and clinical manifestations

C. pneumoniae is a common cause of acute respiratory infection, including community-acquired pneumonia (CAP) [69], pharyngitis, bronchitis, sinusitis, and exacerbations of chronic bronchitis [89]. The ubiquity of *C. pneumoniae* infection is evidenced by an antibody prevalence of 50% in individuals by the age of 20 years and of 80% at the age of 60 years. Primary infection occurs mainly in school-aged children, while reinfection is observed in adults. In addition, this bacterial pathogen is reported to account for a relatively large number of cases (6 – 20%) of CAP, which means about 1000 cases per 100,000 U.S. inhabitants [90]. The exact mode of transmission is unknown but spread via droplets has been proposed. Most patients with *C. pneumoniae* infection are asymptomatic but the course of respiratory illness can vary widely from mild to severe disease. Upper respiratory tract symptoms, such as rhinitis, sore throat, or hoarseness, may be reported initially, followed by fever, myalgia and chills [91]. After gradual onset, symptoms may continue over extended periods, with persistence of cough and malaise for several weeks or months despite appropriate antibiotic therapy. Especially individuals with severely compromised respiratory function suffer from severe *C. pneumoniae* induced CAP [92]. CAP has also been associated with severe acute respiratory exacerbations in patients with cystic fibrosis and *C. pneumoniae* infection can even become life-threatening in patients with acute leukemia and treatment-induced neutropenia [93, 94]. *C. pneumoniae* is also found to be involved in the development of extra-pulmonary pathologies

such as atherosclerosis, multiple sclerosis and Alzheimer disease [90]. Next to the host's immunological status, environmental factors such as poor hygienic conditions and smoking can increase the susceptibility for developing *C. pneumoniae* infection. Finally, as is the case with many infectious diseases, an individual's genetic background is a major factor contributing to *C. pneumoniae* infection susceptibility.

So far, tetracyclines, erythromycin, and doxycycline are the most commonly employed antibiotic drugs in the first-line treatment of acute *C. pneumoniae* infections. Although they are very effective against *C. pneumoniae* viability, antibiotic drugs cause some adverse (gastrointestinal) side-effects and the use of it should be preferably avoided. Therefore, ongoing research should provide more insight in *C. pneumoniae* microbiology and pathophysiology to develop more customized therapies or prophylactic vaccination with minimal side-effects.

4.2.2 Pathophysiology of *C. pneumoniae* bacterial infection

C. pneumoniae *microbiology and replication cycle*

C. pneumoniae are Gram-negative aerobic bacteria with an obligate intracellular replication cycle. This bacterial species lacks the machinery for providing its own ATP supply and is therefore fully dependent on the host's intracellular ATP stock for survival and reproduction. They are typically coccoid or rod-shaped bacteria surrounded by a cell wall with inner and outer membranes. The cell wall contains an outer LPS-membrane but lacks peptidoglycan. It instead contains cysteine-rich proteins that are likely the functional equivalent of peptidoglycan. Furthermore, typical surface-associated chlamydial macromolecules, including major outer membrane protein (MOMP) and OmcB (OMP2) are contained within the cell wall. This unique cell wall structure allows for intracellular division and extracellular survival. The bacterial nucleoid contains circular DNA and plasmid DNA. Ribosomes for protein synthesis are scattered around in the bacterial cytoplasm.

Chlamydophila has a very unique biphasic life-cycle in which it alternates between a non-replicating, infectious elementary body (EB) and a replicating, non-infectious reticulate body (RB). The EBs are pear-shaped structures containing a periplasmic space and a loose outer membrane [95] and have closely associated periplasmic minibodies whose functional significance is unknown [96]. The EB is the metabolic inert and dispersal form of the pathogen

and is analogous to spore structures. Therefore, *C. pneumoniae* is perfectly capable of extracellular survival. Furthermore, the EB is devoid of peptidoglycan and maintains structural integrity via a network of disulfide cross linkages involving MOMP. In the form of an EB, the bacterium induces its own endocytosis upon contact with host cells. The OmcB-protein, present at the EB-surface, binds heparin and this may be related to mammalian host cell adhesion and entry [97]. Once inside the cell, *C. pneumoniae* blocks the host cell response, including phagolysosomal fusion, which could be detrimental to survival. Within the phagosome, EBs germinate through the interaction with glycogen and transform into the replicative, non-infectious RBs, which are capable of DNA, RNA and protein synthesis, and starts to divide by binary fission. RB multiplication results in the formation of an intracellular microcolony of *Chlamydophila* bacteria that is referred to as the inclusion. Subsequent interactions with the individual host cell lead to either a productive or a non-productive infection. In productive infections, RB multiplication slows down and eventually leads to a second round of differentiation where RB reverts to EB. EBs are then again released into the extracellular space in order to produce another round of host cell invasion and RB replication. In non-productive infections, specific stimuli derived from the host's immune response initiate persistent *C. pneumoniae* development. This persistent form of the bacteria is characterized by an aberrant morphology, absence of capability to induce infection [98, 99], decreased surface antigen expression [100] and increased synthesis of proteins, such as chlamydial heat shock protein (Hsp)60, which may contribute to disease pathogenesis [101]. Since persistent *C. pneumoniae* are metabolically less active than rapidly dividing typical RBs, this persistent form may not be susceptible to antimicrobial killing and exhibits antibiotic resistance.

Host response to* C. pneumoniae *infection

Invading *C. pneumoniae* bacteria are initially encountered by the airway epithelium and rAM. As already mentioned, *C. pneumoniae* are Gram-negative bacteria and contain LPS in their cell wall which induces signalling through both TLR-2 and TLR-4 [102]. However, since chlamydial LPS contains nonhexaacyl lipid A structures (instead of the conventional hexaacyl lipid A structures), the host defence response is not effectively activated via TLR-4, which may allow bacterial growth and even promote persistence [103]. In addition, chlamydial Hsp60, which functions as a cytoplasmatic chaperone molecule, has been suggested to act as a ligand for both TLR-2 and TLR-4 [104] but only signalling through TLR-4 is demonstrated [105]. TLR-2

and TLR-4 engagement both result in the activation and nuclear translocation of NF-κB through the MyD88-dependent signalling pathway. The importance of the acute pro-inflammatory host response is illustrated by the fact that MyD88-deficient mice develop severe inflammation and lung injury due to the defective initial clearance of the bacteria [106]. The NF-κB dependent expression of TNF-α, IL-6, CCL2, RANTES and IL-8 promotes the recruitment and activation of especially neutrophils and monocytes to the infected lung where these cells contribute to the clearance of the pathogen by ingesting and killing the bacteria [107]. However, monocytes and neutrophils are found to augment the bacterial replication as well. Recruited macrophages and monocytes can act as fresh reservoirs for newly generated infectious EBs [108]. Depletion of $Gr1^+$-positive polymorphonuclear neutrophils resulted in a decreased chlamydial burden in the lungs of infected mice, thus illustrating their function as a bacterial reservoir [109]. Additionally, it was found that *C. pneumoniae* can even hide inside apoptotic neutrophils to silently infect and propagate within macrophages [110]. TIR-domain containing adaptor-inducing IFN-β (TRIF), an adaptor recruited as a consequence of TLR-3 activation, was suggested to be responsible for IFN-β production during *C. pneumoniae* infection [111]. However, as dsRNA is absent in *Chlamydophila* spp., TRIF-signalling is likely to occur through another TLR-molecule. Although *C. pneumoniae* infection promptly and strongly induces secretion of type I IFN, its function during infection appears to be complex. While *in vitro* studies showed that type I IFNs inhibit chlamydial growth in bone marrow derived macrophages [112], *in vivo* studies using IFNAR-deficient mice demonstrated that type I IFNs enhanced susceptibility for chlamydial infection by inducing local rAM apoptosis [113]. Recently, another chlamydial lipopeptide, referred to as the macrophage infectivity potentiator, exposed on the surface of EBs was demonstrated to induce TLR-2, TLR-1/TLR-6 mediated signalling in human macrophages [114], suggesting that besides LPS and Hsp60, other chlamydial components can act as ligands for TLRs. Also Nod-like receptors (NLRs), such as NOD1 and NOD2 are thought to be involved in the intracellular recognition of *C. pneumoniae* [115]. Recent research revealed that also NLRP3-dependent IL-1β secretion was critical for bacterial clearance and host survival [116].

Besides innate cells, also T-lymphocytes are essential in the immune defence against *C. pneumoniae*. In particular, $CD8^+$ T-cells play a predominant role in *C. pneumoniae* protective immune responses, mainly through the secretion of IFN-γ. The central role of IFN-γ in the resistance to *C. pneumoniae* was already highlighted by the fact that IFN-$\gamma^{-/-}$ mice were completely unprotected against infection [117]. Protective mechanisms mediated by IFN-γ

involve stimulatory effects on the expression and activity of inducible nitric oxide synthase (iNOS), secretion of cytokines and chemokines by leukocytes and tissue resident cells, and regulation of T-cell activity [118]. However, in this context, it is noteworthy to emphasize the more adverse effect of IFN-γ. It is namely demonstrated that IFN-γ induces the conversion of chlamydial RBs towards the persistent form of the pathogen. Via the stimulation of indolamine 2,3-dioxygenase (IDO), the presence of IFN-γ results in the depletion of the tryptophan pool, an essential amino acid which is indispensable for intracellular chlamydial growth [107]. Thus, IFN-γ can contribute to the establishment of the chronic persistent form of the disease. Next to their role as cellular IFN-γ source, CTLs exhibit antichlamydial growth activity by performing potent cytotoxic activity upon MHC I restricted recognition of infected cells [119]. CTL-responses are executed largely independent of Th1-cells. However, Th1-cells deliver a substantial contribution to host protection against *C. pneumoniae* infection through the secretion of IFN-γ [117]. Th17-lymphocytes contribute to the host defence against *C. pneumoniae* infection as well. Indeed, IL-17 deficient mice showed significantly delayed clearance of bacteria and more severe disease. Neutralization of IL-17 led to decreased DC-activation which in turn resulted in a diminished induction of antibacterial type I immune responses [120]. Activated NKT-cells were found to increase CD40 expression and IL-12 production by DCs as well. Therefore, NKT-cells are crucial for enhancing type I immunity during *C. pneumoniae* infection as well [121]. Several studies evidenced that *C. pneumoniae* can modulate the cellular immune response for its own benefit. The chlamydial MOMP, for instance, has been found to promote Th2- rather than Th1-responses by stimulating IL-10 production by APCs [122]. In addition, this chlamydial antigen skews the humoral immune responses in mice from a Th1-associated IgG2 antibody response towards a Th2-associated IgG1 antibody response [123]. Although substantial secretion of *C. pneumoniae* specific lung IgA and serum IgG2 is found in infected individuals, different studies demonstrated no major role of antibodies in the control of *C. pneumoniae in vivo* [102, 124, 125].

4.2.3 Asthma and *C. pneumoniae* infection: the chicken or the egg? part II

An association between asthma and *C. pneumoniae* infection was first put forward by Hahn and co-workers in the early 1990s. From 19 wheezing adult asthmatic patients, nine had serologic evidence of current or recent infection with the pathogen [126]. This observation was followed by many other cases in which *C. pneumoniae* specific IgA- and IgG-molecules were

found in the serum of asthmatic patients. These findings are reviewed in [127]. As is the case with respiratory viral pathogens, it is now believed that respiratory bacterial pathogens, including *C. pneumoniae*, play an important role in the aetiology of asthma. For instance, pulmonary infection of neonatal mice with *C. muridarum*, the natural mouse strain which is frequently used to model the human case of chlamydial infection, resulted in a more severe asthma phenotype later on in life. Notably, this early-life infection increases mucus-secreting cell numbers, IL-13 expression, and AHR [128]. There are different plausible mechanisms by which *C. pneumoniae* infection could contribute to the induction of asthma. Respiratory chlamydial infection causes airway epithelial damage which in turn provides endogenous danger signals for subsequent allergen sensitization. In order to circumvent the anti-bacterial effects of a Th1-mediated immune response, *C. pneumoniae* has evolved strategies to skew the host's response towards a Th2-reaction which is beneficial for the pathogen's viability and propagation. In support of this concept, mouse studies showed that pulmonary infection with *C. muridarum* leads to the enhanced production of the Th2-cytokine, IL-13, by a variety of cell types [129]. Moreover, Kaiko and co-workers demonstrated that antigen-pulsed *C. muridarum* infected bone-marrow derived DCs induced a significant bias of naïve $CD4^+$ T-cells towards a Th2-phenotype while IFN-γ secretion was inhibited [130]. In addition, early-life infection may impair lung function and may cause irreversible damage to pulmonary structure because the lungs, unlike most other organs, continue to mature during the first two years of life. Combined, these observations suggest that chlamydial infection of the lungs of healthy individuals creates a structural and immunological niche which is more prone for developing eosinophilic, Th2-mediated allergic airway disease.

Evidence for an association between *C. pneumoniae* infection and asthma does not necessarily indicate a causative role for the bacterial infection. It could rather indicate an increased susceptibility of asthmatic individuals to develop chlamydial infection, although few data are currently available to support this hypothesis. However, studies with the mouse variant *C. muridarum* evidenced that IL-13, a major Th2-cytokine which is abundantly secreted during asthma pathogenesis, enhances the susceptibility of the respiratory tract to chlamydial infection by reducing bacterial clearance [129]. Other research has shown that overt production of another Th2-cytokine, IL-10, can lead to enhanced bacterial dissemination and disease sequelae [131]. Interestingly, BALB/c mice, which are biased towards Th2-mediated responses, are markedly more susceptible to chlamydial lung infection compared to the Th1-predisposed C57BL/6 strain [132]. Furthermore, *in vitro* studies demonstrated that expression of

the alternative macrophage activation marker, mannose receptor (CD206), plays a pivotal role in determining susceptibility to *C. pneumoniae* infection [133]. Thus, it is clear that a pronounced pulmonary Th2-environment can create a niche in which the viability and propagation of *C. pneumoniae* bacteria are promoted. The presence of Th2-dominant conditions, including increased levels of IL-13 and IL-10, and of alternatively activated macrophages in the lungs of asthmatic individuals may therefore promote susceptibility and may contribute to the prevalence of chlamydial infection in these patient populations. Regardless of the fact whether asthmatic patients are more susceptible for developing chlamydial infection or not, *C. pneumoniae* is at least a significant cause of acute asthmatic exacerbations and a considerable determinant of disease severity. Indeed, a high proportion of clinical studies have reported the presence of an acute *C. pneumoniae* infection in human subjects hospitalized for acute exacerbation of bronchial asthma [134-139]. Based on a variety of *in vitro* and *in vivo* studies, several mechanisms responsible for *C. pneumoniae*-induced asthma exacerbations are proposed. First, chlamydial infection of airway cells induces a cascade of cytokine production, including TNF-α and IL-8, and ROS-production through TLR-stimulation and through certain *C. pneumoniae* specific stress-response proteins, like Hsp60 and Hsp10. Secretion of these inflammatory mediators leads to the subsequent recruitment and activation of several immune cells. Bacterial induced pulmonary accumulation of recruited leukocytes eventually results in inflammation and tissue damage which in turn can provoke an acute asthma exacerbation [127]. In agreement herewith, Huittinen and co-workers further confirmed the association of *C. pneumoniae* derived Hsp60 with asthma pathogenesis by illustrating the presence of Hsp60 specific IgA antibodies in a significant portion of asthma patients [140]. *C. pneumoniae* infection also induces the production of IL-6, IFN-β, and basic fibroblast growth factor (bFGF) in human bronchial smooth muscle cells *in vitro* [141, 142]. Because IFN-β and bFGF mediate smooth muscle cell proliferation, these data provide a mechanism by which *C. pneumoniae* infection might contribute to airway remodelling in patients with asthma. In addition, *C. pneumoniae* infection increases the secretion of matrix metalloproteinases (MMPs) by different hematopoetic and non-hematopoetic cells [143], which actively contribute to tissue remodelling and eventually to exacerbations in asthmatic individuals [144]. Thus, although there are relatively few data available, the body of evidence is sufficient to make the biologically plausible assumption that pulmonary infection with *C. pneumoniae* is likely to be associated with increased airway inflammation, thereby inducing asthmatic exacerbations and augmenting asthma severity.

Collectively, clinical and experimental studies investigating the association between asthma and *C. pneumoniae* pathogens have provided biological evidence that could account for this association but also provided conflicting data. Studies on the potential association between *C. pneumoniae* and asthma are greatly hampered by the lack of standardized, sensitive and specific methods for the detection of atypical respiratory pathogens in patients [145]. In addition, the difficulty (both in practical and ethical terms) in sampling the lower respiratory tract in representative populations of patients with asthma and control subjects constitutes a second major barrier.

A schematic overview of the correlation between *C. pneumoniae* infections and allergic asthma is depicted in figure 1.

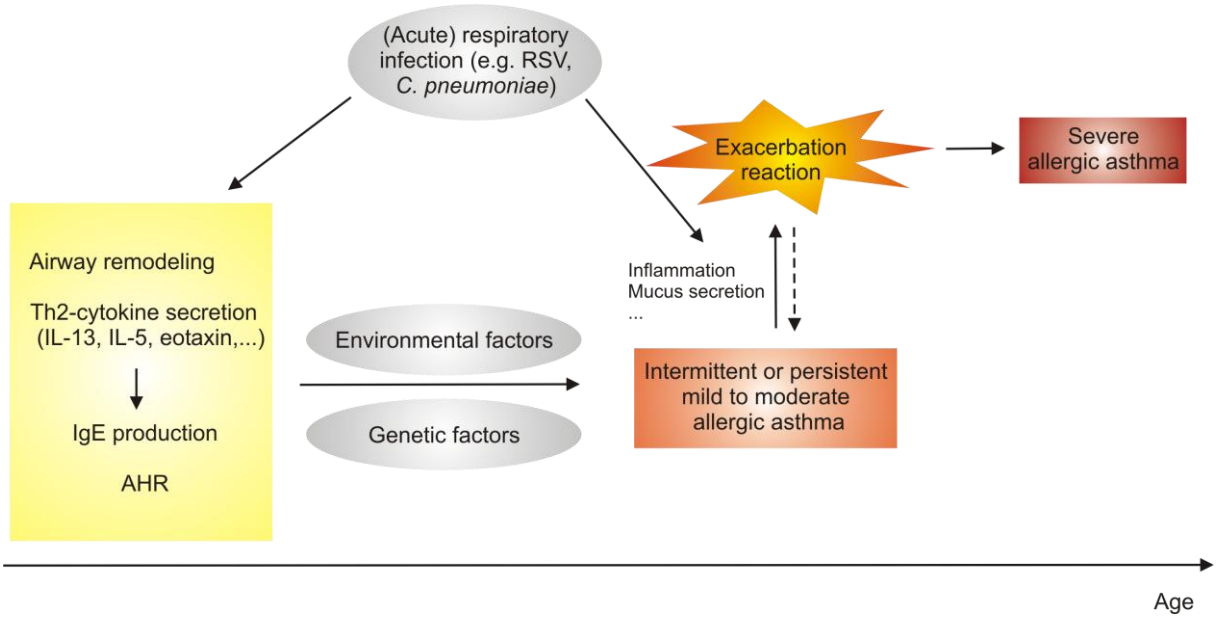

Figure 1: Overview of the correlation between (acute) respiratory infections and the development and/or progress of allergic asthma.
In addition to environmental and genetic factors, respiratory infections in early life can significantly contribute to the development of intermittent or persistent mild to moderate allergic asthma at later age. During this stage of the disease, acute respiratory infections can provoke exacerbation reactions by inducing pulmonary inflammation and mucus secretion. The succession of exacerbation reactions can eventually result in disease progression towards a severe allergic asthma phenotype.

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PART II

AIMS OF THE THESIS

Resident alveolar macrophages (rAM) exist in an environment high in antigenic material of which the majority must be ignored except when the antigen represents an infectious threat. When the activation threshold of rAM is exceeded, the development of a pulmonary inflammation is inevitable. During the course of a pulmonary inflammation, macrophage responses display a dynamic character. As a result of the rapid secretion of chemokines and cytokines by different activated resident pulmonary cells like rAM, monocytes are recruited to lung. In contrast to the rAM-population, the local inflammatory cytokine environment is the major determinant of the maturation of recruited alveolar monocytes/macrophages which might imply that the latter contribute to the inflammatory response in a different way than rAM. So it is clear that both the interplay between rAM and recruited alveolar macrophages and the fate of either population during the inflammation can influence the propagation and resolution of immune respiratory responses. In this context, it was already found that rAM are rapidly replaced by recruited macrophages during the course of a pulmonary inflammation induced by LPS and *Streptococcus pneumoniae*.

rAM and recruited monocytes/macrophages significantly contribute to the onset, propagation and resolution of pulmonary inflammatory responses during allergic asthma. However, the dynamics of both macrophage populations during these different stages of the allergic bronchial inflammation are still poorly characterized. Therefore, we initially analyzed the dynamics of rAM during the course of an allergic bronchial inflammation. By using an OVA/alum-based mouse model of asthma, featuring a Th2-biased sensitization and an eosinophilic airway inflammation reminiscent of the immunopathology of mild to moderate asthma, we found that the rAM-subset disappeared from the alveoli during the acute stages of the eosinophilic inflammation. These results imply that a new post-inflammation rAM resided in the airways after the clearance of the allergic bronchial inflammation. Compared to naïve rAM, post-inflammation rAM are possibly derived from a different monocyte-subset. In addition, it is not unlikely that the differentiation of these recruited monocytes to mature post-inflammation rAM is largely biased by the altered local microenvironment. In line with this premise, recent research already provided evidence that the functional status of post-influenza rAM are imprinted by the preceding inflammatory reaction. We therefore performed a phenotypical and functional comparison between naïve rAM and post-inflammation rAM to determine to what extent post-inflammation rAM were subjected to innate imprinting by the preceding allergic bronchial inflammation.

As rAM fulfill a pivotal role in the host's defence against invading respiratory viral and bacterial pathogens, observed alterations in the post-inflammation rAM functional status may have detrimental effects on the lung tissue integrity during subsequent respiratory syncytial virus (RSV) and *Chlamydophila* (*C.*) *pneumoniae* infection. Today, these respiratory pathogenic microorganisms are considered as the principal cause of the most severe exacerbations in asthmatic individuals. Thus, as a final aim of the thesis, we determined the antiviral and antibacterial immune responses of post-inflammation rAM during subsequent RSV- and *C. muridarum* (the mouse biovar of *C. trachomatis*) infection respectively, and addressed their potential contribution to the immunopathology of these respiratory pathogens in the post-inflammation lung.

PART III

RESULTS

Chapter 1

Innate Imprinting of Murine Resident Alveolar Macrophages by an Allergic Bronchial Inflammation Causes a Switch from Hypo- to Hyperinflammatory Reactivity

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INTRODUCTION

The mucosal surfaces of the respiratory tract are continuously exposed to environmental antigens and must therefore restrain excessive inflammatory responses to fulfil their role of gaseous exchange and to prevent bystander tissue damage. Powerful mechanical and immunosuppressive mechanisms protect the lung against the development of inappropriate immune reactivity and inflammation. When these defence mechanisms fail, chronic airway inflammatory diseases like allergic asthma may develop [1]. In allergic asthma, the infiltration of the bronchial mucosa by leukocytes, mainly eosinophils, along with subepithelial fibrosis, globlet cell hyperplasia and airway hyperresponsiveness leads to reversible loss of lung function and in the long term to irreversible tissue remodelling [2].

Through the past decades, immunosuppressive mechanisms that inhibit or limit the development of maladaptive pulmonary inflammatory responses have been identified. Allergen uptake and presentation by pulmonary plasmacytoid DCs provide intrinsic protection against inflammatory responses to harmless antigen by skewing T-cell differentiation towards the tolerogenic $CD4^+$ $CD25^+$ regulatory T-cell phenotype [3]. Immunomodulatory cytokines such as IL-10 and TGF-β are known to possess anti-inflammatory activities in the development of allergic asthma. Next to their inhibitory effect on pro-inflammatory cytokine secretion and leukocyte maturation, IL-10 and TGF-β are commonly implicated in the generation of the inducible regulatory T-cell subsets Tr1 and Th3 respectively [4]. Being important sources of pulmonary IL-10 and TGF-β [5], resident alveolar macrophages (rAM) have been shown to exert immunosuppressive activities on T-lymphocytes and DCs [6]. Several studies demonstrated a significant increase in allergic inflammation and T-cell reactivity in antigen challenged lungs after depletion of rAM [7, 8]. The increased sensitivity of rAM-depleted lungs to antigen exposure observed in these studies was attributed to the loss of rAM-mediated suppression of DC-maturation, -function and -trafficking to mediastinal LNs [9, 10].

Following resolution of inflammation, the increased numbers and altered differentiation of long-lived antigen-reactive lymphocytes, a hallmark of the adaptive immune branch, stands in strong contrast to the back to basal cell numbers and differentiation commonly reported for innate immune cells such as tissue macrophages. Recent reports however, increasingly challenge the paradigm of innate cells retaining no memory of prior inflammatory insults [11].

Murine rAM have been reported to display a sustained desensitization to bacterial Toll-like receptor (TLR)-ligands after the resolution of respiratory influenza infection [12]. In a mouse model of Sendai virus infection, a lasting effect on rAM was observed that persisted after the clearance of the virus. In this model for RSV-induced pathology, rAM-activation persisted, resulting in a chronic lung condition with pathological features resembling asthma and chronic obstructive pulmonary disease [13]. These observations indicate that infection may educate also innate immune cells altering the way they respond to a subsequent inflammatory insult. This concept of innate imprinting has been documented in several mouse models of infection [11]. However, the extent to which innate imprinting also occurs following non-infectious, allergic inflammation and the nature of its functional outcome remain largely unknown.

We now provide evidence for a pronounced innate imprinting of rAM as a consequence of allergic bronchial inflammation in mouse models of eosinophilic, Th2-biased mild to moderate asthma and of neutrophilic, Th1- and Th17-biased severe refractory asthma. The altered functional maturation of post-inflammation rAM was evidenced by an enhanced responsiveness of the cells to TLR-ligands and a newly acquired capacity to produce the type-I IFN, IFN-β. Mechanistically, we provide evidence that the switch from a restrained to an unrestrained rAM inflammatory response is the consequence of allergic inflammation-induced rAM-turnover accompanied by the appearance after the resolution of inflammation of a new population of secondary rAM with increased reactivity to inflammatory insults.

MATERIALS AND METHODS

Mice

6- to 8-week old female C57BL/6 mice were purchased from Janvier (Le Genest St.Isle, France). This wt strain expresses the CD45.2 alloantigen and also served as recipient for the generation of CD45-chimeric mice. 12-week old female B6.SJL-*Ptprca Pep3b*/BoyJ mice, expressing the CD45.1 alloantigen were obtained from Charles River (Brussel, Belgium) and served as bone marrow donor for the generation of CD45-chimeric mice. Both mice strains were kept under specific pathogen free conditions. All experiments performed in this study were approved by the local ethical committee.

Mouse models of allergic airway inflammation

For the allergic asthma model, C57BL/6 mice were immunized intraperitoneally with 20µg of grade V chicken egg OVA (Sigma-Aldrich, St.Louis, MO, USA), adsorbed on 1mg $AlOH_3$ (alum; Sigma-Aldrich) in endotoxin-free PBS (Lonza, Walkersville, MD, USA). To generate a model for a non-eosinophilic severe refractory Th1-/Th17-mediated allergic bronchial inflammation, C57BL/6 mice were immunized subcutaneously with 20µg of OVA in PBS emulsified in 75µl CFA (Sigma-Aldrich). In both mouse models, OVA-sensitized mice were exposed to OVA-aerosols, consisting of either 1% (allergic asthma model) or 0,1% (neutrophilic Th1-/Th17-mediated allergic inflammation model) of grade III OVA (Sigma-Aldrich) in PBS.

Generation of chimeric CD45.2 alloantigen-expressing recipient mice

Bone marrow cells were isolated under sterile conditions from the tibias and femurs of sex-matched CD45.1 donor mice. Briefly, tibias and femurs were flushed with sterile PBS and the cell suspension was filtered through 70µm nylon meshes (BD Biosciences, San Diego, CA, USA) to remove cell aggregates. RBC lysis was performed before transplantation by incubation of the single cell suspension in ACK Lysing Buffer (Lonza) for 3 min at room temperature. Recipient CD45.2 alloantigen-expressing C57BL/6 mice received 8 Gy of total body irradiation using 5 MV photons of a linear accelerator (SL-75, Elekta, Crawley, UK). This radiation dose depleted nearly completely the bone marrow but did not induce depletion of rAM and memory T-cells in previously OVA-alum sensitized mice. A total of $8x10^6$ CD45.1 donor bone marrow cells suspended in 250µl sterile PBS were transplanted via lateral

tail vein injections into CD45.2 recipient mice. The drinking-water of the CD45.2 recipient mice was supplemented with 0.2% neomycin trisulfate antibiotics (Sigma-Aldrich) 5 days before until 14 days after the irradiation.

Alveolar cell isolation and culture

Mice were anesthetized with avertin (2,2,2-tribromethanol; 2,5% in PBS; Sigma-Aldrich). BAL was performed by making a small incision in the trachea, to allow passage of a lavage canulae. Lungs are flushed 4 times with 1ml Ca^{2+}- and Mg^{2+}-free Hank's Balanced Salt Solution (HBSS; Invitrogen, Carlsbad, CA, USA), supplemented with 0.05mM EDTA (ethylenediaminetetraacetic acid). Optionally, a prior lavage with 0,5ml HBSS-EDTA was performed and BAL fluid was isolated by centrifugation and collection of the supernatant. BAL cells were washed and resuspended in PBS for further use. Naïve and post-inflammation rAM isolated via BAL were cultured in complete culture medium (RPMI 1640 containing 1% heat-inactivated FCS, 25mM HEPES (N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid), 2mM L-glutamine, 1mM pyruvate, 100U/ml penicilline/streptomycin (Invitrogen), and 55µM 2-ME (Sigma-Aldrich). All cultures were enriched for macrophages by plastic adhesion for 1h at 37°C. Naïve and post-inflammation rAM were then stimulated for the indicated times with LPS (*Escherichia coli* 0111:B4; Sigma-Aldrich), polyriboinosinic:polyribocytidylic acid (poly(I:C); Invivogen, San Diego, CA, USA) or imiquimod (Invivogen) at 37°C.

Flow cytometry

The expression of alveolar macrophage maturation markers was assessed on naïve and post-inflammation rAM by flow cytometry. Briefly, BAL cells were counted and suspended at a concentration of 10^6 cells/ml. High affinity FcγRs were blocked by incubation with purified anti-mouse CD16/CD32 (Fc-Block) for 15 min at 4°C and stained with CD11c-allophycocyanin (APC), DEC-205-PE, F4/80-biotin, CD11b-PE, CD115-PE, CD16/CD32-PE and CD36-PerCP for 1h at 4°C. Biotinylated F4/80 Ab was detected by an additional incubation step with streptavidin-PE for 20 min at 4°C. All antibodies and the streptavidin-PE were purchased from BD Biosciences. Autofluorescence was detected in the FL-1 channel.

Turnover of the naïve rAM during the course of the bronchial inflammation elicited in both models was determined using CD45-chimeric mice. Naïve rAM were identified by recipient specific CD45.2 expression and by uptake of latex Fluoresbrite plain Yellow-Green [14] 1

micron microspheres (Polysciences, Warrington, PA, USA), administered by i.t. route 48 h before the first OVA-aerosol exposure. Elicited cells were identified as microsphere$^-$ cells expressing donor CD45.1. Anti-mouse CD45.1-PE and CD45.2-PerCP-Cy5.5 antibodies (BD Biosciences) were used according the manufacturer's instructions. Pre-incubation of the cells with Fc-Block was used to prevent unwanted binding to FcRs.

All samples were measured on a FACSCalibur flow cytometer (BD Biosciences) and analyzed using CellQuest software.

BAL total and differential cell counts

BAL cell counts and cell type composition was analyzed by flow cytometry. Cells, pre-incubated with Fc-Block were classified as monocytes (alveolar macrophages, elicited monocytes and DCs), neutrophils, eosinophils or lymphocytes based on forward and side scatter gating and fluorescence intensities for anti-mouse CD3ε-Alexa488, B220-FITC, CCR3-PE, CD11c-APC and I-A^b-biotin which was recognized by streptavidin-PerCP. All antibodies and streptavidin-PerCP were purchased from BD Biosciences, except CCR3-PE (R&D Systems, Abingdon, UK). Additionally, the total number of BAL cells was calculated from the measured total cell count relative to the number of Flow-Count beads (Beckman Coulter, Brea, CA, USA) of which a constant amount of was added to the sample. Total numbers of BAL cells were counted by use of a Bürker-chamber (Marienfeld, Lauda-Königshofen, Germany). Trypane blue was added to exclude dead cells. Differential cell counts obtained by flow cytometry were confirmed by morphological examination of cytospin preparations using a Shandon cytocentrifuge (Techgen, Zellik, Belgium) and stained with May-Grünwald-Giemsa (Sigma-Aldrich). The percentage of monocytes/macrophages, neutrophils, and eosinophils was determined by counting at least 400 cells. Both analyses were performed on an Olympus BX51 microscope, equipped with X4, X10, X20, X40 and X100 lenses.

Total RNA preparation and real-time quantitative PCR

RNA isolation was performed using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's protocol. cDNA was synthesized using a Superscript II Reverse Transcription Reagent Kit (Invitrogen). Real-time quantitative PCR (qPCR) was performed on a Lightcycler 480 (Roche Molecular Systems) using a qPCR kit for SYBR Green I (Roche

Molecular Systems). Real-time qPCR amplification was performed in triplicate reactions under the following conditions: a preincubation step at 95°C for 5min, followed by 50 cycles at 95°C for 10s and at 60°C for 30s. The following forward and reverse primers were used: 5'-TGAACACGG CAGTGGCTTTA-3' and 5'-GCATTCACAGTCACT TAGGTGGTTTA-3' (murine *arg*-1); 5'-CAGCTGGGCTGTACAAACCTT-3' and 5'-CATTGGAAGTGAAGC GTTTCG-3' (murine *inos*) 5'-AGCCCTCATGGTCTGGTTGGTT-3' and 5'-GCACTCCGA GGCACTGTTATCC-3' (murine *usp18*); 5'-ATCTCTCCCTACTC TGCCCTCCTA-3' and 5'-GCGTATAAATCAGCAATCCCTTCA-3' (murine *ifit*2); 5'-CCC TGGGCCCTTCCTGT-3' and 5'-CCCGGGGGCACTTGTCT-3' (murine *oas1*); 5'-GGATA GAAGTTGTGGGGAG TGGC-3' and 5'-CAGCCTTGGTGACCTTGACGA-3' (murine *ifi205*) 5'-TAGTCCTTCCT ACCCCATTTCC-3' and 5'-TTGGTCCTTAGCCACTCCTTC-3' (murine *il6*); 5'-AACCAG GGCCTTCTTTAG-3' and 5'-GATCTGCCTGCCTTGGTCT-3' (murine *il12p40*); 5'-CCTG CTGCTCTCAAGGTTGTT-3' and 5'-TGGCTGTCACTGCC TGGTACTT-3' (murine *rpl13a*). *mRPL13a* mRNA was used as reference housekeeping gene for normalization. All primers were purchased from Invitrogen.

In vitro phagocytosis assay

Uptake of YG^+ microspheres by naïve and post-inflammation rAM was imaged with a Leica TCS SP5 AOBS confocal microscope (Leica, Wetzlar, Germany) using 488-nm Multi Argon laser line. Cytoplasm was stained with CellTracker Orange (Invitrogen) and excited with 543-nm HeNe laser. Nuclei were stained with 500nM DAPI (4,6 diamidino-2-phenylindole) (Invitrogen) and excited with the 405-nm line of an UV diode laser. Stained cells were mounted in 1% N-propylgallate in glycerol before image acquisition. Images were acquired with LAS AF software (Leica) and subsequently analyzed with Volocity software (Perkin-Elmer, Coventry, UK).

Cytokine/chemokine measurement

Protein levels of mouse TNF-α, IL-6, IL-12p70, CXCL1 and CXCL2 in culture supernatant or BAL fluid were quantified with the Bioplex suspension array system (Biorad, Hercules, CA) for simultaneous detection of cytokines, according to the manufacturer's protocol. The analytes were measured with the Bioplex protein array reader and the Bioplex manager software, using recombinant cytokine standards (all from Biorad).

Culture supernatant levels of IFN-β were determined via the VeriKine Mouse IFN Beta ELISA Kit (PBL interferon source, Piscataway, NJ, USA) according to the manufacturer's protocol.

Statistics

Statistics were performed using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA). Following outlier statistics in order to choose between performing a one-way ANOVA or Kruskal-Wallis nonparametric test, Gaussian distribution of parameters was checked using a Kolmogorov-Smirnov test. Differences in mean between each two independent experimental groups were analyzed using an unpaired t-test or the nonparametrical Mann-Whitney U test at 95% confidence interval. No statistic analysis was done for gene expression data, as this concerned data of pooled samples.

RESULTS

Surface marker profile and basal differentiation of naïve and post-inflammation rAM

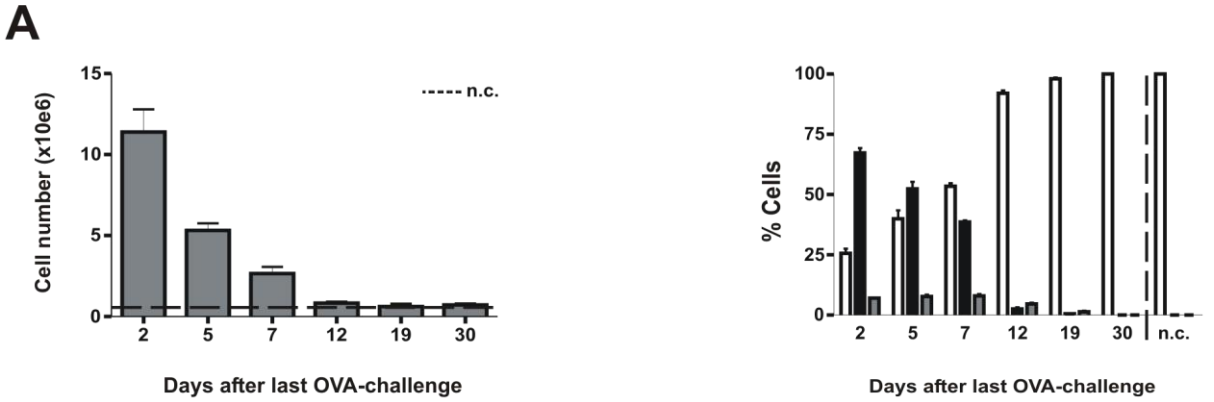
In this study we used a mouse model of allergic asthma in which a Th2-biased sensitization of C57BL/6 mice against the model allergen OVA is elicited by repeated intraperitoneal immunization using aluminiumhydroxide (alum) as adjuvant. Exposure of the sensitized mice to nebulized OVA then generated an eosinophilic airway inflammation reminiscent of the immunopathology of mild to moderate asthma (figure 1A, right panel). The clearance of the allergic pulmonary inflammation was verified by harvesting BAL samples at different time points after the last of seven OVA-challenges. This showed that the alveoli regained a new steady-state condition within 12 days. At this time point, absolute cell numbers returned to basal levels (figure 1A, left panel) and cytospin analysis showed that the cellular composition of the alveoli again consisted for 90% of macrophages (figure 1A, right panel). These macrophages form the new rAM-population of the post-inflammation lungs and can therefore be considered as post-inflammation rAM. In addition, at this time point Th2-associated inflammatory cytokines were no longer detectable in the BAL fluid (*data not shown*).

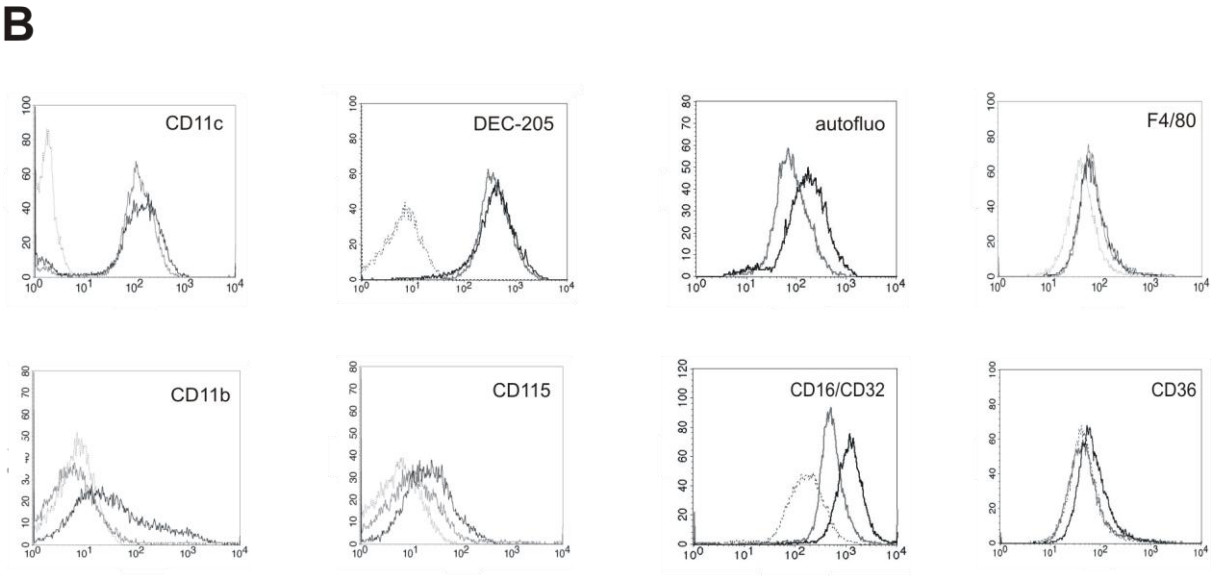
In order to determine to what extent the post-inflammation rAM-population exhibited a characteristic alveolar macrophage phenotype, we analyzed the expression levels of alveolar macrophage markers by flow cytometry. Naïve and post-inflammation rAM-populations were isolated via BAL from naïve and OVA-challenged mice 15 days after the last OVA-exposure. CD11c and DEC-205, hallmark surface markers of rAM [15-20], were equally and uniformly expressed on both rAM-populations (figure 1B). High intrinsic fluorescence intensity, which constitutes as a general phenotypic characteristic of rAM [21], was present in both rAM-populations. Also F4/80, a broad macrophage marker [22], was nearly equally and uniformly expressed on both cell populations. In contrast, naïve rAM were uniformly negative for the expression of CD11b, whereas post-inflammation rAM expressed medium to high levels of CD11b. Also the monocyte marker CD115 (M-CSFR) and phagocytosis receptors FcγRIII/II (CD16/CD32) and SR-A (CD36) showed uniformly elevated expression levels at rAM from post-inflammation mice (figure 1B). Thus, although post-inflammation rAM exhibit a characteristic alveolar macrophage marker profile – autofluo^{high} CD11c^{+} DEC205^{+} F4/80^{+} – they differ from naïve rAM in the expression levels of macrophage/monocyte maturation and phagocytosis markers.

Alveolar macrophages typically exhibit high phagocytic activity [23]. To verify to what extent post-inflammation rAM had retained this functional trait, naïve and post-inflammation rAM were incubated *ex vivo* with fluorescent latex microspheres for up to 6h. Phagocytosis of the latex microspheres was assessed by confocal microscopy. Fluorescent microspheres were readily engulfed by naïve rAM, whereas microsphere uptake was strongly reduced in post-inflammation rAM at all time points (figure 1C). After 6h of incubation, nearly 85% of naïve rAM were positive for uptake of fluorescent microspheres. In contrast, only 10% of the post-inflammation rAM exhibited microsphere uptake. In addition, microsphere$^+$ naïve rAM consistently featured higher numbers of microspheres per cell compared to microsphere$^+$ post-inflammation rAM (figure 1D).

A reduced phagocytic activity is often observed in macrophages that have been alternatively differentiated [24]. These so-called M2-macrophages feature in addition to a low phagocytic capacity an arginine metabolism differing from M1- or classical differentiated macrophages by an increased arginase/iNOS expression ratio [25]. Analysis of *arg-1* and *inos* mRNA levels indeed confirmed this shift towards a M2-characteristic arginine metabolism. The expression of *arg-1*, the prototypic M2-marker, was up to 50-fold higher in post-inflammation rAM compared to naïve rAM while the expression of the M1-marker, *inos*, was hardly different between both macrophage populations (figure 1E).

Figure 1. Marker profile and basal differentiation of post-inflammation rAM.
(A) OVA/alum sensitized C57BL/6 mice were exposed to 7 OVA-aerosols or left untreated as naïve controls. Average total (*left panel*) and differential (*right panel*; white bars: macrophages; black bars: eosinophils; grey bars: neutrophils) BAL cell counts were determined via flow cytometry and Giemsa and May-Grünwald-stained cytospin analyses respectively at the indicated time points after the last OVA-exposure (n=5) (nc = naïve control). Error bars represent SEM. (B) Flow cytometry analysis of the expression pattern of alveolar macrophage maturation markers. OVA/alum sensitized C57BL/6 mice were exposed to 7 OVA-aerosols (n=7) or left untreated (n=7). BAL samples were taken at d15 after the last OVA-exposure and naïve (grey line) and post-inflammation (black line) rAM were analyzed for intrinsic fluorescence intensity and for expression of CD11c, DEC-205, F4/80, CD11b, CD115, CD16/CD32 and CD36. Dotted line: unstained control. (C) Naïve and post-inflammation rAM isolated from C57BL/6 mice (n=5) were incubated for the indicated time with YG$^+$ microspheres (10 microspheres/cell). Microsphere uptake was determined by confocal analysis of (C) the percentage of microsphere$^+$ cells and (D) the number of microspheres per cell. The percentage of microsphere$^+$ rAM represents the average of 10 randomly selected microscopic fields from triplicate cultures. Error bars represent SEM. Confocal pictures in D are an overlay of green (YG$^+$ microsphere), red (CellTracker Orange) and blue (DAPI nuclear staining). Bars: 20µm (E) Naïve (white bars) and post-inflammation (grey bars) rAM were isolated (n=7) and the basal levels of *arg-1* and *inos* mRNA transcripts were assessed by RT-qPCR. Results are expressed as the mean n-fold induction in mRNA expression compared to naïve rAM ± SD of triplicate PCR reactions. All data presented in this figure are representative for two independent experiments.


A
Cell number (x10e6)
----- n.c.
Days after last OVA-challenge
% Cells
Days after last OVA-challenge
n.c.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DEC-205
autofluo
F4/80
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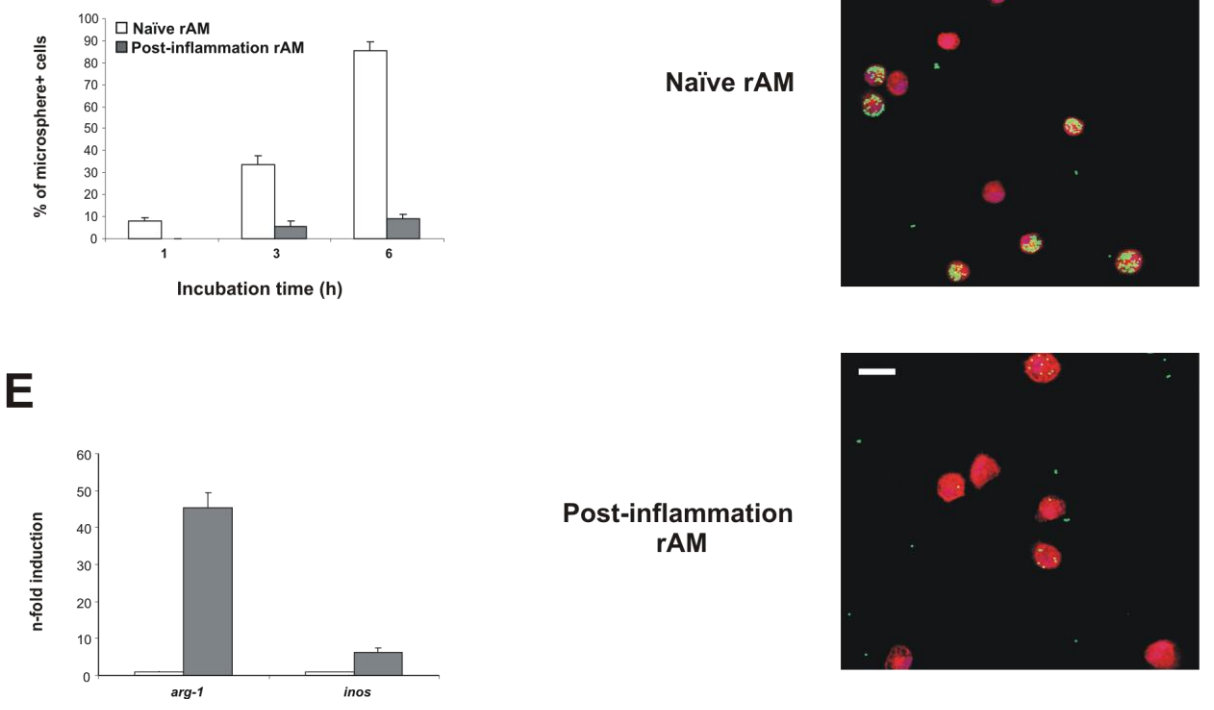
CD115
CD16/CD32
CD36


C
Naïve rAM
Post-inflammation rAM
% of microsphere+ cells
Incubation time (h)
D
Naïve rAM
Post-inflammation rAM
E
n-fold induction
arg-1
inos

Differential inflammatory cytokine and IFN-β response after TLR-stimulation

We next investigated to what extent TLR-signalling in post-inflammation rAM is affected. We therefore compared the response of naïve and post-inflammation rAM to ligation of the anti-bacterial TLR, TLR-4, and the anti-viral TLRs, TLR-3 and TLR-7. rAM were isolated from naïve and post-inflammation lungs and cultured *ex vivo* for 6h in the presence of *E. coli* LPS (0,1µg/ml), poly I:C (10µg/ml) or imiquimod (10µg/ml) respectively. Levels of inflammatory cytokines and chemokines were subsequently analyzed via the Bioplex suspension array system. As shown in figure 2, compared to naïve rAM, post-inflammation rAM secreted markedly increased protein levels of TNF-α, IL-6, IL-12(p70), CXCL1 (KC) and CXCL2 (MIP-2) after stimulation with LPS and imiquimod. Upon poly I:C stimulation however, these cytokines and chemokines remained undetectable or near basal levels (CXCL2) in the supernatant of both rAM cell cultures (figure 2).

rAM differ from other tissue macrophages in their failure to autonomously produce IFN-β in response to TLR-3 and TLR-4 triggering [26]. Strikingly, post-inflammation rAM showed a switch from an IFN-β production defective to an IFN-β production competent phenotype after LPS and poly I:C stimulation but failed to do so in response to imiquimod (figure 3). This discrepancy was also confirmed at the level of autocrine IFN-β bioactivity as apparent from the strongly increased transcript levels of *usp18*, *ifit2*, *oas1* and *ifi205* in LPS- and poly I:C-treated post-inflammation rAM (figure 3). The IFN-β biomarker function of these genes was confirmed by performing a similar analysis on the post-inflammation rAM from *ifn-β* KO mice [27], which developed a normal Th2-mediated eosinophilic inflammation *(data not shown)*. Post-inflammation rAM of these KO-mice no longer displayed the strong induction of *usp18*, *ifit2*, *oas1* and *ifi205* in response to LPS (*data not shown*), thus confirming the dependence of these genes on the expression of IFN-β.

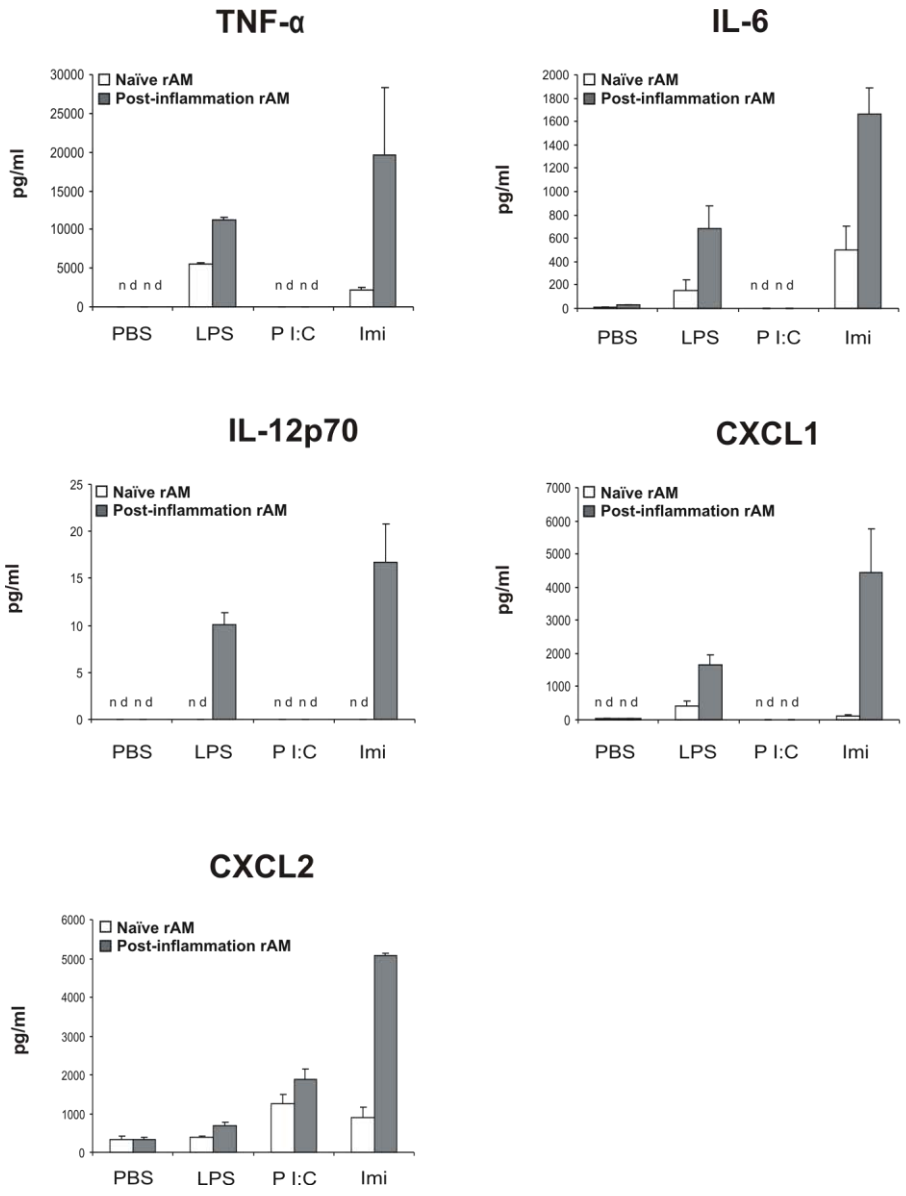


Figure 2. Inflammatory cytokine and chemokine response of naïve and post-inflammation rAM to TLR-3, TLR-4 and TLR-7 engagement.
Naïve and post-inflammation rAM isolated from C57BL/6 mice (n=8) were *ex vivo* stimulated with 0.1µg/ml *E. coli* LPS, 10µg/ml poly I:C (P I:C) or 10µg/ml imiquimod (Imi) for 6h or left untreated as control. Protein levels of TNF-α, IL-6, IL-12(p70), CXCL-1 and CXCL-2in the culture supernatant were measured using the Bioplex suspension array system. Data represent the average protein concentration ± SD of triplicate culture conditions. Data are representative for three experimental repeats.

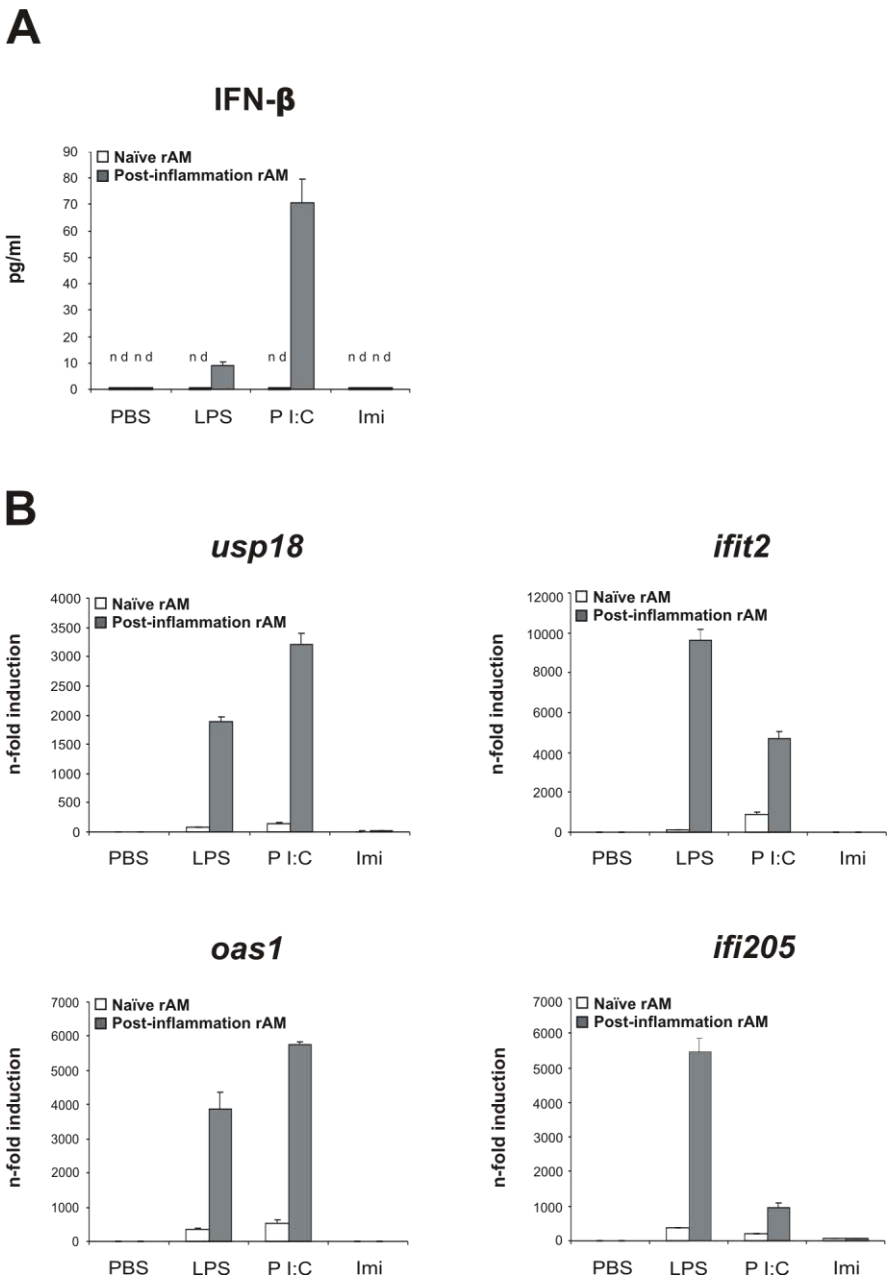


Figure 3. IFN-β response of naïve and post-inflammation rAM to TLR-3, TLR-4 and TLR-7 engagement. Naïve and post-inflammation rAM were isolated from C57BL/6 mice (n=8) and stimulated *ex vivo* with 0,1µg/ml *E. coli* LPS, 10µg/ml poly I:C (P I:C) or 10µg/ml imiquimod (Imi) for 6h or left untreated as control. (A) IFN-β protein levels in the culture supernatant were determined by ELISA. Results represent the average IFN-β levels ± SD of triplicate culture conditions. (B) RT-qPCR was used to analyze mRNA expression levels of *usp18*, *ifit2*, *oas1* and *ifi205*. Results represent the mean n-fold induction compared to unstimulated naïve rAM ± SD of triplicate PCR reactions. Data presented are representative for three independent experiments.

Increased in vivo inflammatory TLR- reactivity of post-inflammation lungs

The functional outcome of the differential TLR-reactivity observed between both rAM-populations was further verified by analysis of the inflammatory BAL infiltrate 16h after instillation of LPS (10ng), poly I:C (800ng) or imiquimod (800ng). In contrast to the

relatively weak response observed in naïve lungs, inflammation-experienced lungs showed a more pronounced alveolar infiltration of inflammatory leukocytes after challenge with LPS, poly I:C or imiquimod (figure 4A). Although macrophages/monocytes and neutrophils constituted the main components of the inflammatory infiltrate in both groups, higher cell numbers of both leukocyte populations were found in the BAL isolated from LPS-challenged post-inflammation lungs compared to naïve lungs (figure 4B). Furthermore, in naïve lungs the instillation of poly I:C or imiquimod resulted in an alveolar infiltration of mainly monocytes/macrophages. In contrast, in post-inflammation lungs poly I:C or imiquimod challenge caused in addition a significant recruitment of neutrophils as well as eosinophils to the bronchoalveolar lumen (figure 4B).

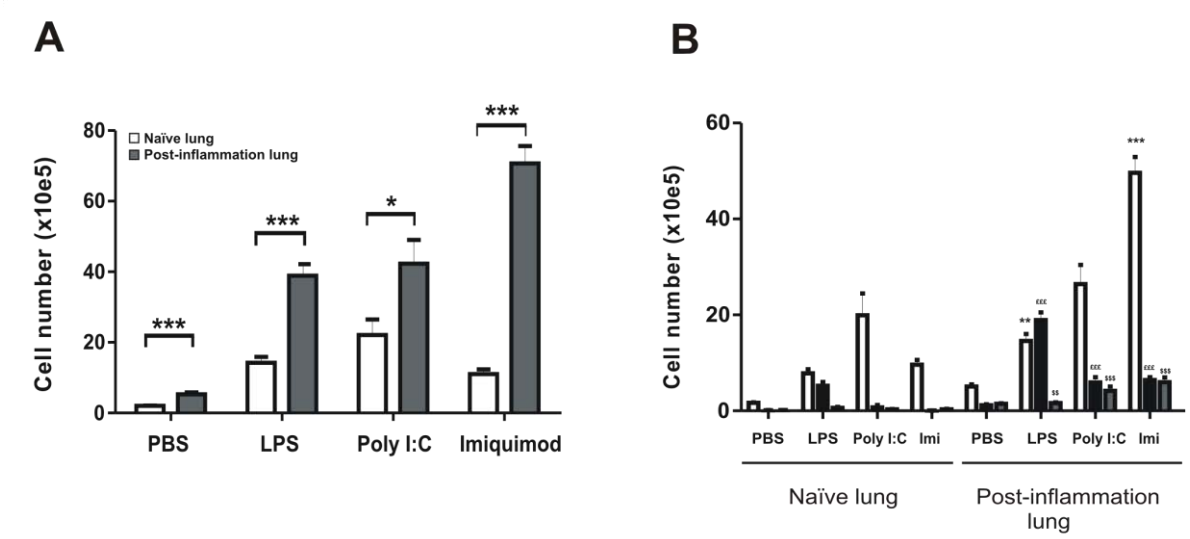


Figure 4. *In vivo* LPS, poly I:C and imiquimod challenge of post-inflammation lungs.
(A) Post-inflammation (d15 after the last OVA-challenge) and naïve C57BL/6 mice (n=7) were challenged i.t. with 10ng *E. coli* LPS, 800ng poly I:C, 800ng imiquimod or PBS as a control and BAL was collected 16h later. Average BAL cell counts ± SEM (A) and average BAL cellular composition ± SEM (B) were determined by flow cytometry and Giemsa and May-Grünwald-stained cytospin analyses. White bars: macrophages, black bars: neutrophils and grey bars: eosinophils. (A): * $p<0.05$ and *** $p<0.0001$. (B): ** $p<0.01$ and ***$p<0.001$ compared to the corresponding macrophage numbers in LPS, poly I:C and imiquimod stimulated naïve lungs; $^{£££}p<0.001$ compared to the corresponding neutrophil numbers in LPS, poly I:C and imiquimod stimulated naïve lungs; $^{\$\$}p<0.01$ and $^{\$\$\$}p<0.001$ compared to the corresponding eosinophil numbers in LPS, poly I:C and imiquimod stimulated naïve lungs.

Inflammation-induced rAM-turnover is crucial for the development of the post-inflammation rAM-phenotype

We next addressed the question to what extent the observed post-inflammation rAM-phenotype arose either from naïve rAM that underwent an education process during pulmonary inflammation or from monocytes that were freshly recruited during or after

bronchial inflammation and whose differentiation was subsequently influenced by the prevailing lung environment. We therefore followed the fate of the naïve rAM-population in the course of the allergic bronchial inflammation. To facilitate the discrimination between rAM and newly recruited macrophages/monocytes, OVA/alum sensitized C57BL/6 mice, which express the CD45.2 leukocyte alloantigen, were first irradiated and transplanted with CD45.1 alloantigen-expressing bone marrow cells (figure 5A). In combination with intratracheal delivery of fluorescent (YG^+) latex microspheres (1µm), flow cytometry analysis allowed to identify rAM as microsphere$^+$ CD45.2$^+$ cells and recruited macrophages/monocytes as microsphere$^-$ CD45.1$^+$ cells (figure 5B). At the moment of intratracheal delivery of YG^+-microspheres, a small fraction of newly recruited macrophages from the donor type were present and had taken up the instilled microspheres. This cell fraction accounted only for 2% in the naïve condition (figure 5B, upper right panel, green gate). The pulmonary response to nebulised OVA was not influenced by the preceding irradiation process (supplemental figure S1). Furthermore, intratracheal administration of microspheres did not elicit inflammatory cell recruitment as such and did not alter the expression of typical alveolar macrophage markers (CD11c, DEC-205 and F4/80) (*data not shown*).

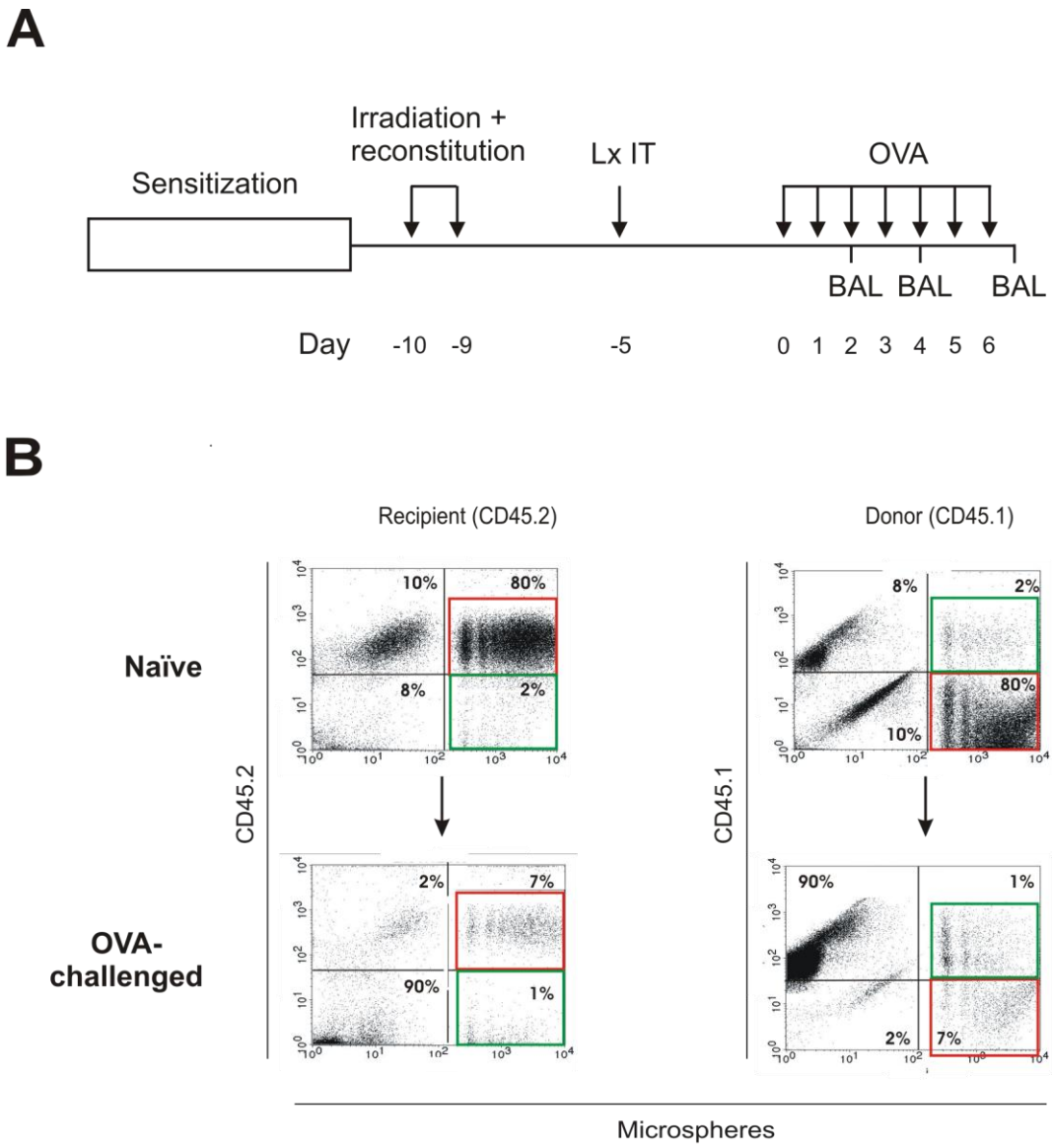
As illustrated in figure 5B, exposure to OVA-aerosol provoked pronounced inflammatory cell recruitment to the bronchoalveolar lumen. As a consequence, the relative number of the microsphere$^+$ CD45.2$^+$ rAM decreased with increasing numbers of OVA-challenges. To determine the inflammation-induced rAM-turnover, we therefore calculated the absolute numbers of microsphere$^+$ CD45.2$^+$ cells present in the BAL from the percentage of the cell population and the total alveolar cell infiltrate. This analysis revealed that absolute rAM-numbers remained constant after 2 OVA-challenges (figure 5C). However, microsphere$^+$ CD45.2$^+$ numbers sharply dropped after 4 OVA-exposures and nearly completely disappeared after 7 OVA-exposures (figure 5C). In order to find out whether also fewer OVA-exposures would lead to rAM-disappearance from the alveoli, OVA/alum sensitized CD45-chimeric mice were exposed to only 2 OVA-aerosols. As expected, absolute rAM-numbers remained constant for the first two days of treatment but then decreased dramatically from day 3 after the last (2nd) OVA-challenge (figure 5D).

We next verified to what extent the phenotype of the post-inflammation rAM was a consequence of the preceding allergic inflammation per se or just of the accompanying rAM-

turnover. We therefore mimicked the enhanced rAM-turnover and their replacement by newly recruited macrophages/monocytes by a sterile, non-inflammatory depletion of naïve rAM via i.t. administered clodronate liposomes [28]. This depletion was followed by a spontaneous gradual reconstitution of the rAM-population, reaching its initial number at day 14 (*data not shown*). Strikingly, this depletion-induced secondary rAM-population showed, when stimulated *ex vivo*, a LPS-hyporesponsive phenotype highly similar to the hyporesponsiveness of naïve rAM. As illustrated in figure 6A, no differences in mRNA expression levels of the inflammatory cytokines *il6* and *il12p40* were found between LPS-stimulated naïve and depletion-induced secondary rAM. Also the expression of the IFN-β responsive genes, *usp18* and *ifit2*, did not differ between both rAM-populations (figure 6A).

The dependence of the altered post-inflammation rAM-reactivity on inflammation-induced rAM-turnover rather than on rAM-turnover per se, raised the issue to what extent the nature of the bronchial allergic inflammation may affect this phenomenon of innate imprinting. We therefore applied the same experimental set-up but now in a mouse model of non-eosinophilic severe refractory asthma. In this model, systemic sensitization against OVA in the presence of CFA leads to a Th1- and Th17-biased neutrophilic bronchial inflammation after exposure to nebulised OVA [29]. Also in this model, bronchial inflammation resulted in a drastic decrease in absolute microsphere$^+$ CD45.2$^+$ rAM-numbers from 4 OVA-challenges on (figure 6B) which was similar to the rAM-turnover observed in the course of a Th2-biased eosinophilic bronchial inflammation. When assayed for reactivity to LPS-stimulation, these post-inflammation rAM showed the enhanced inflammatory reactivity characteristic for post-inflammation rAM from the eosinophilic asthma model, namely increased expression levels of NF-κB and IFN-β responsive genes after *ex vivo* stimulation with LPS (figure 6A).

Figure 5. Naïve rAM-turnover in response to allergen exposure.
(A) OVA/alum sensitized wt C57BL/6 mice (CD45.2) (n=7) were irradiated and reconstituted intravenously with CD45.1-expressing donor bone marrow cells at d-10 and d-9 respectively. YG^{+}-microspheres were administered i.t. at d-5 followed at d0 by exposure of the mice to OVA-aerosol, or left unchallenged as control. (B) Naïve rAM from unchallenged CD45-chimeric mice were identified by flow cytometry as $CD45.2^{+}$ $microsphere^{+}$ cells (red gate) within the macrophage/granulocyte gate. Elicited AM were identified as $CD45.1^{+}$ $microsphere^{-}$ cells. (C) The presence of naïve rAM ($microsphere^{+}$ $CD45.2^{+}$ cells) in BAL samples collected after two, four and seven OVA-exposures on d3, d5 and d8 respectively, were determined by flow cytometry as in B. Average absolute naïve rAM-numbers were calculated from the percentages of $microsphere^{+}$ $CD45.2^{+}$ cells and absolute alveolar cell numbers as determined by flow cytometry, and compared to the unchallenged control group (broken line) (D) In a second set-up, OVA/alum sensitized CD45-chimeric mice (n=7) were exposed to two OVA-aerosols. BAL was taken one, three and six days after the second OVA-exposure. Average absolute naïve rAM-numbers were obtained as in C and compared to that of unchallenged control mice (broken line). Bars in C and D represent SEM. ** $p<0.01$; *** $p<0.0001$. Data presented are representative for two independent experiments.


A
Irradiation +
reconstitution
Lx IT
OVA
Sensitization
BAL BAL BAL
Day -10 -9 -5 0 1 2 3 4 5 6
B
Recipient (CD45.2)
Donor (CD45.1)
Naïve
OVA-
challenged
CD45.2
CD45.1
10%
80%
8%
2%
2%
7%
90%
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10%
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Microspheres
C
7x OVA
Cell number (x10e5)
1.25
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Cell number (x10e5)
2
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1
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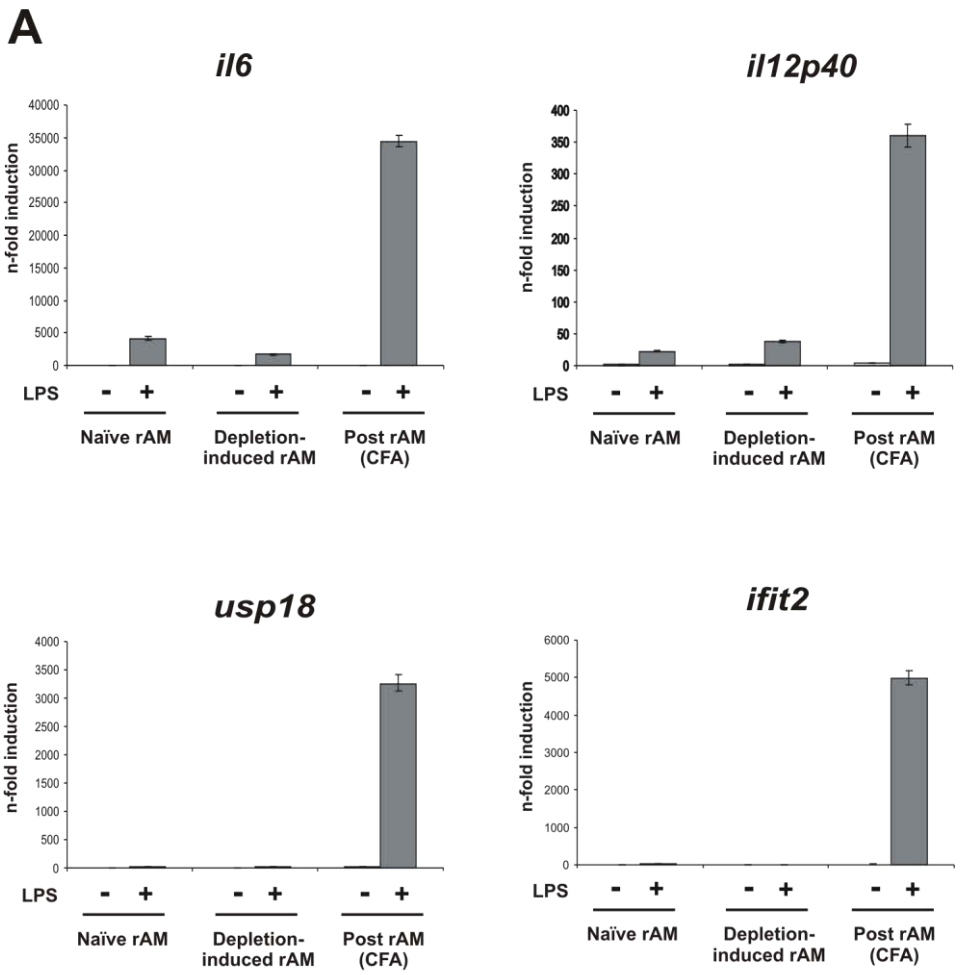
2
3
6
OVA-
challenges
days after 2nd
OVA-challenge

Figure 6. Inflammation-induced rAM-turnover is crucial for the development of the post-inflammation rAM-phenotype.
(A) Naïve rAM from C57BL/6 mice were depleted by i.t. instillation of 30% clodronate liposomes (CL) at d0. OVA/CFA sensitized C57BL/6 mice were exposed to 7 OVA-aerosols [29]. BAL was performed at d15 after clodronate treatment or instillation of mock liposomes or at d15 after the last (7^{th}) OVA-challenge respectively (n=7). Mock-treated naïve rAM, CL depletion-induced secondary rAM and post-inflammation rAM from the non-eosinophilic severe refractory asthma model were isolated and stimulated *ex vivo* with 0,1µg/ml LPS for 6h. RT-qPCR analysis was used to determine the mRNA transcript levels of *il6*, *il12p40*, *usp18* and *ifit2*. Results represent mean n-fold induction levels compared to unstimulated rAM from mock treated mice ± SD from triplicate PCR reactions. (B) OVA/CFA sensitized wt C57BL/6 mice (CD45.2) (n=7) were irradiated and reconstituted intravenously with CD45.1-expressing donor bone marrow cells at d-10 and d-9 respectively. YG^+ microspheres were administered i.t. at d-5 followed at d0 by exposure of the mice to OVA-aerosol, or left unchallenged as control. BAL samples were collected after two, four and seven OVA-exposures. The presence of naïve rAM (microsphere$^+$ CD45.2$^+$) in the BAL was determined as in figure 5B and C. Error bars represent SEM. Dotted line represents the naïve control. *** $p<0.0001$. All data presented are representative for two independent experiments.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il6
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usp18
ifit2
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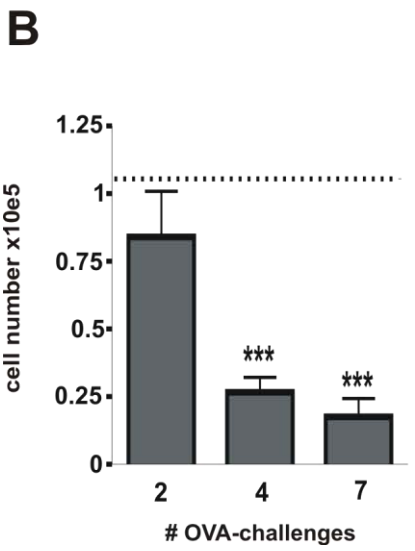
LPS
- +
Naïve rAM
Depletion-induced rAM
Post rAM (CFA)


B
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2
4
7
OVA-challenges

DISCUSSION

Inflammatory responses are characterized as highly dynamic processes. Once local innate immune cells are activated by the inflammatory insult, cytokine and chemokine secretion results in different waves of leukocyte recruitment to the site of inflammation. After the elimination of the antigenic threat, inflammation is cleared and the tissue eventually strives to regain steady-state conditions. In the present study, we used a mouse model of eosinophilic asthma in which OVA/alum sensitized mice are exposed to nebulised OVA, resulting in the infiltration of the bronchoalveolar lumen with eosinophils, $CD4^+$ T-lymphocytes, predominantly Th2-cells, and monocytes. Once allergen exposure was arrested, the bronchial inflammation dampened and cleared eventually. Absolute cell numbers returned to basal levels and the cellular composition of the alveoli again consisted nearly exclusively of rAM. Phenotypic analysis of this post-inflammation rAM-population showed few differences between naïve and inflammation-experienced rAM. CD11c and DEC-205, rAM surface markers not found on other macrophage populations and expressed mainly by DCs and DC-subpopulations [15-20], were equally expressed on both naïve and post-inflammation rAM. High intrinsic fluorescence intensities were also found on both rAM-populations. Yet, post-inflammation rAM exhibited higher levels of CD11b, another member of the integrin family which is historically considered as the canonical macrophage marker [22], and of CD115, the receptor for the monocyte/macrophage growth factor M-CSF [30].

In spite of post-inflammation rAM being phenotypically nearly indistinguishable from naïve rAM, both populations differed dramatically in innate functionality. rAM readily engulf opsonised and non-opsonised particulate matter [23]. This high phagocytic capacity is also illustrated by the high uptake of latex microspheres that we observed in cultures of naïve rAM. However, post-inflammation rAM appear to have lost this phagocytic capacity, showing a near 10-fold decrease in latex beads engulfed per cell after 6h and a similar decrease in the number of cells having engulfed latex beads. Contrary to this reduced phagocytic capacity, *ex vivo* TLR-stimulation of post-inflammation rAM showed a strongly enhanced inflammatory cytokine and chemokine response. Secreted levels of inflammatory mediators like TNF-α, IL-6, IL-12p70, CXCL1 and CXCL2 were increased after LPS and imiquimod stimulation compared to naïve rAM. These data indicate a shift from a naïve rAM-phenotype exerting phagocytic clearance of inhaled microparticles with a minimal inflammatory reactivity to a post-inflammation rAM-phenotype possibly responding to a microbial insult by secreting a

full range of inflammatory mediators. Further evidence for a shift towards a less tightly controlled inflammatory phenotype derives from the strikingly different regulation of IFN-β expression observed in post-inflammation rAM. Although naïve rAM possess functional IFNAR-signalling which renders them fully responsive to exogenous type I IFN, these cells characteristically fail to autonomously secrete IFN-β after engagement of TLR-3 or TLR-4 [26]. However, when assayed for this trait, post-inflammation rAM produced significant levels of bioactive IFN-β following TLR-4 and TLR-3 stimulation by LPS and poly I:C respectively. TLR-4 is the only TLR family member that uses all four known downstream adaptor molecules (TIRAP, MyD88, TRIF and TRAM) [31]. Engagement of TLR-4 induces the expression of NF-κB target genes via the MyD88-dependent pathway while the type I IFN response (via IRF-3) is induced mainly via the TRIF-dependent pathway. Triggering of TLR-3 however, engages predominantly TRIF-dependent signalling and expression of type I IFNs [31]. In agreement herewith, TLR-3 triggering by poly I:C selectively induced a potent IFN-β response in post-inflammation rAM whereas naïve rAM failed to do so. Finally, triggering exclusively the complementary MyD88-signalling pathway by using the TLR-7 ligand imiquimod, further confirmed the strongly increased inflammatory reactivity of post-inflammation rAM as opposed to the hypoinflammatory reactivity of naïve rAM. Thus, innate imprinting of rAM by allergic bronchial inflammation causes a switch from a highly phagocytic, hypoinflammatory to a low phagocytic, hyperinflammatory phenotype which can contribute to the increased total pulmonary response to microbial and viral infectious agents.

A shift in the GM-CSF/M-CSF balance may be at the origin of the differential type I IFN responsiveness of post-inflammation rAM. It has been shown before that priming of bone marrow derived macrophages (BMDM) with M-CSF or GM-CSF affects the cytokine repertoire produced after LPS-stimulation. GM-CSF-priming of BMDM enhanced the expression of genes induced by the MyD88-dependent pathway like *tnf-α*, *il-12p40* and *il-23p19*, while M-CSF-primed BMDM expressed increased levels of IRF-3 and IFN-β-induced genes like *ifn-β* and *ccl5* [32]. The healthy lung constitutes a GM-CSF rich environment [33]. Imprinting by GM-CSF of naïve rAM could therefore be at the basis of the defective IFN-β production observed in this rAM-population. A shift towards M-CSF as a result of the preceding allergic bronchial inflammation could then contribute to the enhanced TRIF-dependent TLR-signalling seen in post-inflammation rAM. This proposition is further supported by the observed dependence of the post-inflammation rAM-phenotype on a preceding allergic bronchial inflammation, rather than on the recruitment of monocytes per se.

Thus, sterile depletion of naïve rAM by clodronate liposomes followed by a spontaneous repopulation of the lung with a new population of secondary rAM did not result in the characteristic inflammatory and IFN-β positive phenotype as represented by post-inflammation rAM. Yet, also an accelerated rAM-turnover and replacement by newly elicited mononuclear phagocytes may be part of the mechanism underlying this phenomenon of innate imprinting. Applying the genetically stable and activation-independent CD45.1 and CD45.2 alloantigen expression system to generate chimeric mice expressing the CD45.1 alloantigen on peripheral blood leukocytes and the CD45.2 alloantigen on rAM, we observed a rapid clearance of naïve rAM from the bronchoalveolar lumen starting after four OVA-aerosol challenges and their replacement by newly recruited monocytes/macrophages. Similar findings have been reported using a mouse model for acute respiratory distress syndrome in which a rapid rAM-turnover is induced by a single bolus instillation of *E. coli* LPS [34]. A study of the rAM-turnover kinetics in mice infected with *Streptococcus pneumoniae* also revealed a brisk replacement of rAM by elicited macrophages during the innate phase of the infection [35]. Thus, an inflammation-driven clearance of rAM may facilitate their replacement by a novel population of rAM that have acquired an altered innate response profile as a result of an altered inflammatory cytokine profile in the local microenvironment. The generic nature of this mechanism of innate imprinting is further illustrated by the similar rAM-clearance kinetics and the similar post-inflammation rAM functional phenotype observed in a recently established mouse model of Th1- and Th17-biased non-eosinophilic severe refractory asthma [29]. Nevertheless, the nature of the inflammatory response may exert some fine-tuning of the basal differentiation of the post-inflammation rAM. Thus, in the eosinophilic asthma model, Th2-associated cytokines like IL-4 may promote the M2-differentiation state of post-inflammation rAM, as suggested by the increased *arg-1* to *inos* ratio we observed in this model.

In conclusion, alveolar macrophages exist in an environment high in antigenic material of which the majority must be ignored except when the antigen represents an infectious threat [36, 37]. This rAM-phenotype provides a basal level of restraint that can be elegantly overridden in the presence of infection. We demonstrate that this tightly controlled cell population rapidly disappears from the alveoli following a brief exposure to allergen and is subsequently replaced by a new post-inflammation rAM-population which exhibits altered innate characteristics. Besides a reduced phagocytic clearance of (inhaled) microparticles and an increased inflammatory TLR-signalling, the acquisition of an autonomous IFN-β secretion

capacity is among the most striking features of this case of innate imprinting. Until now, most reports on innate imprinting reported TLR-desensitization and/or a more restrained inflammatory phenotype as main features of the imprinted macrophages [11, 12]. Here we demonstrate an opposite outcome, namely an 'immunologically released' rAM-phenotype, possibly contributing to the increased sensitivity of the allergic lung towards bacterial and viral infections and renewed exposure to allergen.

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AUTHOR CONTRIBUTIONS

T. Naessens wrote the paper and performed the major part of the experimental work.
S. Vander Beken, P. Bogaert and S. Lienenklaus performed research.
N. Van Rooijen provided clodronate liposomes.
S. Lienenklaus, S. Wiess, S. De Koker and J. Grooten designed research.

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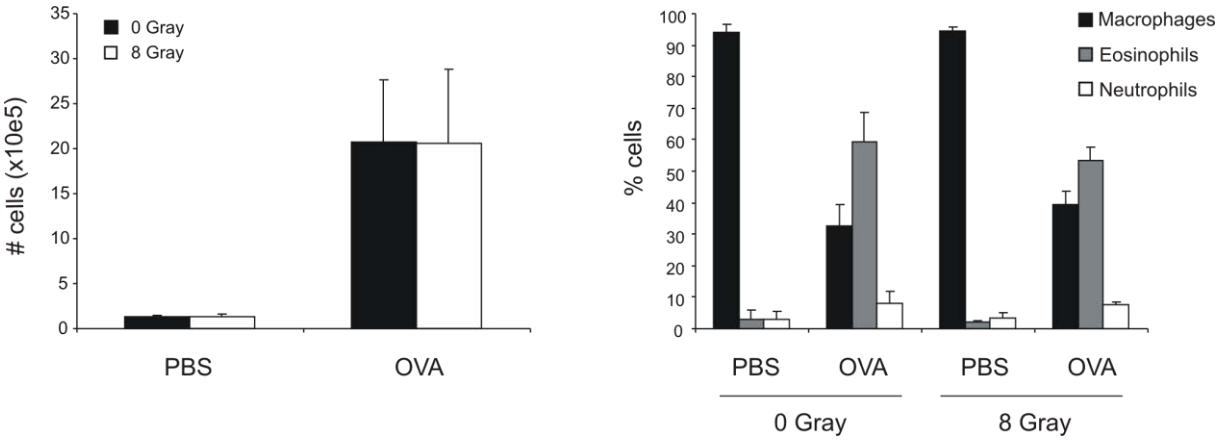
SUPPLEMENTAL FIGURES



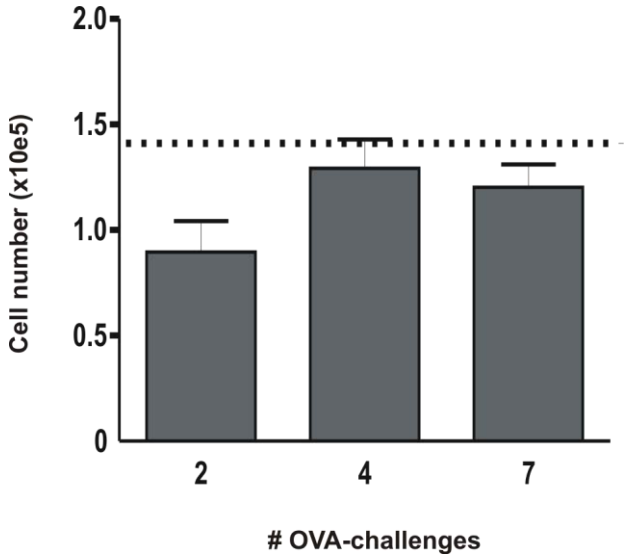
Supplemental figure S1. The pulmonary response of irradiated recipient mice to nebulised OVA.
Sensitized C57BL/6 mice (n=5) were exposed to a total body irradiation dose of 8 Gray or left untreated at d0 and reconstituted by intravenous injection of bone marrow cells or PBS respectively at d1. Subsequently, mice were exposed to 2 OVA-aerosols or left unchallenged as control at d10 and d11. BAL was performed at d12 and Average BAL cell counts +/- SEM (left panel) and average BAL cellular composition +/- SEM (right panel) were determined by flow cytometry and Giemsa and May-Grünwald-stained cytospin analyses.

ADDITIONAL DATA

Inflammation-induced rAM-turnover is ATP-dependent

In order to investigate which mechanism is involved in the observed allergic inflammation-induced naïve rAM-turnover, a chimeric mouse model, as described in figure 5 of the manuscript body, was applied in hCD39-transgenic mice. In these mice, the human *cd39* transgene is controlled by the Clara Cell (CC)-promoter which results in the constitutive overexpression of human CD39 by lung epithelial cells. CD39 is a surface-located ectopyrase responsible for the conversion of ATP/ADP to AMP. Its constitutive overexpression in the lungs of mice leads to the immediate depletion of local pulmonary produced ATP/ADP (Théâtre et al, *accepted for publication in The Journal of Immunology*).

In contrast to naïve rAM-numbers of wt mice, naïve rAM-numbers in hCD39-mice remained constant during the total course of seven OVA-exposures (additional figure A1).



Additional figure A1. Naïve rAM-turnover after allergen exposure of hCD39-transgenic mice.
OVA/alum sensitized hCD39 mice (CD45.2) (n=7) were irradiated and reconstituted intravenously with CD45.1-expressing donor bone marrow cells at d-10 and d-9 respectively. YG^+ microspheres were administered i.t. at d-5 followed at d0 by exposure of the mice to OVA-aerosol, or left unchallenged as control. BAL samples were collected after two, four and seven OVA-exposures. The presence of naïve rAM (microsphere$^+$ CD45.2$^+$) in the BAL was determined as in figure 5B and C of the manuscript body. Error bars represent SEM. Dotted line represents the naïve control.

These data imply that the inflammation-induced naïve rAM-turnover observed in wt mice is an ATP-dependent process. As ATP is a potent co-stimulator of inflammasome/caspase-1

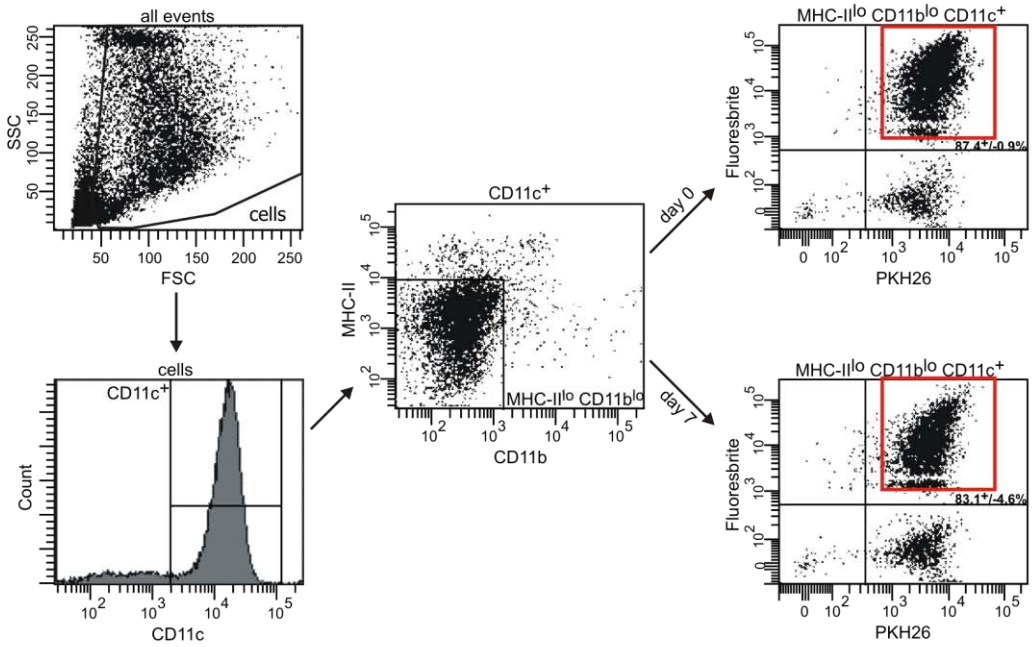
mediated apoptosis, referred to as pyroptosis [38], this pathway of cell death constitutes a plausible mechanism for the allergic inflammation-induced naïve rAM-turnover. However, further research, such as the assessment of caspase-1 activity and cell death markers in naïve rAM after two or three OVA-exposures, is needed to confirm these findings.

Inflammation-induced rAM-turnover is not influenced by the underlying bone-marrow chimeric approach

It is known that total body irradiation can accelerate constitutive rAM-turnover [39, 40]. Therefore, the use of a bone-marrow chimeric approach to study the allergic inflammation-induced rAM-turnover can bias the basic findings described earlier in the manuscript. To exclude any possible side-effect of the prior irradiation on naïve rAM-turnover, we applied an alternative approach to positively identify naïve rAM. We intratracheally administered the fluorescent phagocytic cell label, PKH26. This fluorescent label has been shown before to specifically label rAM after intratracheal or intranasal administration [41]. In combination with intratracheal delivery of fluorescent latex microspheres, flow cytometry analysis allowed to identify naïve rAM as microsphere^{+} PKH26^{+} cells. In this way, up to 80% of the total naïve rAM-population ($CD11c^{+}CD11b^{-}MHCII^{low}$ cells) was labelled (additional figure A2A). In addition, this dual *in situ* labelling of naïve rAM with fluorescent latex microspheres and the fluorescent PKH26 dye remained stable over a time period of seven days both at the level of fluorescence intensity and the number of labelled cells (additional figure A2A). Finally, this dual intratracheal administration of fluorescent latex microspheres and the fluorescent PKH26 dye did not elicit inflammation (additional figure A2B).

As illustrated in additional figure A3, absolute numbers of naïve rAM, identified as $CD11c^{+}$ microsphere^{+} PKH26^{+} cells, dropped sharply after 4 exposures to nebulised OVA (additional figure A3). This result is in line with data obtained from the bone marrow chimeric experiment depicted in figure 5 of the manuscript body. Thus, the observed allergic inflammation-induced naïve rAM-turnover is not due to an accelerated reconstitution of pulmonary macrophages after total body irradiation and bone marrow transfer but represents a feature intrinsic of allergic pulmonary inflammation per se.

A



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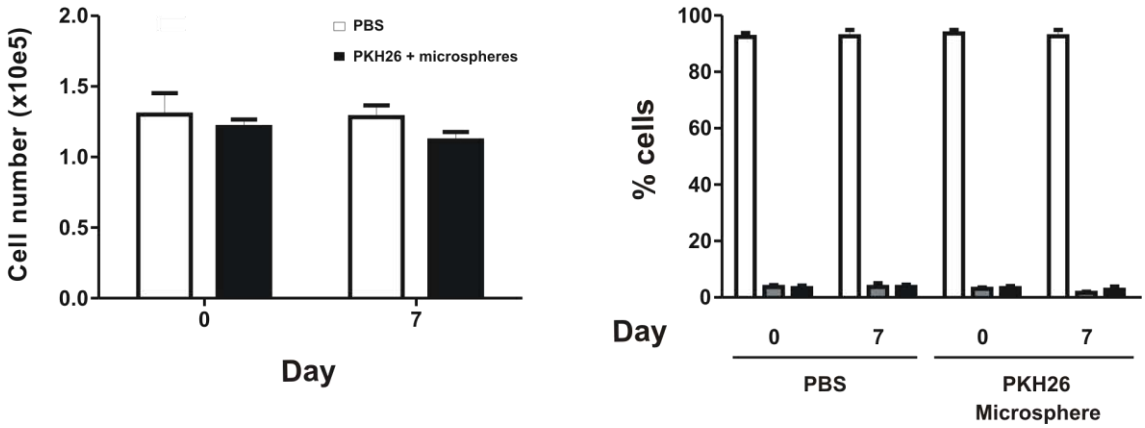
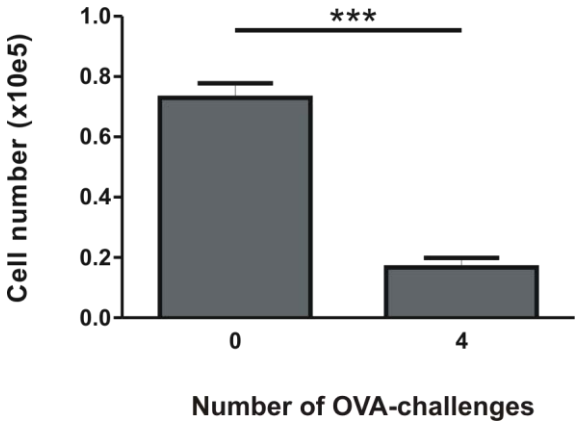


Additional figure A2. Labelling of naïve rAM with the fluorescent PKH-26 dye and fluorescent latex microspheres.

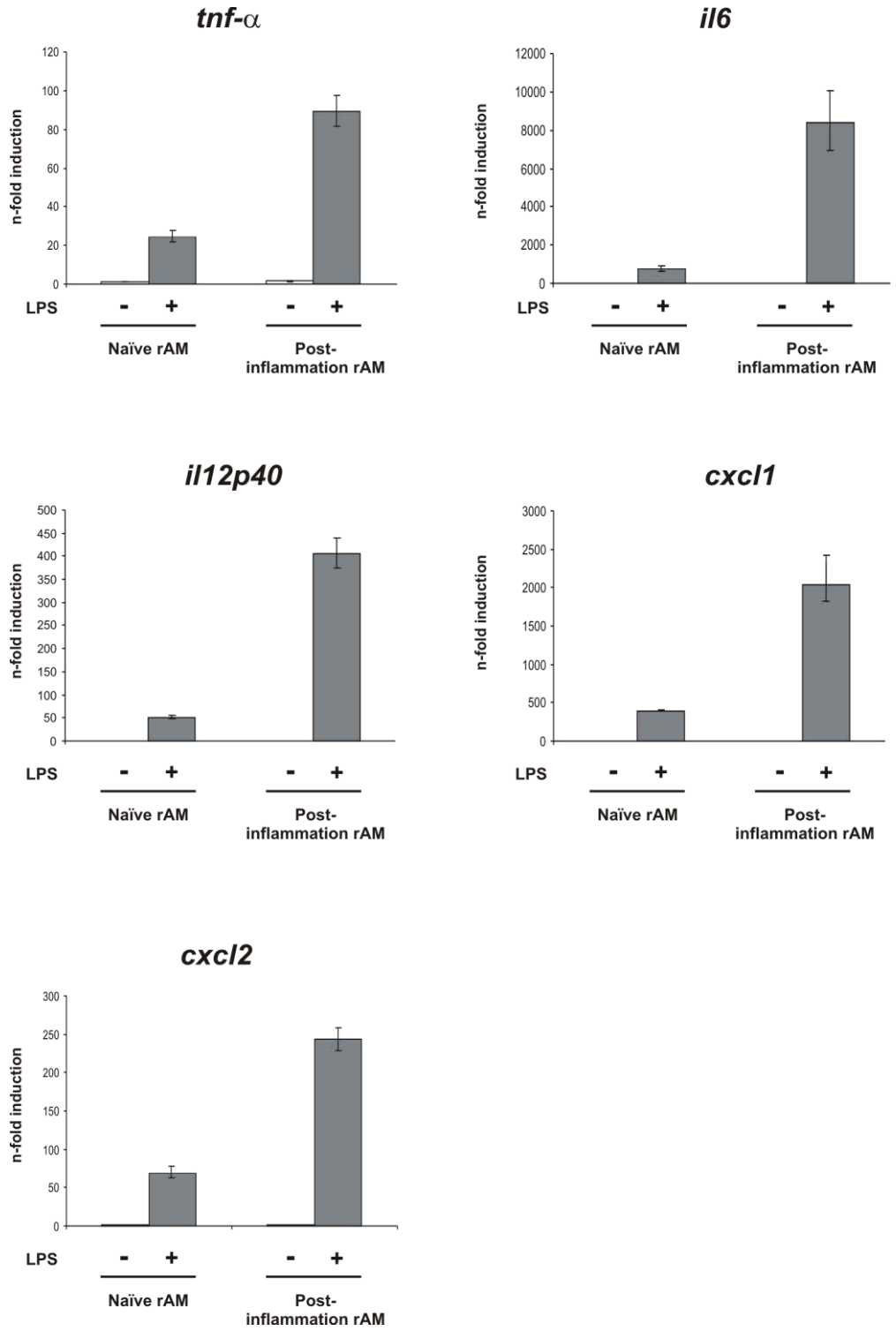
C57BL/6 mice were intratracheally instilled with PKH-26 (10µM) and fluorescent microspheres ($5x10^7$) at d-5 and d-2 respectively. (A) BAL was performed at d0 and d7 and naïve rAM were identified by flow cytometry as $CD11c^+CD11b^-MHCII^{low}$ cells within the cell gate. Finally, the portion of $PKH26^+microsphere^+$ naïve rAM was determined (red gate). (B) Average BAL cell counts +/- SEM (left panel) and average BAL cellular composition +/- SEM (right panel) from PBS-instilled and PKH26/microsphere-instilled mice were determined by flow cytometry and Giemsa and May-Grünwald-stained cytospin analyses. White bars: Macrophages, grey bars: neutrophils and black bars: eosinophils.



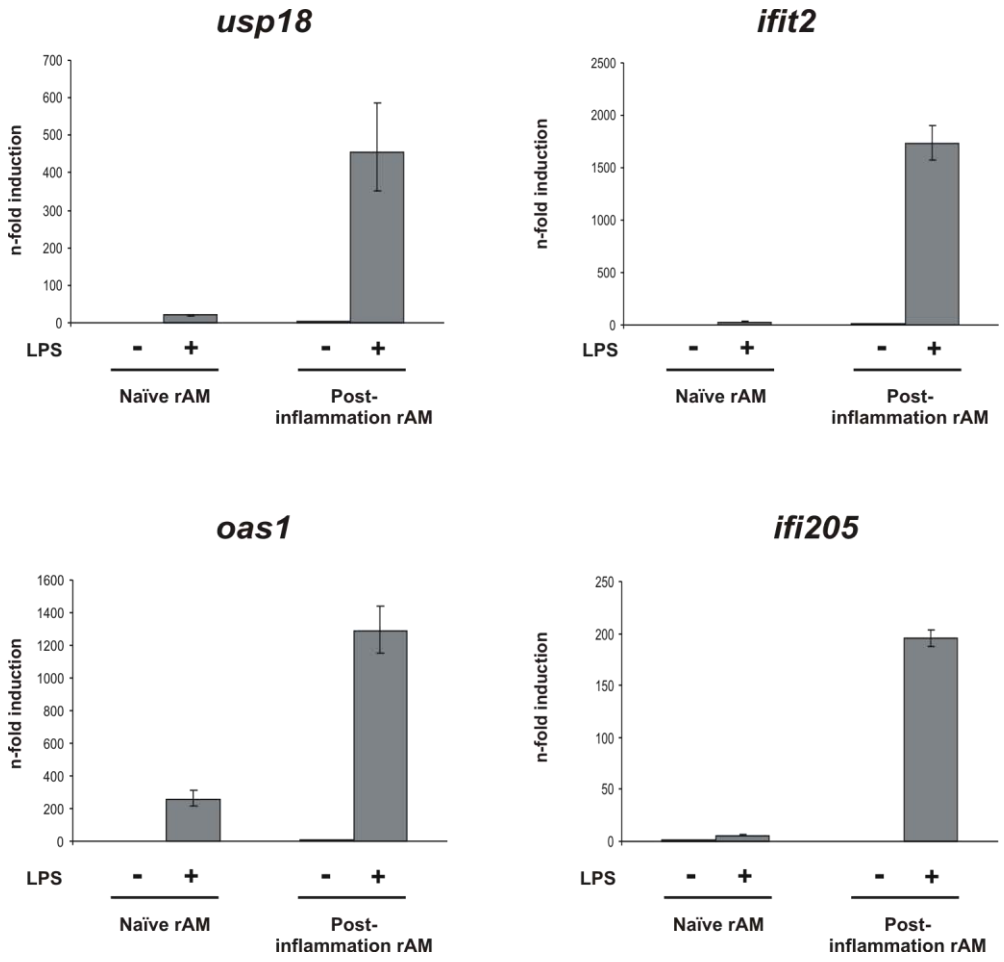
Additional figure A3. Turnover of naïve rAM, labelled as PKH26$^+$microsphere$^+$ cells, in response to allergen exposure.
C57BL/6 mice were immunized with OVA/alum at d-14 and d-7. Subsequently, the PKH26 dye (10µM) and fluorescent microspheres ($5x10^7$) were intratracheally administered at d-5 and d-2 respectively. At d0 till d3 mice were exposed to 1% OVA-aerosol (n=7) or left untreated (n=7) and BAL was performed at d4. The presence of naïve rAM (PKH26$^+$ microsphere$^+$ cells) in BAL samples was determined by flow cytometry as in additional figure A2. Average absolute naïve rAM-numbers were calculated from the percentages of PKH26$^+$ microsphere$^+$ cells by flow cytometry and the total BAL cell count. Bars represent SEM. *** p<0, 0001.

Differential inflammatory cytokine and IFN-β response of post-inflammation rAM after TLR-stimulation in BALB/c mice

We next investigated to what extent the increased TLR-signalling in post-inflammation rAM is affected by the genetic background of the mouse strain. We therefore compared the *in vitro* response of naïve and post-inflammation rAM from BALB/c mice to LPS. rAM were isolated from naïve and post-inflammation lungs of BALB/c mice and cultured *ex vivo* for 6h in the presence of *E. coli* LPS (0,1µg/ml). Expression of genes encoding the pro-inflammatory cytokines and chemokines TNF-α, IL-6, IL-12, CXCL1 and CXCL2, was subsequently assessed by RT-qPCR. As shown in additional figure A4, post-inflammation rAM showed increased expression of these pro-inflammatory genes after LPS-stimulation compared to naïve rAM (additional figure A4). In addition, autocrine and paracrine IFN-β activity was measured by determining the transcript levels of the IFN-β responsive genes, *usp18*, *ifit2*, *oas1* and *ifi205*. Compared to naïve rAM, post-inflammation rAM expressed largely increased levels of *usp18*, *ifit2*, *oas1* and *ifi205* after LPS-stimulation (additional figure A5). Differences in gene expression levels between both LPS-stimulated rAM-populations of BALB/c mice were of the same magnitude as those observed in C57BL/6 mice. Therefore, we suggest that innate imprinting of rAM by a preceding allergic bronchial inflammation is independent of the strain genetic background.



Additional figure A4. Inflammatory cytokine and chemokine response of naïve and post-inflammation rAM of BALB/c mice to TLR-4 engagement.
Naïve and post-inflammation rAM isolated from BALB/c mice (n=8) were *ex vivo* stimulated with 0.1µg/ml *E. coli* LPS for 6h or left untreated as control. Gene expression levels of *tnf-α*, *il6*, *il12p40*, *cxcl1* and *cxcl2* were measured using RT-qPCR. Results represent mean n-fold induction levels compared to unstimulated rAM from mock treated mice ± SD from triplicate PCR reactions.



Additional figure A5. IFN-β response of naïve and post-inflammation rAM from BALB/c mice to TLR-4 engagement.
Naïve and post-inflammation rAM were isolated from BALB/c mice (n=8) and stimulated *ex vivo* with 0,1µg/ml *E. coli* LPS for 6h or left untreated as control. RT-qPCR was used to analyze mRNA expression levels of *usp18*, *ifit2*, *oas1* and *ifi205*. Results represent the mean n-fold induction compared to unstimulated naïve rAM ± SD of triplicate PCR reactions.

Chapter 2

Innate Imprinting of Resident Alveolar Macrophages by an Allergic Bronchial Inflammation Affects the Outcome of a Subsequent RSV Lung Infection

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Manuscript in preparation

INTRODUCTION

Resident alveolar macrophages (rAM) are the predominant cell population in the alveolar spaces of healthy individuals and serve as important sentinels in the recognition of invading pathogens and other airborne particles. In combination with their key innate effector function, rAM are thought to have an immunosuppressive effect in the lung as well, limiting excessive inflammation in order to prevent disturbance of the pulmonary gas exchange function [1]. rAM have been shown to exert immunosuppressive activities on T-lymphocytes and dendritic cells (DCs) in different pathologies, including allergic asthma. Allergic asthma is a chronic inflammatory disease of the airways associated with a predominant Th2-response to inhaled allergens and results in the airway infiltration of eosinophils and mast cells, goblet cell hyperplasia, and airway hyperreactivity (AHR) [2]. By acting as significant pulmonary sources of the immunosuppressive cytokines, IL-10 and TGF-β, rAM are able to inhibit pro-inflammatory cytokine secretion and leukocyte maturation during allergic bronchial inflammation. In addition, these two cytokines are commonly implicated in the generation of the inducible regulatory T-cell subsets Tr1 and Th3 [3]. Several studies demonstrated a significant increase in allergic inflammation and T-cell reactivity in antigen challenged lungs after depletion of rAM [4-6]. Increased sensitivity of rAM-depleted lungs to antigen exposure observed in these studies was attributed to the loss of rAM-mediated suppression of DC-maturation, function and trafficking to mediastinal LNs [7, 8].

In asthmatics, pulmonary accumulation of inflammatory leukocytes and cytokines together with lung structural remodelling and AHR eventually lead to recurrent episodes of airway obstruction, wheezing and shortness of breath. These macroscopic observable symptoms are referred to as asthmatic exacerbations. Acute exacerbations are the major cause of morbidity, mortality and healthcare costs for individuals with asthma while current preventive and therapeutic options are limited. Today, mechanisms of asthmatic exacerbations are still poorly understood. Yet, it is increasingly clear that the causative agent of these exacerbations has very diverse origins. However, the majority of asthmatic exacerbations are associated with respiratory viral infections, including rhinoviruses, influenza, parainfluenza, and adenoviruses. The most severe asthmatic exacerbations in children [9] and adults [10] are often induced by respiratory syncytial virus (RSV) infections.

RSV is a non-segmented, negative strand RNA virus of the *Paramyxoviridae* family. RSV is the leading cause of infant hospital admission and causes 70% of the bronchiolitis hospitalizations in the developed world [11]. Infection of airway epithelial cells (AECs) by RSV induces an antiviral immune cascade in which different cellular and protein components are found to be crucially involved in clearance of the virus. Cytotoxic $CD8^+$ T-lymphocytes (CTLs) [12, 13] and neutralizing immunoglobulin (Ig)-molecules [13-15] are pivotal in the clearance of (recurrent) RSV-infections. However, a variety of studies emphasized the importance of rAM as key players in the early immune responses to RSV-infection [16]. It is even suggested that rAM, rather than adaptive immune cells, are critical determinants of the severity of RSV-induced bronchiolitis [17]. As professional phagocytes, rAM are able to efficiently engulf and eliminate high doses of invading viral particles [17]. In addition, rAM are the primary type I IFN producers during RNA-virus pulmonary infection [18].

In a mouse model of allergic bronchial inflammation, we recently demonstrated that, after the clearance of the eosinophilic inflammation, a new secondary rAM-population resides in the airways. Strikingly, this post-asthma rAM-phenotype displayed increased pro-inflammatory reactivity in response to *in vitro* TLR-4, TLR-3 and TLR-7 stimulation. Additionally, the increased pro-inflammatory *in vitro* TLR-reactivity of these post-asthma rAM was combined with a decreased phagocytic activity (*Naessens et al. accepted for publication in AJP, 2012*) Therefore, alteration of the rAM's functional phenotype due to a preceding allergic bronchial inflammation may affect the pathophysiological outcome of subsequent respiratory (RSV) viral infections. We now provide evidence that the RSV antiviral immune response of post-asthma rAM, present in the alveolar lumen after the resolution of the allergic bronchial inflammation, is largely modified. Specifically, we show that in contrast to rAM from naïve mice, post-asthma rAM exhibit strong pro-inflammatory reactivity during RSV-infection. Furthermore, compared to naïve rAM, post-asthma rAM showed a reduced contribution to viral clearance. Our observations reveal that post-asthma rAM may be implicated in the increased susceptibility of asthma patients to secondary RSV-infections and contribute to the onset of exacerbate reactions associated with asthmatic individuals.

MATERIALS AND METHODS

Mouse model

6- to 8-week old female BALB/c mice, purchased from Janvier (Le Genest St.Isle, France) and kept under specified pathogen free conditions were immunized intraperitoneally with 20µg of grade V chicken egg OVA (Sigma-Aldrich, St.Louis, MO, USA), adsorbed on 1mg $AlOH_3$ (alum; Sigma-Aldrich) in endotoxin-free PBS (Lonza, Walkersville, MD, USA). OVA-sensitized mice were then exposed to OVA-aerosols, consisting of 1% of grade III OVA (Sigma-Aldrich) in PBS. To establish a subsequent RSV lung infection, 1.10^7 PFU of mouse-adapted RSV A2 (propagated on Hep-2 cells) was administered intranasally to mice that were slightly anesthetized by isoflurane. For the depletion of rAM, 100 µl of a 30% clodronate (dichloromethylene-diphosphonate) liposome solution (in PBS) was administered intratracheally 3 days prior mock- or RSV-infection to mice that were fully anesthetized by a ketamine/xylazine mixture. All experiments performed in this study were approved by the local ethical committee.

Virus and viral plaque assay

RSV A2 was propagated on Hep-2 or Vero cells which were grown in DMEM medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 100U/ml penicilline/streptomycin (PS; Invitrogen) and 2mM L-glutamine at 37°C in the presence of 5% CO_2. Subsequently, RSV A2 was stored in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (HBSS; Invitrogen, Carlsbad, CA, USA) supplemented with 20% sucrose. RSV titers in stock solutions and BAL fluid were determined by plaque assay on Vero cells. RSV plaques were stained with anti-RSV goat serum (AB1128, Chemicon International, Billerica, MA, USA). For the BAL fluids in which no virus could be detected, the viral titer was set as the detection limit of the used assay as indicated. Inactivation of RSV was performed by heat inactivation (30 min. at 56°C).

Alveolar cell isolation and culture

Mice were anesthetized with avertin (2,2,2-tribromethanol; 2,5% in PBS; Sigma-Aldrich). BAL was performed by making a small incision in the trachea, to allow passage of a lavage canulae. Lungs are flushed 4 times with 1ml Ca^{2+}- and Mg^{2+}-free HBSS, supplemented with 0.05mM EDTA (ethylenediaminetetraacetic acid). Optionally, a prior lavage with 0,5ml

HBSS-EDTA was performed and BAL fluid was isolated by centrifugation and collection of the supernatant. BAL cells were washed and resuspended in PBS for further use. Naïve and post-asthma rAM isolated via BAL were cultured in complete culture medium (RPMI 1640 (Invitrogen) containing 1% heat-inactivated FCS, 25mM HEPES (N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid), 2mM L-glutamine, 1mM pyruvate, 100U/ml PS, and 55µM 2-ME (Sigma-Aldrich)). All cultures were enriched for macrophages by plastic adhesion for 1h at 37°C. Naïve and post-asthma rAM were then stimulated for the indicated time with RSV A2 at 37°C in the presence of 5% CO_2.

Lung isolation and homogenization

Mice were anesthetized with avertin and the lungs were removed from the thorax. Lung tissue was first minced and incubated for 30 min at 37°C in RPMI 1640 medium containing 150 U/ml collagenase II (Sigma-Aldrich) and 0.02 mg/ml DNase I (Roche Diagnostics, Vilvoorde, Belgium). Minced lungs were then passed through 70 µm nylon meshes (BD Biosciences, San Jose, CA, USA) to obtain single cell suspensions. Red blood cells were lysed using ACK red blood cell lysis buffer (Lonza).

Total and differential cell counts

Total numbers of BAL and lungs cells were counted by use of a Bürker-chamber (Marienfeld, Lauda-Königshofen, Germany). Trypane blue was added to exclude dead cells. BAL cell type composition and pulmonary NK-cell levels were analyzed by flow cytometry. Briefly, BAL cells, pre-incubated with Fc-Block were classified as monocytes (alveolar macrophages, elicited monocytes and DCs), neutrophils, eosinophils or T-lymphocytes based on forward and side scatter gating and fluorescence intensities for anti-mouse MHC II-eFluor450, CD3ε-Alexa488, CCR3-PE, CD4-PerCP, CD8-PE-Cy7, CD11c-APC and CD11b-APC-Cy7. Lung NK-cells were identified based on forward and side scatter gating and fluorescence intensities for anti-mouse CD3ε-Alexa488, CD11b-APC-Cy7 and CD49b-V450. Pre-incubation of the lung cells with Fc-Block was used to prevent unwanted binding to FcRs. All antibodies were purchased from BD Biosciences, except CCR3-PE (R&D Systems, Abingdon, UK). All samples were measured on a FACS LSRII flow cytometer and analyzed using FACS Diva software (both from BD Biosciences).

Cytokine/chemokine measurement

Protein levels of mouse TNF-α, IL-6, MCP-1 and IL-10 in culture supernatant were quantified with the Bioplex suspension array system (Biorad, Hercules, CA, USA) for simultaneous detection of cytokines, according to the manufacturer's protocol. The analytes were measured with the Bioplex protein array reader and the Bioplex manager software, using recombinant cytokine standards (all from Biorad).

Serum IL-6 bioactivity was assessed through an in-house developed 7TD1 bio-assay according to [19].

IFN-γ enzyme-linked immunospot (ELISPOT) assay

IFN-γ producing lung draining lymph node (LDLN) $CD8^+$ T-cells were quantified by IFN-γ ELISPOT assay kit (Diaclone, Besançon, France), according to the manufacturer's instructions. Briefly, LDLN of mice were passed through 70 µm nylon meshes to obtain single cell suspensions. Red blood cells were lysed using ACK red blood cell lysis buffer. LDLN cells were subsequently cultured in complete culture medium (RPMI 1640 containing 10% heat-inactivated FCS) on anti-IFN-γ antibodie (Diaclone) pre-coated 96-well ELISPOT plates (U-Cytech Biosciencs, Utrecht, The Netherlands) in the presence of RSV-derived MHC I binding F-peptide (KYKNAVTEL) (provided by Prof. Dr. K. Gevaert, Ghent University, Ghent, Belgium). ELISPOT plates were analyzed using an automated ELISPOT plate reader (AID, Strassberg, Germany)

Intracellular cytokine staining (ICS)

IFN-γ, IL-5 and IL-17 producing LDLN $CD4^+$ T-cells were quantified by ICS and flow cytometry. Briefly, LDLN of mice were passed through 70 µm nylon meshes to obtain single cell suspensions. Red blood cells were lysed using ACK red blood cell lysis buffer. LDLN cells were subsequently cultured in complete culture medium (RPMI 1640 containing 10% heat-inactivated FCS) in the presence of heat-inactivated RSV (MOI 0.5). After 20h of restimulation, Golgiplug (BD Biosciences) was added to the cultures for 6h. Subsequently, cultured LDLN cells, pre-incubated with Fc-Block, were stained with CD3ε-PacificBlue and CD4-PerCP (all from BD Biosciences). After fixation with 2% paraformaldehyde and permeabilization with saponine (Cytofix/Cytoperm kit, BD Bioscience), LDLN were finally

stained with IFN-γ-APC, IL-5-PE or IL-17-APC (all from BD Biosciences). All samples were measured on a FACS LSRII flow cytometer and analyzed using FACS Diva software.

RSV-based enzyme-linked immunosorbent assay (ELISA)

The presence of RSV specific antibodies in sera was detected by ELISA using RSV virions. 96-well MaxiSorp immunoplates (Nunc, Roskilde, Denmark) were coated with 55 000 PFU of RSV (propagated on Vero cells) per well in PBS. After RSV-coating, the wells were washed 3 times with PBS and blocked with 4% skimmed milk in PBS buffer. All subsequent steps were performed in a volume of 100 µl per well, and the wells were washed three times between incubation steps with PBS + 0.05% Tween 20. The presence of RSV-specific IgG, IgG1 and IgG2a in the samples was determined by incubating 1/3 serial dilutions of serum samples in the RSV-coated wells, starting with a 1/100 dilution, for 1 h. After washing, RSV-specific total IgG, IgG1 and IgG2a were respectively detected with horseradish peroxidase-conjugated anti-mouse total IgG (Amersham Biosciences, Buckinghamshire, UK), IgG1 or IgG2a serum (Southernbiotech, Birmingham, Alabama, US). After washing, plates were incubated for 5 min with tetramethylbenzidine substrate (Sigma–Aldrich). The peroxidase reaction was stopped by adding an equal volume of 1 M H_3PO_4. Antibody titers are defined as the reciprocal of the highest dilution with an OD_{450} that is at least three times the value obtained with pre-immune serum.

In vitro neutralization assay

Different dilutions of sera were incubated with RSV in serum free medium for 30 minutes at 37°C. Subsequently these mixtures were used to infect, Vero cells grown in a 96-well plate. Three hours later the cells were washed 3 times with growth medium containing 2% FCS. Thereafter the cells were incubated for 3 days in growth medium containing 2% FCS and 0.6% avicel RC-851 (FMCbiopolymers, Philadelphia, PE, USA). Viral infection was tested by immunostaining of the viral plaques with anti RSV goat serum.

Statistics

Statistics were performed using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA). Following outlier statistics in order to choose between performing a one-way ANOVA or Kruskal-Wallis nonparametric test, Gaussian distribution of parameters was checked using a Kolmogorov-Smirnov test. Differences in mean between each two

independent experimental groups were analyzed using an unpaired t-test or the nonparametrical Mann-Whitney U test at 95% confidence interval. No statistic analysis was done for data of pooled samples.

RESULTS

Post-asthma rAM exhibit altered antiviral immunity during RSV-infection

To examine the influence of a preceding allergic bronchial inflammation on the antiviral immune response of post-asthma rAM, a mouse model of allergic bronchial inflammation was set up. First, a Th2-biased sensitization of BALB/c mice against the model allergen OVA was elicited by repeated intraperitoneal immunization using aluminiumhydroxide (alum) as adjuvant. Subsequent exposure of sensitized mice to nebulized OVA generated an eosinophilic airway inflammation reminiscent of the immunopathology of mild to moderate asthma (figure 1A). Within 12 days after the last of seven OVA-exposures, the allergic eosinophilic bronchial inflammation was cleared. Absolute cell numbers returned to basal levels (figure 1B, left panel) and the alveoli again consisted for 90% of macrophages (figure 1B, right panel). In addition, at this time point Th2-associated inflammatory cytokines were no longer detectable in the BAL fluid (*data not shown*).

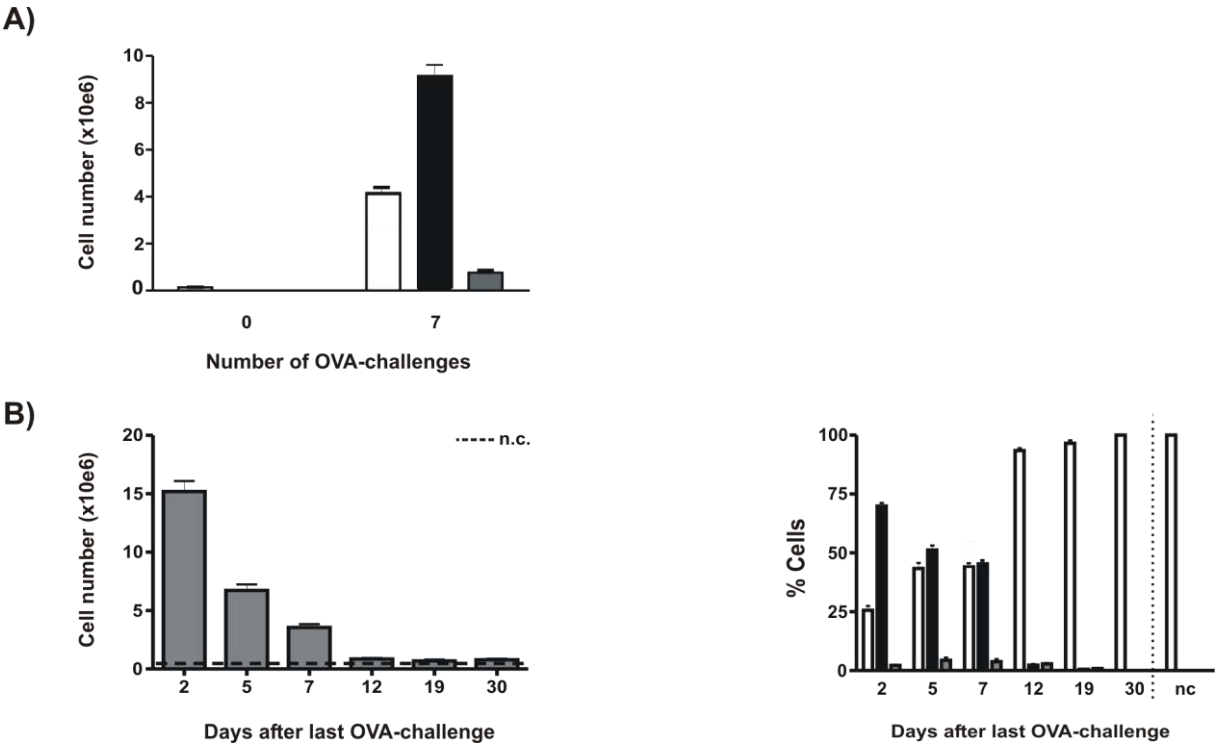

Figure 1. Exposure of OVA/alum sensitized BALB/c mice to nebulized OVA elicits a pulmonary eosinophilic inflammation reminiscent of the immunopathology of mild to moderate asthma.
OVA/alum sensitized BALB/c mice were exposed to 7 OVA-aerosols or left untreated as naïve controls (n=5). (A) Average absolute differential BAL cell counts determined 24h after the indicated number of OVA-exposures. (B) Average total (*left panel*) and relative differential (*right panel*) BAL cell counts determined at the indicated time points after the last OVA-exposure. Total and differential BAL cell numbers were determined via flow cytometry. White bars: macrophages; black bars: eosinophils; grey bars: neutrophils. nc = naïve control. Error bars represent SEM.

Subsequent RSV-infection was achieved by intranasal (i.n.) administration of the mouse-adapted RSV-strain A2 at d15 after the last OVA-exposure. In order to determine the *in vivo* role of post-asthma rAM during the subsequent RSV-infection, this rAM-population was depleted prior to infection through the intratracheal (i.t.) delivery of clodronate liposomes (CL) three days before the viral challenge. BAL was performed three days post-infection and alveolar inflammation was assessed via flow cytometry.

RSV-infection of naïve rAM^+ mice resulted in a relatively weak pulmonary inflammatory response. This inflammatory response was largely increased in the case of prior naïve rAM-depletion (figure 2A). In contrast, post-asthma rAM^+ lungs mounted a more severe inflammatory response against RSV which decreased when post-asthma rAM were depleted prior to infection (figure 2A). As shown in figure 2B, differences in total BAL cell numbers between the different mouse groups were mainly due to differences in macrophage/monocyte, neutrophil and $CD4^+$ T-cell numbers. Increased inflammatory responses of the post-asthma lungs and the pronounced pro-inflammatory reactivity of post-asthma rAM were however not correlated with increased viral clearance. Naïve and post-asthma rAM^+ lungs showed no difference in RSV-titers at d3 post-infection (figure 3A). Furthermore, depletion of naïve rAM prior to infection resulted in increased RSV-titers in the alveolar lumen (figure 3A). In contrast, depletion of post-asthma rAM prior to infection had no effect on the alveolar RSV-titers (figure 3A). In addition, it is worth mentioning that increased viral titers observed in RSV-infected rAM^- lungs could not be attributed to impaired natural killer (NK-)cell recruitment to the lungs. On the contrary, most NK-cell numbers were seen in RSV-infected, rAM^- lungs (figure 3B).

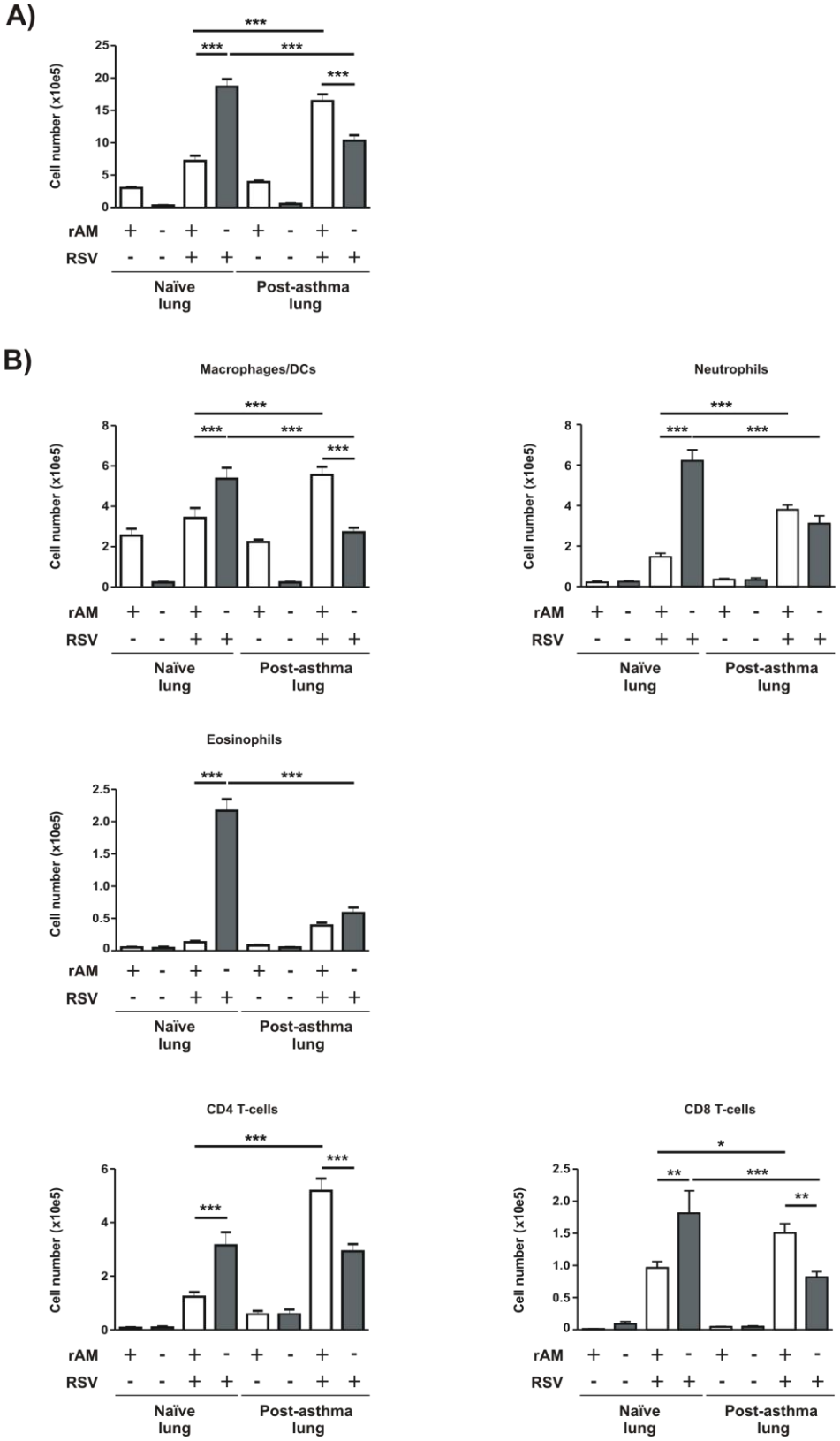

Figure 2. Alveolar inflammatory response to RSV-infection in naïve and post-asthma rAM$^+$ and rAM$^-$ mice at d3 post-infection.

Post-asthma (d15 after the last OVA-challenge) and naïve BALB/c mice (n=7) received CL i.t. three days before inoculation with 1.10^7 PFU RSV to deplete post-asthma and naïve rAM respectively or received PBS as a control. BAL was collected at d3 post-infection. (A) Average BAL cell counts ± SEM and (B) average BAL cellular composition ± SEM were determined by flow cytometry. * $p<0.05$; ** $p<0.01$ and *** $p<0.0001$.

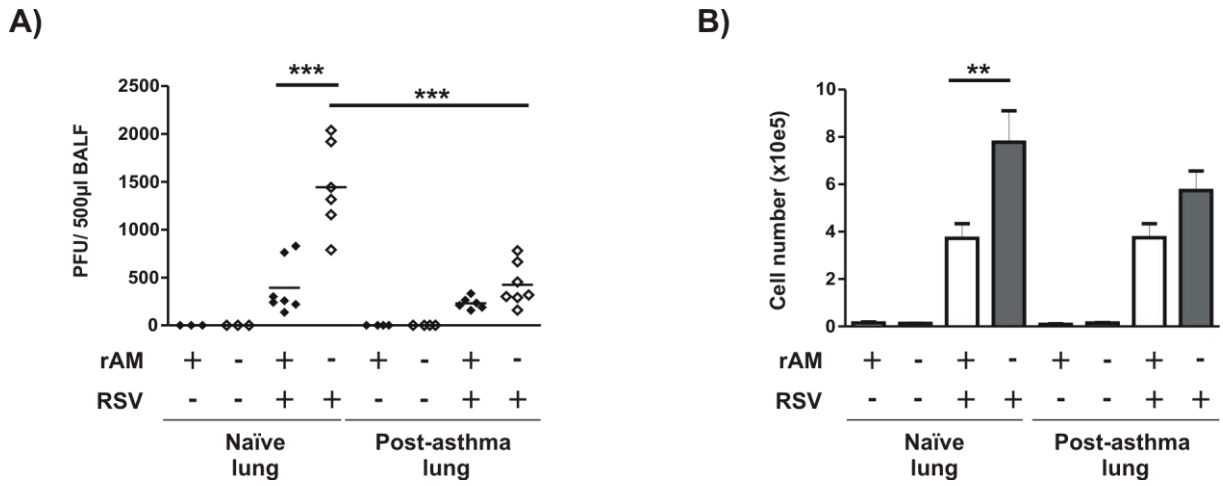

Figure 3. Alveolar RSV-titers in naïve and post-asthma rAM$^+$ and rAM$^-$ mice at d3 post-infection.
Post-asthma (d15 after the last OVA-challenge) and naïve BALB/c mice (n=7) received CL three days before inoculation with 1.10^7 PFU RSV to deplete post-asthma and naïve rAM respectively or PBS as a control. (A) On d3 post-infection, BAL fluid was collected and virus recovery was assessed by plaque assay. (B) Average NK-cell numbers ± SEM in whole-lung tissue specimens were determined as CD3ε$^-$CD49b$^+$CD11b$^+$ cells within the lymphocyte FSC-SSC gate by flow cytometry. ** $p<0.01$ and *** $p<0.0001$.

Post-asthma rAM exhibit increased cytokine and chemokine production after in vitro RSV-infection

We next investigated whether the observed differences in *in vivo* antiviral immune responses between naïve and post-asthma rAM were due to intrinsic differences between both rAM-populations or rather reflected secondary effects elicited by other cell types in the lung. To address this issue, naïve and post-asthma rAM were isolated and cultured *in vitro* for 24h and 48h in the presence or absence of RSV. RSV failed to induce a productive infection in both rAM-populations. Non-productive RSV-infections of isolated rAM have also been reported by others [20]. This was probably due to the deficient capacity of the experimental RSV-strain to infect rAM *in vitro* rather than to inhibition of viral replication performed by the rAM. However, compared to naïve rAM, post-asthma rAM showed markedly increased secretion of the pro-inflammatory cytokines IL-6 and TNF-α, the chemokine MCP-1 and, to a lesser extent of the anti-inflammatory cytokine IL-10 (figure 4).

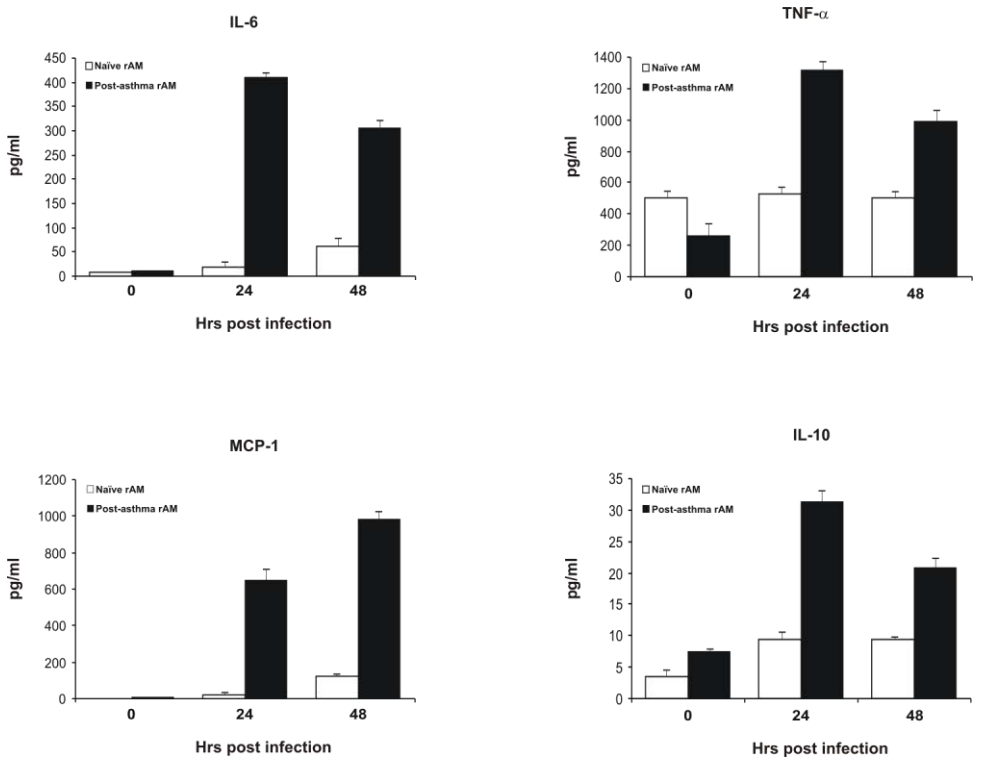

Figure 4. Inflammatory cytokine and chemokine response of naïve and post-asthma rAM to *in vitro* RSV-infection.
Naïve and post-asthma rAM were isolated from BALB/c mice (n=8) and infected *ex vivo* with RSV (MOI 25) for 24h or 48h or were mock-infected as a control. IL-6, TNF-α, MCP-1 and IL-10 protein levels in the culture supernatant were determined by the Bioplex suspension array system. Results represent the average cytokine levels ± SD of triplicate culture conditions.

Post-asthma rAM determine the late-phase pulmonary immune responses to RSV-infection

It is suggested that the initial encounter between pulmonary innate immune cells, like rAM, and respiratory viruses are critical for determining the pathophysiological outcome of the infectious disease at later stages [17]. Therefore, we analyzed the effect of the post-asthma rAM's initial antiviral immune response on the subsequent global late-phase antiviral immune responses to RSV-infection. Naïve and post-asthma lungs were i.n. infected with RSV, with or without prior CL-induced rAM-depletion. BAL samples were collected at d6 post-infection and alveolar inflammation was assessed via flow cytometry. Compared to d3, RSV-infection of naïve rAM^+ mice resulted in an augmented pulmonary inflammatory response at d6, which showed even further increases when naïve rAM were initially depleted (figure 5A). Compared to naïve rAM^+ lungs, post-asthma rAM^+ lungs showed a more severe inflammatory response against RSV-infection which again showed a significant decrease when the post-asthma rAM-population was first depleted (figure 5A). At this time point however, observed differences in total BAL cell numbers between the different mouse groups were mainly due to differences in

macrophage/monocyte and $CD8^+$ T-cell numbers (figure 5B), instead of neutrophil numbers as seen on d3 (figure 2B). Similar to d3, naïve and post-asthma rAM^+ lungs showed no difference in alveolar RSV-titers at d6 post-infection (figure 6). However, as opposed to d3, depletion of naïve rAM no longer affected the alveolar viral load (figure 6). In contrast, depletion of post-asthma rAM slightly accelerated viral clearance from the post-asthma lung as in the vast majority of this mouse group the presence of RSV-virus was no longer detectable (figure 6).

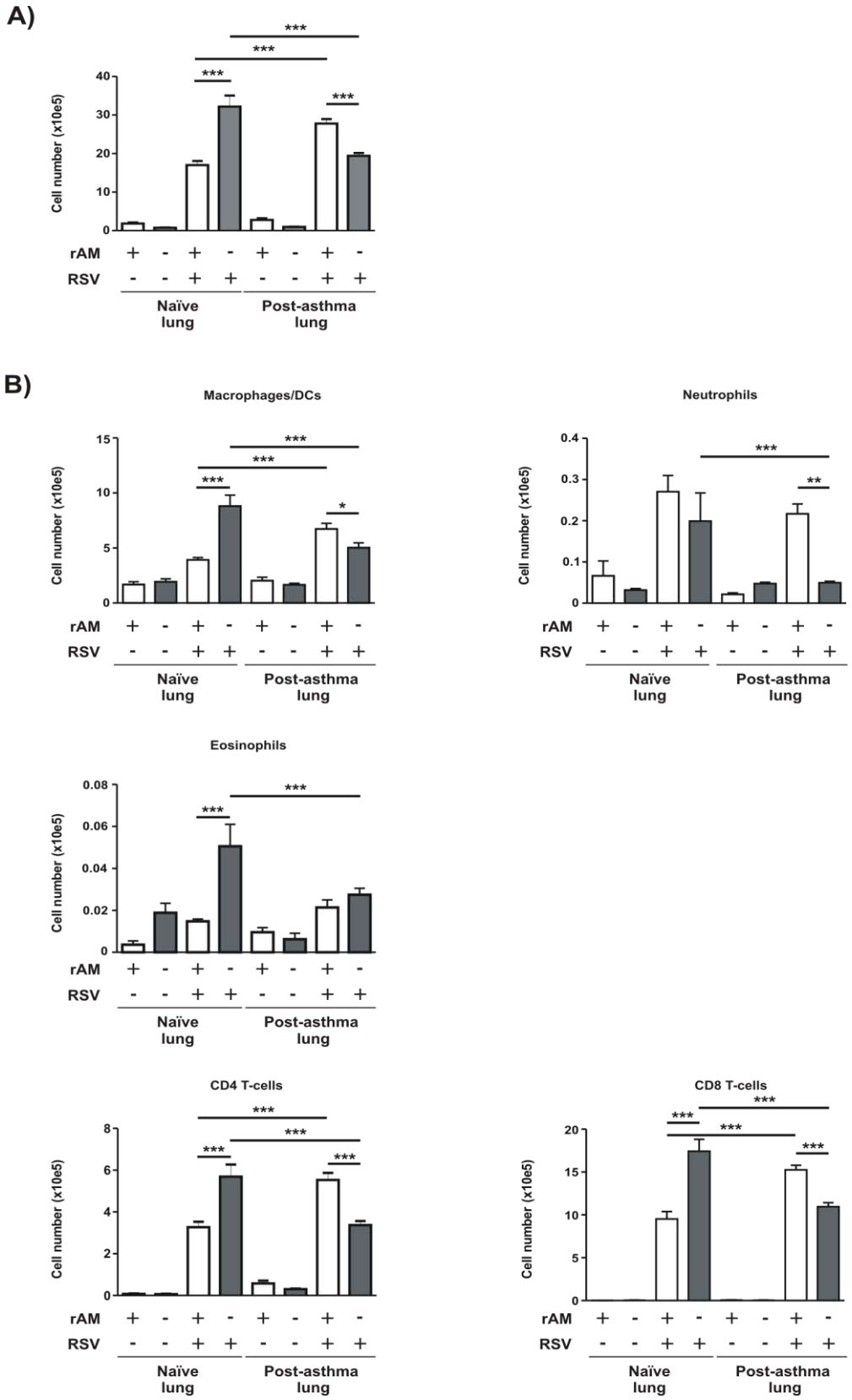

Figure 5. Alveolar inflammatory response to RSV-infection in naïve and post-asthma rAM$^+$ and rAM$^-$ mice at d6 post-infection.

Post-asthma (d15 after the last OVA-challenge) and naïve BALB/c mice (n=7) received CL i.t. three days before inoculation with 1.10^7 PFU RSV to deplete post-asthma and naïve rAM respectively or received PBS as a control. BAL was collected at d6 post-infection. (A) Average BAL cell counts ± SEM and (B) average BAL cellular composition ± SEM were determined by flow cytometry. * $p<0.05$; ** $p<0.01$ and *** $p<0.0001$.

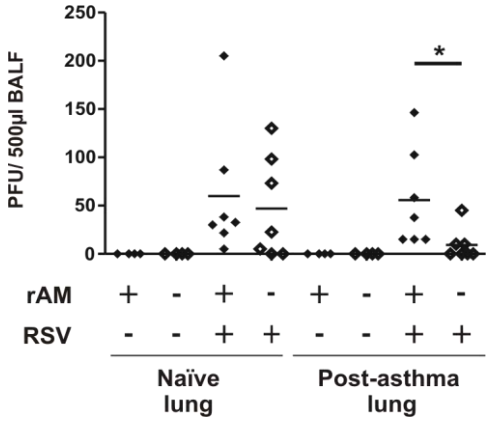

Figure 6. Alveolar RSV-titers in naïve and post-asthma rAM$^+$ and rAM$^-$ mice at d6 post-infection.
Post-asthma (d15 after the last OVA-challenge) and naïve BALB/c mice (n=7) received CL three days before inoculation with 1.10^7 PFU RSV to deplete post-asthma and naïve rAM respectively or PBS as a control. On d6 post-infection, BAL fluid was collected and virus recovery was assessed by plaque assay. * $p<0.05$

Effect of post-asthma rAM on systemic disease parameters during later stages of RSV-infection

Next to alveolar inflammation and viral titers, we also analyzed systemic infection parameters, including morbidity and CD8$^+$ T-cell responses in the lung draining lymph nodes (LDLN). Naïve and post-asthma lungs were i.n. infected with RSV, with or without prior CL-induced rAM-depletion and body weight loss was determined at d5 and d6 post-infection. Compared to mock-infected control groups, all RSV-infected groups exhibited significant weight loss at d6 post-infection but no differences were observed between RSV-infected 'naïve' and 'post-asthma' rAM$^+$ mice at this time point (figure 7A). Yet, naïve rAM-depletion prior to infection resulted in extra loss of body weight at d6 post-infection as compared to RSV-infected naïve rAM$^+$ lungs (figure 7A). On the other hand, depletion of post-asthma rAM prior to infection did not affect the body weight of the mice (figure 7A). Elevated levels of serum IL-6 are considered as an additional read-out parameter for morbidity. Serum concentrations of this pro-inflammatory cytokine behaved in accordance with the observed differences in body weight loss between the different RSV-infected mice groups at d6 post-infection (figure 7B).

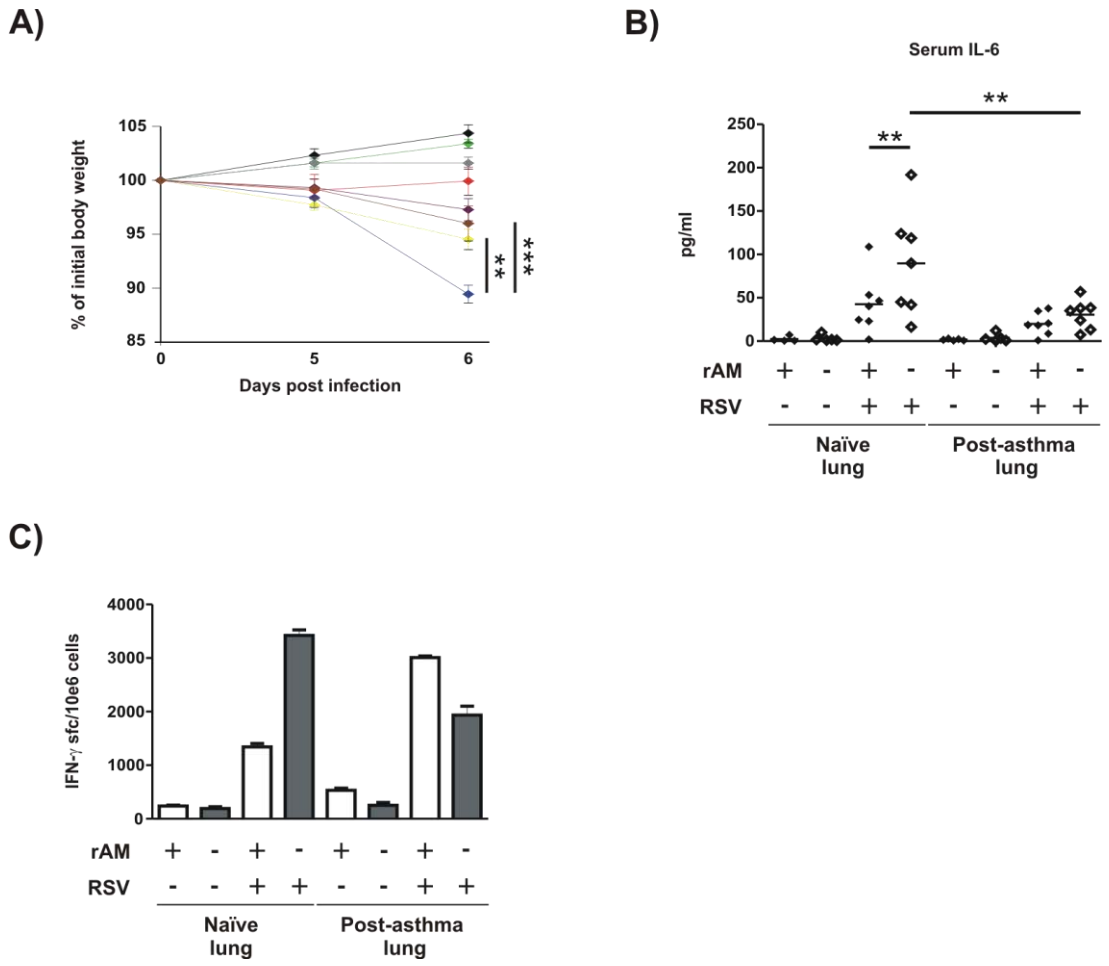

Figure 7. Development of systemic parameters of infection in naïve and post-asthma rAM$^+$ and rAM$^-$ mice at d6 post-infection.
Post-asthma (d15 after the last OVA-challenge) and naïve BALB/c mice (n=7) received CL three days before inoculation with 1.10^7 PFU RSV to deplete post-asthma and naïve rAM respectively or received PBS as a control. (A) Weight loss ± SEM was registered at d5 and d6 post-infection. Green: mock-infected naïve rAM$^+$ mice; red: mock-infected naïve rAM$^-$ mice; yellow: RSV-infected naïve rAM$^+$ mice; blue: RSV-infected naïve rAM$^-$ mice; black: mock-infected post-asthma rAM$^+$ mice; grey: mock-infected post-asthma rAM$^-$ mice; purple: RSV-infected post-asthma rAM$^+$ mice and brown: RSV-infected post-asthma rAM$^-$ mice. (B) Blood serum was collected at d6 post-infection and serum IL-6 protein levels ± SEM were determined via an in-house developed bio-assay [19]. (C) LDLN were isolated at d6 post-infection and 1.10^5 LDLN-cells were restimulated for 24h with 5µg/ml of a MHC-I epitope of the RSV F-protein. The number of IFN-γ secreting CD8$^+$ T-cells was subsequently quantified by ELISPOT. Data in (C) represent averages of triplicate reactions originated from pooled samples.

Finally, CD8$^+$ T-cell responses in LDLN were quantified at d6 post-infection by ELISPOT assay upon restimulation with a MHC I restricted peptide epitope derived from the RSV F-protein. The ELISPOT results shown in figure 7C demonstrate that RSV-infection of post-asthma rAM$^+$ mice resulted in higher F-specific CD8$^+$ T-cell responses compared to naïve rAM$^+$ mice which were infected with RSV. Depletion of naïve rAM prior to RSV-infection increased LDLN CD8$^+$ T-cell immunity (figure 7C). In contrast, depletion of post-asthma rAM decreased the number of IFN-γ secreting LDLN CD8$^+$ T-cells (figure 7C).

Influence on immune memory responses

We next analyzed RSV-specific cellular and humoral immune memory responses in the different RSV-infected mouse groups. Naïve rAM^+ and rAM^- mice and post-asthma rAM^+ and rAM^- mice were i.n. infected with RSV. LDLN were sampled three weeks post-infection and $CD8^+$ T-cell responses were quantified by ELISPOT assay upon restimulation with a MHC I restricted peptide epitope from the RSV F-protein. LDLN $CD4^+$ T-cell responses were determined via intracellular cytokine staining (ICS) and flow cytometry upon restimulation of LDLN-cells with heat-inactivated RSV-virus. LDLN RSV-specific $CD8^+$ T-cell responses were similar in both naïve rAM^+ and post-asthma rAM^+ mice (figure 8A). Depletion of naïve rAM prior to RSV-infection did not alter the subsequent $CD8^+$ T-cell memory response as compared to naïve rAM^+ RSV-infected mice (figure 8A). However, compared to RSV-infected post-asthma rAM^+ mice, depletion of post-asthma rAM prior to RSV-infection augmented the number of IFN-γ secreting $CD8^+$ T-cells as shown by the ELISPOT results in figure 8A. Furthermore, although ICS and flow cytometry analysis revealed that a RSV-specific memory Th1-response was significantly elicited in all RSV-infected mouse groups, no actual differences were observed in the percentage of IFN-γ^+ $CD4^+$ T-cells between all four groups (figure 8B). In addition, no memory Th2-responses (figure 8C) and Th17-responses (figure 8D) were detected in the LDLN of all RSV-infected mouse groups.

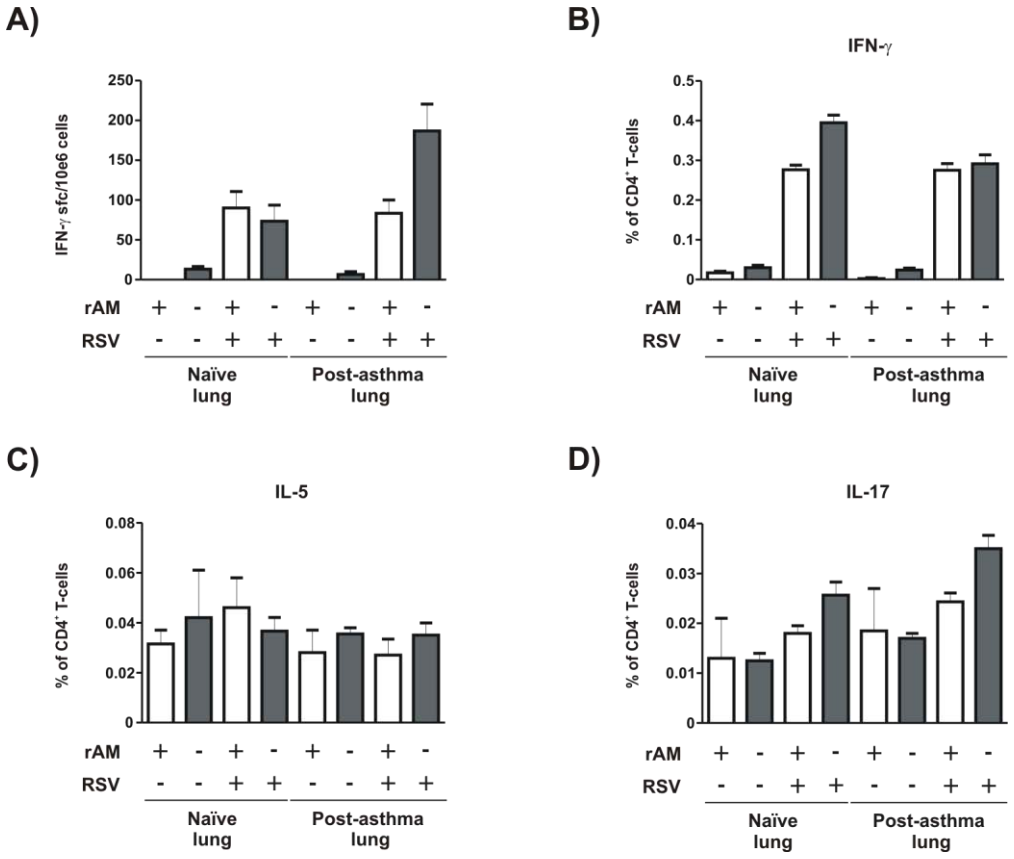

Figure 8. Development of RSV-specific LDLN memory responses in naïve and post-asthma rAM$^+$ and rAM$^-$ mice at 3wks post-infection.
Post-asthma (d15 after the last OVA-challenge) and naïve BALB/c mice (n=7) received CL three days before inoculation with 1.10^7 PFU RSV to deplete post-asthma and naïve rAM respectively or received PBS as a control. (A) LDLN were isolated 3wks post-infection and 1.10^5 LDLN-cells were restimulated for 24h with an MHC-I epitope of the RSV F-protein. The number of IFN-γ secreting CD8$^+$ T-cells was subsequently quantified by ELISPOT. In addition, 1.10^6 LDLN-cells were restimulated for 24h with heat-inactivated RSV (MOI 0.5). The percentage of IFN-γ$^+$ CD4$^+$ T-cells (B), IL-5$^+$ CD4$^+$ T-cells (C) and IL-17$^+$ CD4$^+$ T-cells (D) were quantified within the population of the living CD3ε$^+$ lymphocytic cells by ICS and flow cytometry. All data represent averages of triplicate reactions originated from pooled samples.

The production of virus-neutralizing antibodies has been found to be crucial in the host's protective immunity against RSV-reinfection [21]. Therefore, we characterized the B-cell memory response by measuring serum levels of RSV-specific IgG1- and IgG2a-molecules and their capacity to neutralize the RSV-virus. Naïve rAM$^+$ and rAM$^-$ mice and post-asthma rAM$^+$ and rAM$^-$ mice were i.n. infected with RSV and blood serum samples were collected 3 weeks post-infection. As determined by RSV-based ELISA, infection of naïve rAM$^+$ mice resulted in higher serum levels of both IgG1 (figure 9A; left panel) and IgG2a (figure 9A; right panel) compared to infection of post-asthma rAM$^+$ mice. Furthermore, a reduction in the systemic levels of RSV-specific IgG1 was observed only after depletion of naïve rAM, and not after depletion of post-asthma rAM (figure 9A, left panel). IgG2a serum levels were not

affected by naïve or post-asthma rAM-depletion (figure 9A, right panel). The IgG1/IgG2a ratio, indicative for the Th2/Th1 balance, was practically equal in all four infected mouse groups (figure 9B). Finally, the RSV-neutralization assay revealed that the actual levels of RSV-neutralizing serum antibodies did not differ between all four RSV-infected mouse groups (figure 10).

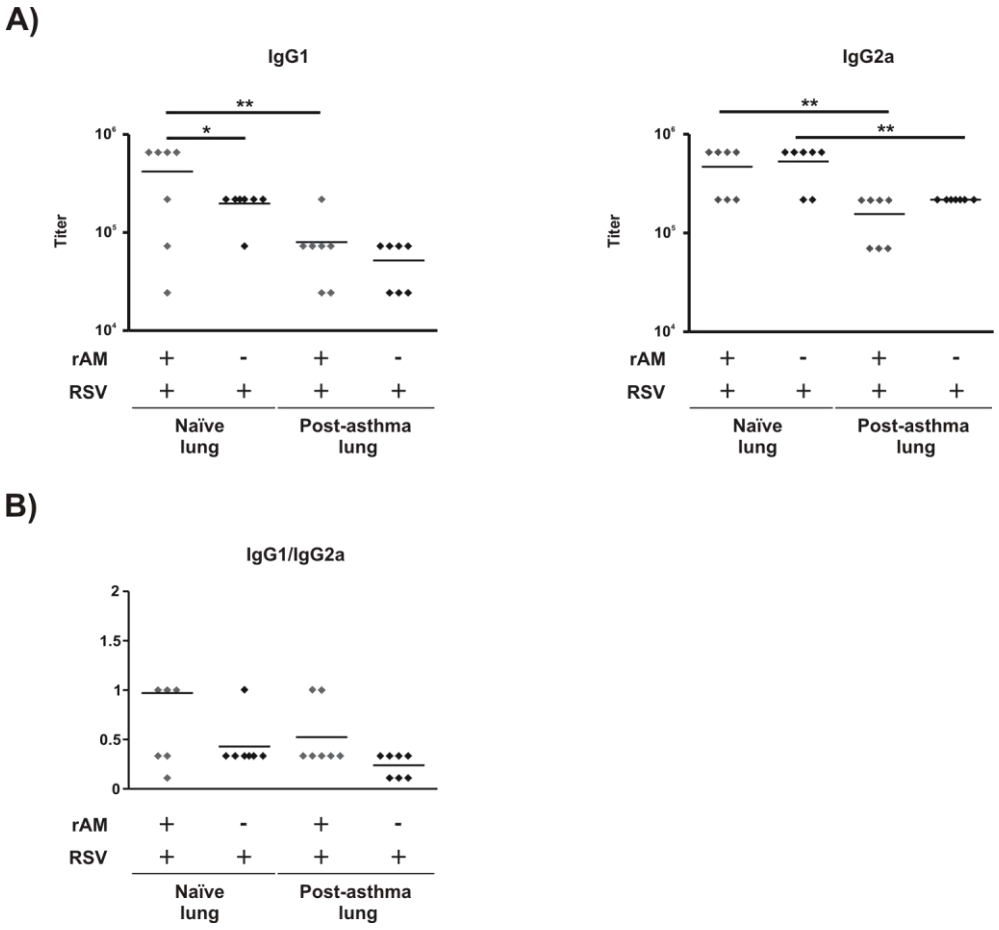

Figure 9. Development of RSV-specific humoral memory responses in naïve and post-asthma rAM$^+$ and rAM$^-$ mice at 3wks post-infection.
Post-asthma (d15 after the last OVA-challenge) and naïve BALB/c mice (n=7) received CL three days before inoculation with 1.10^7 PFU RSV to deplete post-asthma and naïve rAM respectively or received PBS as a control. Blood serum was collected 3 wks post-infection. (A) Serum levels of RSV-specific IgG1 (left) and IgG2a (right) were determined via RSV-based ELISA. (B) Ratio between serum levels of RSV-specific IgG1 and IgG2a. * $p<0.05$; ** $p<0.01$

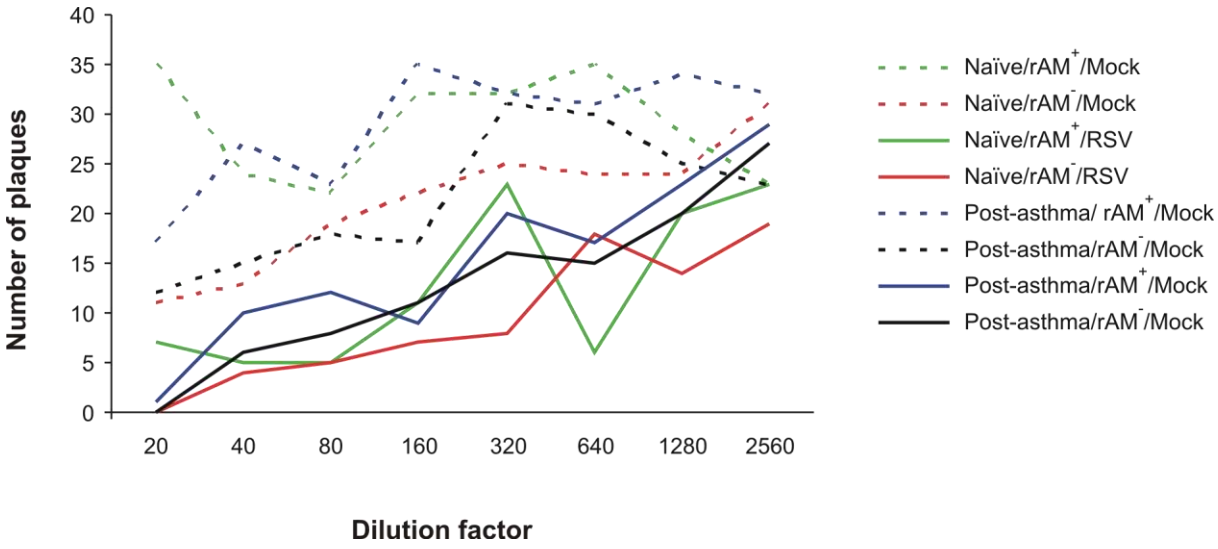

Figure 10. Determination of total RSV-neutralizing serum antibodies in naïve and post-asthma rAM$^+$ and rAM$^-$ mice at 3wks post-infection.
Post-asthma (d15 after the last OVA-challenge) and naïve BALB/c mice (n=7) received CL three days before inoculation with 1.10^7 PFU RSV to deplete post-asthma and naïve rAM respectively or received PBS as a control. Blood serum was collected 3 wks post-infection. Serum levels of RSV-specific neutralizing antibodies were determined via an *in vitro* neutralization assay on pooled sera.

Influence on immune memory responses during RSV-reinfection

We finally investigated the physiological relevance of the observed differences in RSV-specific immunological memory by exposing the different RSV-infected mouse groups to a secondary RSV-infection 3 weeks after the primary viral challenge. As shown in figure 11A, i.n. reinfection with RSV elicited a mild inflammatory response in the lungs of all mouse groups at d3 post-reinfection. However, no to minor (as in the case of RSV-reinfected naïve rAM$^-$ mice compared to RSV-reinfected naïve rAM$^+$ mice) differences in total BAL cell counts were detected between the different RSV-reinfected mouse groups (figure 11A). The minor increase in total BAL cell counts observed in RSV-reinfected naïve rAM$^-$ mice was mainly due to slight changes in the recruitment of CD4$^+$ and CD8$^+$ T-cells to the alveoli (figure 11B).

LDLN CD8$^+$ and CD4$^+$ T-cell responses were also assessed at d3 post-reinfection via ELISPOT assay and ICS respectively. The ELISPOT data show that restimulation with F-peptide of LDLN-cells from RSV-reinfected post-asthma rAM$^+$ mice resulted in higher numbers of IFN-γ secreting CD8$^+$ T-cells compared to RSV-reinfected naïve rAM$^+$ mice (figure 12A). Furthermore, CD8$^+$ T-cell responses were increased in RSV-reinfected naïve rAM$^-$ mice while CD8$^+$ T-cell responses of RSV-reinfected post-asthma rAM$^-$ decreased

(figure 12A). In line with these findings, Th1-responses were found to behave similar to $CD8^+$ T-cell responses (figure 12B). In contrast to the strongly pronounced $CD8^+$ and Th1-responses during RSV-reinfection, only mild Th2-responses (figure 12C) and Th17-responses (figure 12D) were noticed, which did not differ between all RSV-reinfected mouse groups.

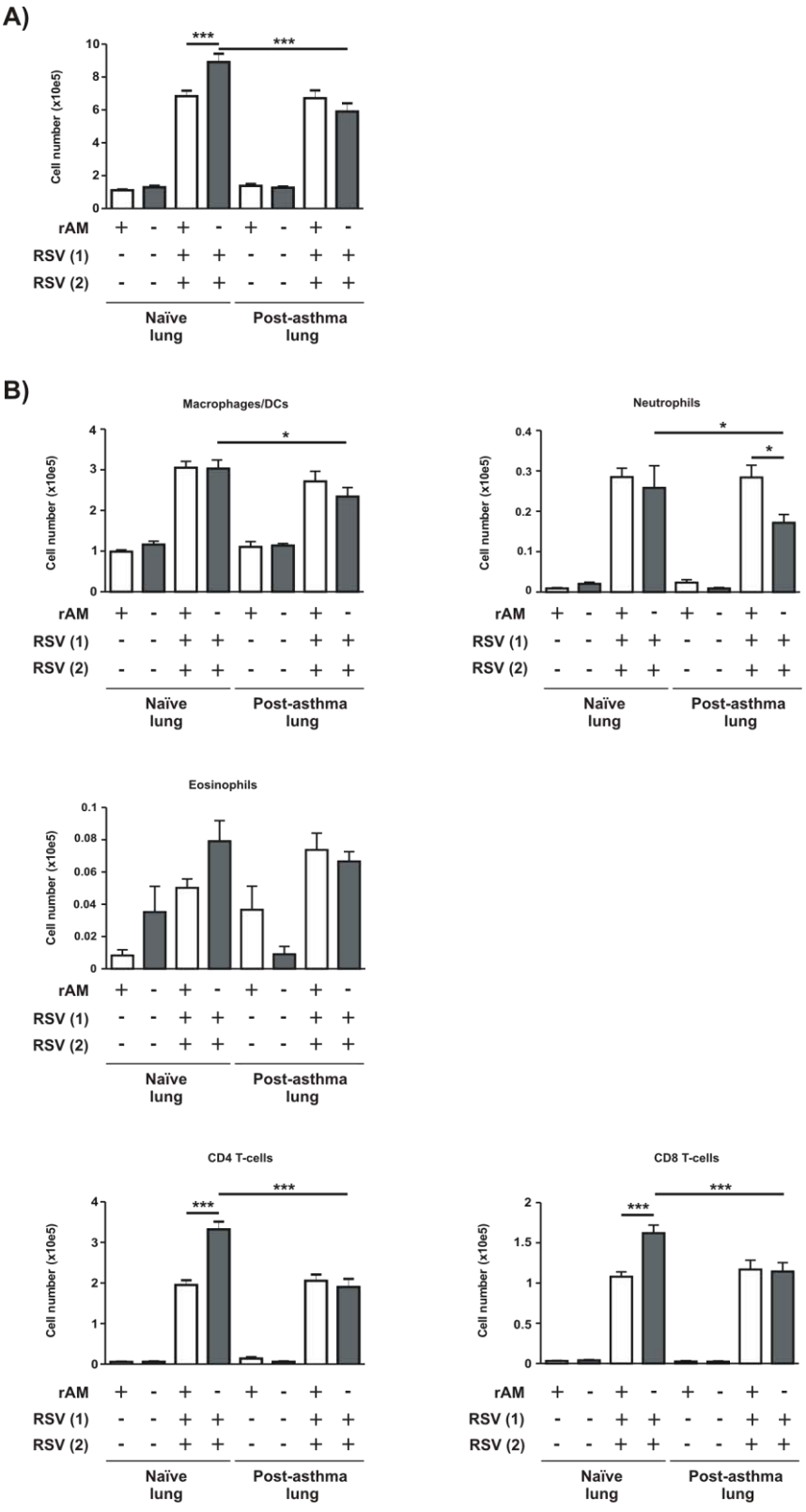

Figure 11. Alveolar inflammation caused by RSV-reinfection of naïve and post-asthma rAM$^+$ and rAM$^-$ mice.

Post-asthma (d15 after the last OVA-challenge) and naïve BALB/c mice (n=7) received CL i.t. three days before inoculation with 1.10^7 PFU RSV to deplete post-asthma and naïve rAM respectively or received PBS as a control. Subsequently, mice exposed to primary infection, referred to as RSV(1), were reinfected with 1.10^7 PFU RSV, referred to as RSV(2), 3 weeks after the primary RSV-challenge. BAL was collected at d3 post-reinfection. (A) Average BAL cell counts ± SEM (B) and average BAL cellular composition ± SEM were determined by flow cytometry. * $p<0.05$ and *** $p<0.0001$.

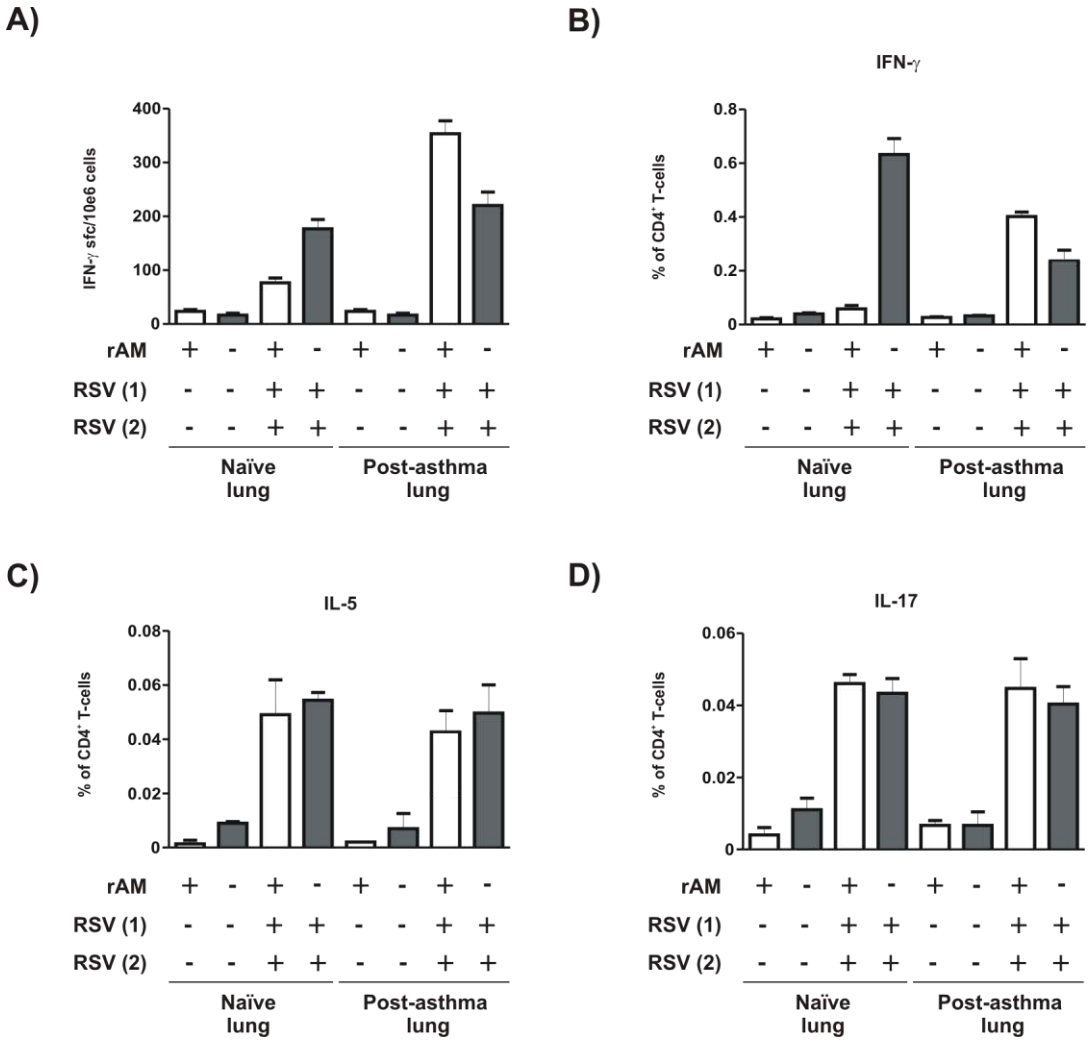

Figure 12. Development of RSV-specific LDLN responses during RSV-reinfection of naïve and post-asthma rAM$^+$ and rAM$^-$ mice.

Post-asthma (d15 after the last OVA-challenge) and naïve BALB/c mice (n=7) received CL three days before inoculation with 1.10^7 PFU RSV to deplete post-asthma and naïve rAM respectively or received PBS as a control. Subsequently, mice exposed to primary infection, referred to as RSV(1), were reinfected with 1.10^7 PFU RSV, referred to as RSV(2), 3 weeks after the primary RSV-challenge. (A) LDLN were isolated at d3 post-reinfection and 1.10^5 LDLN-cells were restimulated for 24h with 5 µg/ml of a MHC-I epitope peptide of the RSV F-protein. The number of IFN-γ secreting CD8$^+$ T-cells was subsequently quantified by ELISPOT. In addition, 1.10^6 LDLN-cells were restimulated for 24h with heat-inactivated RSV (MOI 0.5). The percentage of IFN-γ$^+$ CD4$^+$ T-cells (B), IL-5$^+$ CD4$^+$ T-cells (C) and IL-17$^+$ CD4$^+$ T-cells (D) were quantified within the population of the living CD3ε$^+$ lymphocytic cells by ICS and flow cytometry. All data represent averages of triplicate reactions originated from pooled samples.

DISCUSSION

The pulmonary response to respiratory infections must proceed in a programmed manner in order to clear the pathogen without compromising pulmonary function. This is particularly important in individuals with underlying disease, such as asthma. Several types of viruses, such as rhinoviruses, influenza and RSV, have been detected in respiratory tract secretions from patients with asthma exacerbations. While RSV-infections do not result in dramatic clinical outcomes in a normal adult, it is the instigator of the most severe exacerbation reactions in patients suffering from asthma [10].

In a mouse model of allergic bronchial inflammation, we recently demonstrated that after the clearance of the eosinophilic inflammation, a new secondary rAM population resides in the airways. In our present study we analyzed in which way the presence of this post-asthma rAM-population affects the pathophysiological outcome of a subsequent RSV-infection. Mice that suffered from a prior allergic inflammation developed higher pulmonary innate and adaptive inflammatory responses to subsequent RSV-infection compared to mice in which no prior allergic bronchial inflammation occurred. This increase was largely due to the pro-inflammatory character of post-asthma rAM. Depletion of post-asthma rAM reduced pulmonary inflammation to a level comparable to naïve mice. The pro-inflammatory nature of post-asthma rAM stands in strong contrast with the anti-inflammatory capacities of naïve rAM. Thus, depletion of naïve rAM prior to RSV-infection resulted in an increased pulmonary inflammation. Differences in pulmonary inflammatory responses were possibly due to differences in the recruitment of inflammatory leukocytes to the lungs. This implicates that resident leukocytes, like rAM, and tissue cells, like AECs, would secrete higher levels of pro-inflammatory cytokines and chemokines upon the initial encounter with RSV. In agreement herewith, we now showed that, compared to naïve rAM, post-asthma rAM secreted largely increased levels of NF-κB-inducible inflammatory cytokines in response to *in vitro* RSV-infection. In addition, we previously demonstrated that post-asthma rAM exhibited hyperinflammatory TLR-3, TLR-4 and TLR-7 reactivity *in vitro*. RSV contains several ligands for a variety of PRRs on innate immune cells. TLR-3, expressed by AECs and rAM, contributes to the recognition of RSV by binding to viral RNA [22] while the RSV F-protein has been identified as a TLR-4 and CD14 ligand on human monocytes [23]. Furthermore, TLR-7 was recently acknowledged as another member of the TLR-family involved in the recognition of RSV [24, 25]. In accordance with these data, increased inflammatory TLR-reactivity exhibited

by post-asthma rAM is a reasonable explanation for the way in which post-asthma rAM contributed to the generally increased RSV-induced pulmonary inflammation in post-asthma mice.

Inflammation is considered as a powerful tool of the host to clear pathogens. One would therefore expect diminished alveolar RSV-titers in post-asthma lungs exhibiting an increased inflammatory response. However, RSV-infection of naïve and post-asthma lungs resulted in equal alveolar RSV-titers. Strikingly, depletion of post-asthma rAM prior to RSV-infection had no effect on alveolar viral titers whereas depletion of naïve rAM resulted in a strong increase. In contrast to naïve rAM, which are known for their distinguished capacity to clear respiratory viruses rapidly [17, 18, 26], post-asthma rAM clearly lost their capacity to fulfil early clearance of the virus. In line with these findings, we previously documented a decrease in basal phagocytic activity of post-asthma rAM compared to naïve rAM. The comparable RSV titers observed in post-asthma and naïve lungs at the early stage of infection stresses the fact that the increased pulmonary inflammation observed in post-asthma mice is not simply due to differences in viral titers. Higher levels of secreted TNF-α, MCP-1 and IL-6 detected after *in vitro* RSV-infection of post-asthma rAM confirms an increased inflammatory reactivity of post-asthma rAM. These differences in anti- versus proinflammatory reactivity between naïve and post-asthma rAM were also apparent at later stages of infection through the increase in body weight loss and serum levels of the pro-inflammatory IL-6. Strikingly, post-asthma rAM did not seem to influence these morbidity parameters. Taken together, these results indicate a shift from a naïve rAM-phenotype exerting phagocytic clearance of invading respiratory viruses, such as RSV, with a minimal inflammatory reactivity to a post-asthma rAM-phenotype which responds to a viral insult by secreting a full range of inflammatory mediators without displaying any direct viral clearance. These findings can explain the presence of the severe pulmonary inflammation in RSV-infected post-asthma mice as a necessary evil to take over the post-asthma rAM's job and eventually clear the virus. This hypothesis is in line with the study of Reed and colleagues in which the inherent functional impairment of rAM in New Zealand black mice resulted in greatly enhanced RSV disease compared to BALB/c mice which exhibit normal rAM-function [17]. In addition, the perception of rAM as the silent assassins of the lungs was also confirmed in a study performed by Dockrell and colleagues. In their research they demonstrated that depletion of rAM in a low-dose murine pneumococcal infection model shifted the outcome from complete bacterial

clearance without neutrophil recruitment, to one in which neutrophil recruitment is required for bacterial clearance [27].

However, it is important to consider that the outcome of the interplay between an allergic bronchial inflammation and RSV-infection is largely affected by the timing of RSV-exposure. For instance, Graham and co-workers found that RSV-infection before the allergic inflammation decreased subsequent allergen-induced AHR. In contrast, AHR was increased when the allergic inflammation occurred before the RSV-infection [28]. The importance of the timing of RSV-infection in relation to the allergic inflammation was also illustrated by our additional data showing that RSV-infection during an ongoing allergic bronchial inflammation lead to increased alveolar virus titers. This was in strong contrast with the observation that alveolar RSV-titers were not altered when mice were infected after the clearance of the allergic bronchial inflammation. So it is clear that the actual presence or absence of an ongoing Th2-response, probably through the secretion of Th2-cytokines like IL-13 [29, 30], is a major determinant for virus survival and/or replication.

Post-asthma mice also exhibited altered RSV-specific immune memory responses. At the level of humoral memory responses, lower levels of RSV-specific IgG1- and IgG2a-antibodies were observed in post-asthma mice. IgG-molecules are known to be important for the neutralization of RSV in order to inhibit viral attachment to target cells [21]. However, sera from post-asthma mice did not show impaired *in vitro* neutralization of RSV. In addition, RSV-rechallenge of post-asthma mice did not yield any productive infection which is possibly due to the immediate antibody-mediated neutralization of incoming RSV-particles. In this context however, the presence of lung mucosal IgA is also an important parameter to take into account. Thus, the actual physiological relevance of the observed differences in RSV-specific IgG1- and IgG2a-levels is not defined yet. One possibility is that lower IgG1- and IgG2a-levels in RSV-infected post-asthma mice is reflected in lower amounts of opsonizing IgG1- and IgG2a-molecules in these mice. Impaired opsonization of incoming RSV-particles would eventually result in decreased rAM-mediated uptake and killing of the virus. Reinfection of post-asthma mice with RSV resulted in increased LDLN CTL and Th1-cell responses as well. Both CTL and Th1-responses decreased when post-asthma rAM were initially depleted. In contrast, initial depletion naïve rAM slightly increased RSV-specific CTL-responses and largely increased Th1-memory responses during RSV-reinfection. So it is clear that the initial encounter between both rAM-populations and RSV had a differential influence on the

subsequent development of LDLN cellular memory responses during RSV-reinfection. These data can reflect alterations in the interaction between post-asthma rAM and airway DCs. It is known that naïve rAM suppress airway DC maturation, function and trafficking to mediastinal LNs [7, 8]. Thus, depletion of naïve rAM prior to RSV-infection could increase the presentation of RSV-derived antigens in the LDLN by airway DCs, explaining the increased CTL- and Th1-responses during RSV-reinfection. In contrast, by exhibiting a more pro-inflammatory character, post-asthma rAM probably exerted immune stimulatory rather than immunosuppressive effects on airway DC maturation, function and trafficking during the initial encounter with RSV. This in turn could explain the increased CTL- and Th1-responses in post-asthma rAM^+ mice during RSV-reinfection which were decreased when post-asthma rAM were initially depleted. However, one should take into account that these data may be influenced by alterations in composition of the resident airway DC-population due to the preceding allergic bronchial inflammation. For instance, a change in the ratio between immunosuppressive pDCs and immunogenic mDCs in the airways can significantly affect the outcome of subsequent LDLN T-cell responses. Despite the differences in LDLN memory responses, no differences were found at the level of local alveolar inflammation during RSV-reinfection between all RSV-reinfected mouse groups. Additionally, no virus was detected in the alveoli of these mice as well. Thus, it remains unclear what the actual (patho)physiological significance is of these observed differences in memory T-cell responses. One possibility is that increased memory CTL- and Th1-responses lead to increased levels of pulmonary IFN-γ which in turn can augment general lung tissue pathology and AHR and can induce acute exacerbation reactions [31].

Overall, it is clear that a preceding allergic bronchial inflammation alters different aspects of the immune response to a subsequent acute RSV lung infection. Our observations especially point out a pronounced proinflammatory role for post-asthma rAM during a subsequent acute RSV-infection. In combination with the absence of any direct antiviral activity, this rAM-population can deliver an important contribution to RSV-induced asthma exacerbations, the most severe type of asthmatic exacerbation observed in children [9] and adults [10]. Therefore, post-asthma rAM constitute an interesting target for preventing or treating RSV or, more general, respiratory viral induced asthma exacerbations.

AUTHOR CONTRIBUTIONS

T. Naessens wrote the manuscript and performed the major part of the experimental work.

B. Schepens designed research and performed experimental work (RSV cultures, plaque assays, RSV-based ELISA and *in vitro* neutralization assay).

C. Pollard, P. Bogaert (flow cytometry) and S. De Koker performed technical assistance.

N. van Rooijen delivered clodronate liposomes.

X. Saelens and J. Grooten designed research

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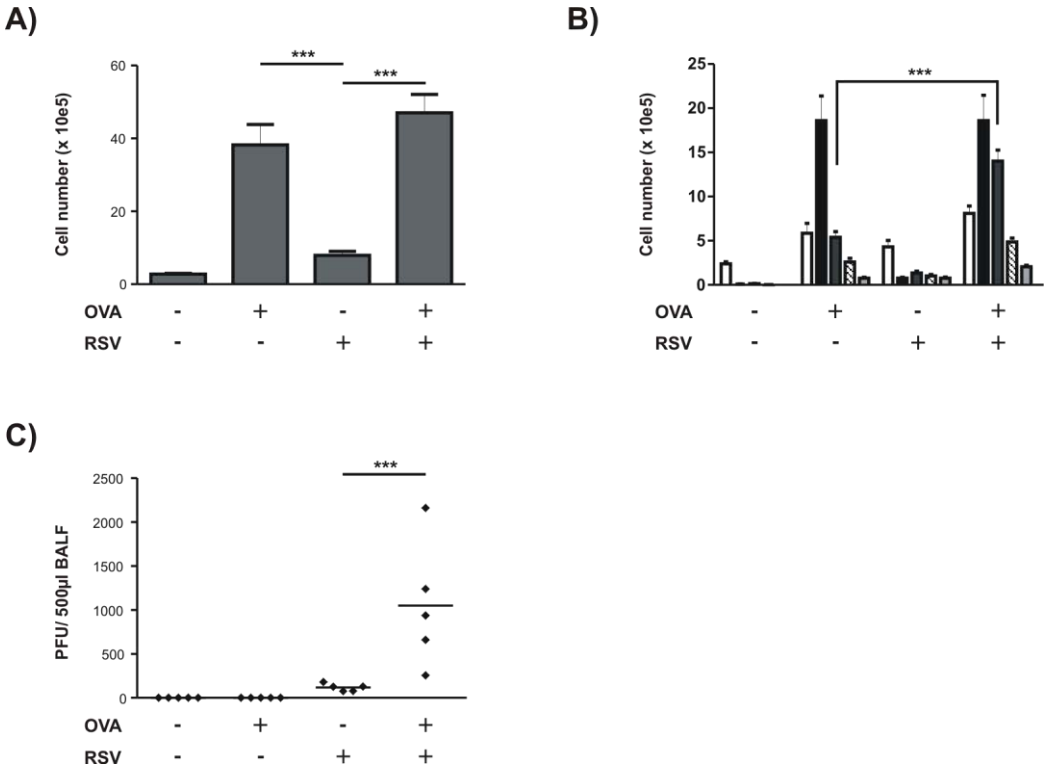
ADDITIONAL DATA

Co-delivery of OVA and RSV in sensitized mice increases alveolar neutrophil numbers and viral titers

Several studies highlighted the fact that the outcome of the interplay between an allergic inflammation and RSV-infection is largely influenced by the timing of infection [32]. To illustrate the importance of the timing of RSV-infection in relation to the allergic inflammation, we now analyzed the outcome of an RSV-infection during an ongoing allergic bronchial inflammation. OVA/alum sensitized mice were i.n. infected with RSV alone or in the presence of OVA. BAL was sampled at d3 post-infection. As shown in additional figure A1A, RSV co-infection did not significantly increase alveolar inflammation when compared to mice that were only challenged with OVA. However, analysis of the cellular composition of the alveolar infiltrate of RSV/OVA co-challenged mice revealed a substantial establishment of a neutrophilic component caused by the virus (additional figure A1B). The most striking observation was however that RSV/OVA co-challenge of mice resulted in increased alveolar RSV-titers compared to mice infected with RSV in the absence of OVA (additional figure A1C). This was in strong contrast with the observation that alveolar RSV-titers were not altered when mice were infected after the clearance of the allergic bronchial inflammation (figure 3 and figure 6 of the manuscript body text).

These data verify previous reports showing that an ongoing pulmonary Th2-response favours RSV viral replication in the lungs. In addition, these results confirm that the timing of RSV-infection in relation to the allergic bronchial inflammation is crucial in determining the antiviral pulmonary immune responses.



Additional figure A1. Effect of the co-delivery of RSV together with OVA on the pulmonary inflammatory response and the alveolar RSV-viral loads.
OVA/alum sensitized BALB/c mice (n=5) were co-challenged with RSV and OVA, challenged with either RSV or OVA alone or left untreated as a control. BAL was performed at d3 post-challenge. (A) Average BAL cell counts ± SEM and (B) average BAL cellular composition ± SEM were determined by flow cytometry. White bars: macrophages; black bars: eosinophils; dark grey bars: neutrophils; striped bars: $CD4^+$ T-cells and light grey bars: $CD8^+$ T-cells. (C) In addition, BAL fluid was collected and virus recovery was assessed by plaque assay. *** $p<0.0001$.

Chapter 3

Innate Imprinting of Resident Alveolar Macrophages by an Allergic Bronchial Inflammation Affects the Outcome of a Subsequent *Chlamydia muridarum* Lung Infection

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INTRODUCTION

Acute exacerbations, defined as episodes of rapidly progressive increases in shortness of breath, cough, wheezing, or chest tightness, in asthmatic individuals represent an important healthcare problem and accounts for a high rate of morbidity and mortality. Many studies have reported a link between *Chlamydophila* (*C.*) *pneumoniae* infection and acute asthma exacerbations (reviewed in [1]). Therefore, it has been suggested that this pathogen may play a significant role in such exacerbations.

C. pneumoniae are Gram-negative obligate intracellular bacteria which infect and survive in resting resident alveolar macrophages (rAM) [2]. By being the bacteria's main target of infection and the host's first line of defence against airborne pathogens, rAM fulfil a pivotal role in the host's protective immunity against *C. pneumoniae*. Upon recognition of the bacterium by PRRs, rAM initiate a gene program that involves the expression and secretion of pro-inflammatory cytokines and chemokines [3]. The propagation of these antibacterial pulmonary inflammatory responses and the possible subsequent lung tissue damage may eventually result in acute exacerbation reactions when infection occurs in asthmatic individuals [2].

In a mouse model of allergic bronchial inflammation, we recently demonstrated that, after the clearance of the allergic inflammation, a new secondary rAM population resides in the airways. Strikingly, these post-asthma rAM displayed several important functional alterations, including increased TLR-reactivity and a decreased basal phagocytic capacity. Therefore, alteration of the rAM-functional phenotype due to a preceding allergic bronchial inflammation can affect the pathophysiological outcome of a subsequent *C. pneumoniae* infection.

We now provide evidence that acute *C. muridarum* (the mouse biovar of *C. trachomatis*) infection of mice that suffered from a preceding allergic bronchial inflammation, exhibited increased pulmonary inflammatory responses along with an increase in alveolar bacterial burden. Furthermore, we show that pronounced pro-inflammatory features of post-asthma rAM, largely contributed to the increased inflammatory reaction in the infected lungs. These observations demonstrate that post-asthma rAM may contribute to the increased susceptibility to secondary *C. pneumoniae* infections and/or the onset of exacerbation reactions associated with asthmatic individuals.

MATERIALS AND METHODS

Mouse model

6- to 8-week old female C57BL/6 mice, purchased from Janvier (Le Genest St.Isle, France) and kept under specified pathogen free conditions were immunized intraperitoneally with 20µg of grade V chicken egg OVA (Sigma-Aldrich, St.Louis, MO, USA), adsorbed on 1mg $AlOH_3$ (alum; Sigma-Aldrich) in endotoxin-free PBS (Lonza, Walkersville, MD, USA). OVA-sensitized mice were then exposed to OVA-aerosols, consisting of 1% of grade III OVA (Sigma-Aldrich) in PBS. To establish a subsequent *C. muridarum* lung infection, 1.10^3 IFU of *C. muridarum* EB's (propagated on HeLa-cells) was administered intratracheally to mice that were anesthetized by a ketamine/xylazine mixture. PBS was instilled as a control. For the depletion of rAM, 100 µl of a 30% clodronate (dichloromethylene-diphosphonate) liposome solution (in PBS) was administered intratracheally 3 days prior mock- or RSV-infection to mice that were fully anesthetized by a ketamine/xylazine mixture. All experiments performed in this study were approved by the local ethical committee.

Bacteria and determination of bacterial titers

C. muridarum (Nigg strain) was propagated on HeLa-cells in MEM-medium (Invitrogen, Ghent, Belgium) supplemented with 10% heat-inactivated FCS (Sigma-Aldrich), 1% L-Glutamine (Invitrogen), 1% vitamin-solution (Invitrogen), 1% streptomycine-sulphate (Invitrogen), 2% vancomycin (Nerum NV, Heusden-Zolder, Belgium), 0,22% cyclohexamide (Sigma-Aldrich) and 5,5 mg/ml D-glucose (Invitrogen) and incubated at 37°C and in 5% CO_2.

For the determination of *C. muridarum* EBs in the BALF, samples were first ultracentrifugated (50.000 g for 60 min). The pellet of bacteria was subsequently resuspended in a sucrose-phosphate-glutamine solution (Sigma-Aldrich), supplemented with 1% streptomycin-sulphate and 2% vancomycin, and inoculated on HeLa-cells. After six days of incubation at 37°C and in 5% CO_2, EB-titers were determined via direct immunofluorescence staining of the bacteria using the IMAGEN Chlamydia Kit (Oxoid Ltd., Hampshire, UK) according to manufacturer's protocol.

Alveolar cell isolation

Mice were anesthetized with avertin (2,2,2-tribromethanol; 2,5% in PBS; Sigma-Aldrich). BAL was performed by making a small incision in the trachea, to allow passage of a lavage canulae. Lungs are flushed 4 times with 1ml Ca^{2+}- and Mg^{2+}-free HBSS, supplemented with 0.05mM EDTA (ethylenediaminetetraacetic acid). Optionally, a prior lavage with 0,5ml HBSS-EDTA was performed and BAL fluid was isolated by centrifugation and collection of the supernatant. BAL cells were washed and resuspended in PBS for further use.

Total and differential cell counts

Total numbers of BAL and lungs cells were counted by use of a Bürker-chamber (Marienfeld, Lauda-Königshofen, Germany). Trypane blue was added to exclude dead cells. BAL cell type composition and pulmonary NK-cell levels were analyzed by flow cytometry. Briefly, BAL cells, pre-incubated with Fc-Block were classified as monocytes (alveolar macrophages, elicited monocytes and DCs), neutrophils, eosinophils or T-lymphocytes based on forward and side scatter gating and fluorescence intensities for anti-mouse MHC II-eFluor450, CD3ε-Alexa488, CCR3-PE, CD4-PerCP, CD8-PE-Cy7, CD11c-APC and CD11b-APC-Cy7. All antibodies were purchased from BD Biosciences, except CCR3-PE (R&D Systems, Abingdon, UK). All samples were measured on a FACS LSRII flow cytometer and analyzed using FACS Diva software (both from BD Biosciences).

Statistics

Statistics were performed using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA). Following outlier statistics in order to choose between performing a one-way ANOVA or Kruskal-Wallis nonparametric test, Gaussian distribution of parameters was checked using a Kolmogorov-Smirnov test. Differences in mean between each two independent experimental groups were analyzed using an unpaired t-test or the nonparametrical Mann-Whitney U test at 95% confidence interval. No statistic analysis was done for data of pooled samples.

RESULTS AND DISCUSSION

To examine the influence of the preceding allergic bronchial inflammation on the antibacterial immune responses of post-asthma rAM during subsequent *C. muridarum* infection, a mouse model of allergic bronchial inflammation was set up. Subsequent *C. muridarum* infection was achieved by i.t. administration of *C. muridarum* elementary bodies (EB) at d15 after the last OVA-exposure. As already described in the first chapter of the results section, at this time point, absolute cell numbers returned to basal levels and the alveoli again consisted for 90% of macrophages. *C. muridarum* is known as the mouse biovar of *C. trachomatis* and is extensively used to study *C. pneumoniae* pathology and immunology [4-8]. To verify the role of post-asthma rAM during the early innate immune responses of post-asthma lungs to a subsequent *C. muridarum* infection, naïve and post-asthma lungs were i.t. infected with *C. muridarum* with or without prior depletion of the respective rAM-population. At d3 post-infection, BAL was performed and alveolar inflammation was assessed by flow cytometry. As shown in figure 1A, the *C. muridarum* infection dose used in this study did not elicit inflammation in the lungs of naïve rAM^+ and rAM^- mice (figure 1A). In contrast, the same infection dose already induced a mild inflammatory response in the lungs of post-asthma rAM^+ mice (figure 1A). Depletion of post-asthma rAM prior to *C. muridarum* infection reduced the total numbers of BAL cells (figure 1A).

The elevated levels of total BAL cells in *C. muridarum* infected post-asthma rAM^+ mice were especially due to an increased recruitment of macrophages/monocytes (figure 1B). It is known that *C. muridarum* stimulates different TLRs at rAM [2]. Since we previously showed an increased *in vitro* TLR-reactivity of post-asthma rAM, hyperinflammatory TLR-signalling exhibited by this rAM-population may underlie the observed increase in innate *C. muridarum* induced pulmonary inflammation. On the other hand, *C. muridarum* has been found to induce apoptosis of rAM in order to spread the infection [8]. This implicates that alveolar BAL cell numbers measured at d3 post-infection is possibly not just due to recruitment of leukocytes but represent a reflection of the recruitment of leukocytes combined with the concurrent apoptosis of macrophages and/or monocytes in the alveoli.

Determination of alveolar bacterial titers at d3 post-infection revealed up to five times higher bacterial loads in the alveoli of post-asthma rAM^+ mice compared to naïve rAM^+ mice (figure 1C). Thus, the preceding allergic bronchial inflammation altered the lung's antibacterial

defence against subsequent *C. muridarum* infection. Possibly the persistence of a Th2-bias rendered the post-asthma lungs more susceptible to subsequent *C. muridarum* infection. Indeed, it is known that BALB/c mice, which are biased towards Th2-mediated responses, are markedly more susceptible to chlamydial lung infection compared to the Th1-predisposed C57BL/6 strain [6]. Alternatively, since rAM are the target cells for *C. muridarum* infection, increased alveolar *C. muridarum* titers in post-asthma rAM^+ mice may be due to altered functionalities of post-asthma rAM. Our earlier research demonstrated that the activation phenotype of post-asthma rAM is biased towards the alternatively activated M2-differentiation status. In contrast to classically activated M1-macrophages, M2-macrophages display impaired bactericidal activities [9]. Moreover, *in vitro* studies have already demonstrated that the expression of alternative macrophage activation markers plays a pivotal role in determining susceptibility to *C. pneumoniae* infection [10]. Furthermore, production of type I IFNs enhances susceptibility to *C. muridarum* lung infection by promoting bacterial dissemination through enhanced apoptosis of local macrophages [8]. As post-asthma rAM displayed altered regulation of IFN-β production, this may contribute to the increased susceptibility of post-asthma mice to a subsequent *C. muridarum* lung infection. As shown in figure 1C, depletion of both naïve and post-asthma rAM prior to *C. muridarum* infection resulted in a five-fold increase in alveolar bacterial burden (figure 1C). This result indicates that post-asthma rAM still exhibited significant protective antibacterial immunity. Yet, the increased bacterial loads in rAM^- mouse groups could also be attributed to a lack of target cells for infection. Strikingly, post-inflammation rAM^- mice still exhibited elevated bacterial loads as compared to infected naïve rAM^- mice. These results clearly indicate that additional alterations in post-inflammation lung cells, like AECs, were beneficial for *C. muridarum* survival and/or replication.

In conclusion, we document a prominent pro-inflammatory role of post-asthma rAM contributing to the increased pulmonary inflammatory reactivity of post-asthma lungs to *C. muridarum* infection. Together with the observation that post-asthma lungs showed higher loads of alveolar *C. muridarum*, these data indicate and support a role of post-asthma rAM in the onset of *C. pneumoniae* induced asthmatic exacerbation. Moreover, increased pulmonary inflammatory responses provoked by the inadequate antibacterial immune function of this rAM-population may compromise the pulmonary gas exchange function and the integrity of the mucosal epithelial barrier, hereby facilitating bacterial dissemination to other body compartments.

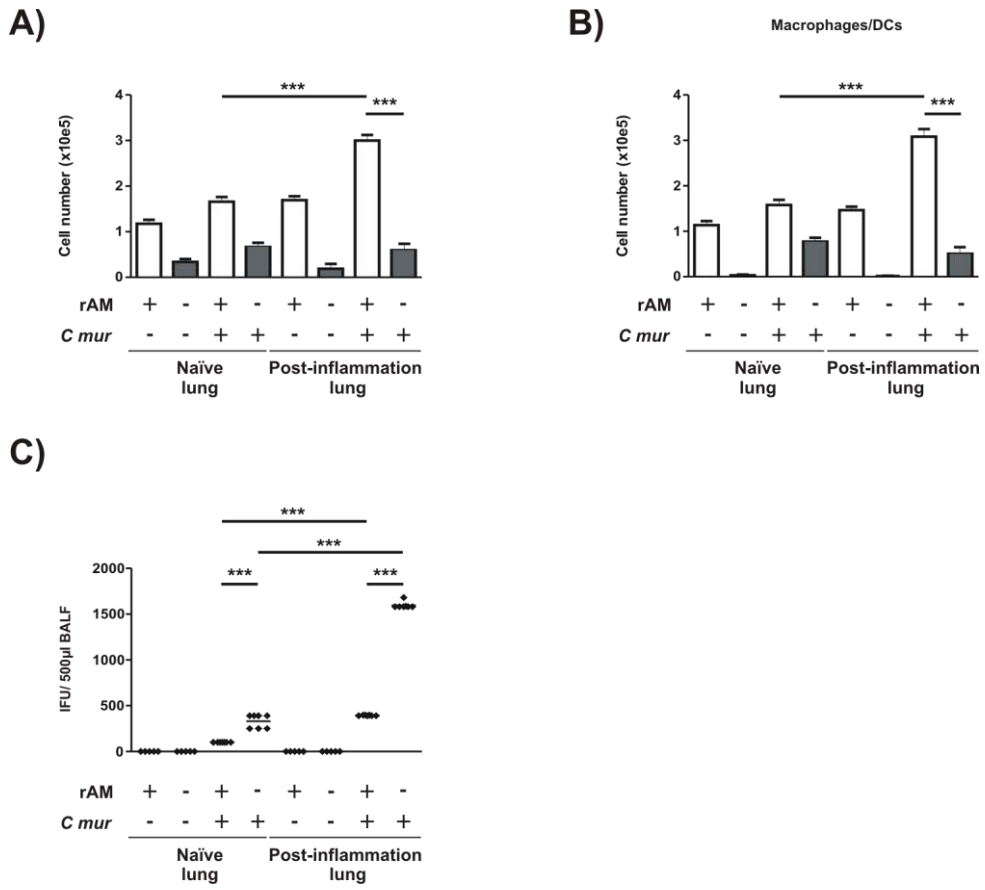

Figure 1. Alveolar inflammatory responses and bacterial titers after *C. muridarum* infection of naïve and post-asthma rAM$^+$ and rAM$^-$ mice at d3 post-infection.
Post-asthma (d15 after the last OVA-challenge) and naïve C57BL/6 mice (n=7) received CL i.t. three days before inoculation with 10^3 IFU *C. muridarum* to deplete post-asthma and naïve rAM respectively or received PBS as a control. BAL was collected at d3 post-infection. (A) Average BAL cell counts ± SEM (B) and average BAL cellular composition ± SEM were determined by flow cytometry. (C) BAL fluid was collected and bacterial recovery was assessed by direct immunofluorescence staining of the bacteria. *** $p<0.0001$

AUTHOR CONTRIBUTIONS

T. Naessens wrote the manuscript and performed the major part of the experimental work.

S. Peirs (*C. muridarum* cultures and titration), C. Pollard and P. Bogaert (flow cytometry) performed technical assistance.

N. van Rooijen provided clodronate liposomes.

D. Vanrompay and J. Grooten designed research.

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PART IV

CONCLUSIONS AND FUTURE PERSPECTIVES

Introduction

Patients with allergic asthma suffer from recurrent episodes of airway obstruction, wheezing, chest tightness, and shortness of breath. These macroscopic symptoms are collectively referred to as asthma exacerbations. From the list of microscopic symptoms, airway inflammation is considered as an important pathophysiological component of allergic asthma. Prolonged allergen exposure elicits Th2-mediated eosinophilic or Th1/Th17-mediated neutrophilic bronchial inflammation in mild to moderate or severe allergic asthma patients respectively [1]. Interactions between inflammatory leukocytes and resident tissue cells of the respiratory tract cause subsequent tissue remodelling. The continuous positive feedback between these elements can eventually result in the development of an asthma exacerbation [2]. The induction of these cascade reactions was initially ascribed to excessive and persistent allergen encounter. However, acute respiratory viral or bacterial infections are now very often detected in hospitalized individuals suffering from an asthma exacerbation. Therefore, acute respiratory viral or bacterial infections are strongly suggested to be the main instigators of asthma exacerbations [3].

Nonetheless, the question which cells and/or molecules that orchestrate acute respiratory viral or bacterial infection induced asthma exacerbation is still not satisfyingly answered.

Resident alveolar macrophages (rAM) are known as the primary immune sentinels of the lung and are major determinants of pulmonary immune responses to viral [4-9] and bacterial [10-13] infections. In order to maintain a sterile non-inflamed microenvironment, this phagocyte population excels in combining efficient removal of respiratory pathogens with suppression of the development of subsequent pulmonary inflammatory responses. However, research of the last decade highlighted the fact that innate imprinting by a preceding pulmonary inflammation can have detrimental effects on the rAM's functional status during subsequent responses of the lung to other, often unrelated, inflammatory stimuli [14-16]. The goal of this study was to investigate to which extent innate imprinting of rAM was induced by a preceding allergic bronchial inflammation. Moreover, the pathophysiological relevance of possible allergic inflammation-induced innate imprinting of rAM during subsequent acute respiratory viral or bacterial lung infections was evaluated.

General overview and interpretation of the experimental observations

As general basic research platform, an established mouse model of allergic asthma was applied. Systemic sensitization against ovalbumin (OVA) in the presence of the Th2-skewing adjuvant aluminiumhydroxide (alum), followed by pulmonary exposure to aerosolized OVA, is a widely established protocol to induce a mild to moderate allergic asthma-like airway inflammatory response in mice [17, 18]. When allergen exposure was arrested, the eosinophilic allergic bronchial inflammation was cleared and steady-state conditions were achieved within a time-frame of 12 days. At this time-point, absolute cell numbers returned to basal levels and the cellular composition of the alveoli again consisted nearly exclusively of rAM.

We initially characterized the phenotype of this post-inflammation rAM-population by assessing the expression of a number of typical alveolar macrophage markers. Among all the macrophage subtypes in the body, naïve rAM exhibit a unique phenotypic marker profile. For instance, naïve rAM express high levels of CD11c and DEC-205, surface markers not found on other macrophage populations and expressed mainly by DCs and DC-subpopulations [19, 20]. In contrast, expression of F4/80 and CD11b, which are historically considered as the canonical macrophage markers, are very low to absent respectively [21]. CD11c and DEC-205 were found to be equally expressed on naïve and post-inflammation rAM. Furthermore, high intrinsic fluorescence intensity, another hallmark of naïve rAM, was also found in post-inflammation rAM. Yet, post-inflammation rAM exhibited higher levels of CD11b and CD115, the receptor for macrophage colony stimulating factor (M-CSF) [22]. In addition, post-inflammation rAM expressed higher levels of FcγRIII (C16/CD32) and equal levels of the scavenger receptor (SR)-A (CD36), which are both receptors involved in phagocytosis [23].

Post-inflammation rAM exhibited only a poor basal *in vitro* phagocytic capacity which is very inconsistent with the conventional reputation of naïve rAM and other macrophage phenotypes of the body which generally excel in phagocytosis of particulate matter [24]. As opposed to their decreased basal *in vitro* phagocytic capacity, post-inflammation rAM showed increased *in vitro* TLR-3, TLR-4 and TLR-7 reactivity. Secreted levels of inflammatory cytokines like TNF-α, IL-6, IL-12p70, CXCL1 and CXCL2 were increased after LPS and imiquimod stimulation compared to naïve rAM. Another striking difference in innate pro-inflammatory signalling between post-inflammation rAM and naïve rAM was found at the level of TLR-induced type I IFN secretion. Post-inflammation rAM produced significant levels of bioactive IFN-β following *in vitro* TLR-4 and TLR-3 stimulation by LPS and poly I:C respectively.

These data stand in strong contrast to our experimental experience and the literature in which it has been demonstrated that naïve rAM characteristically fail to autonomously secrete IFN-β after engagement of TLR-3 and TLR-4 [25]. Naïve rAM possess however functional IFNAR-signalling which renders them fully responsive to exogenous type I IFN and they do upregulate *ifn-β* mRNA transcript levels after *in vitro* TLR-3 and TLR-4 stimulation as well [25]. Therefore, TLR-3 and TLR-4 induced production of IFN-β must be regulated at the posttranscriptional level in naïve rAM. This posttranscriptional regulatory checkpoint is clearly absent in post-inflammation rAM. Together, these data indicate that innate imprinting of rAM by a preceding allergic bronchial inflammation includes a shift from an immunological restrained macrophage phenotype, represented by naïve rAM, towards a less tightly controlled and immunological released phenotype, embodied by post-inflammation rAM.

We next investigated the *in vivo* pathophysiological relevance of the altered innate functionality of post-inflammation rAM by exposing mice, which suffered from a preceding allergic bronchial inflammation, to a subsequent respiratory syncytial virus (RSV) and *Chlamydia* (*C.*) *muridarum* lung infection. These two respiratory pathogens are often appointed as the instigators of asthma exacerbations [3]. Infection of post-inflammation lungs with RSV resulted in increased pulmonary innate and adaptive inflammatory responses compared to RSV-infection of naïve lungs. In contrast, alveolar RSV-titers did not differ between both mouse groups. Prior depletion of both rAM-populations emphasized the strong contrast between the intrinsic pro-inflammatory character of post-inflammation rAM and the anti-inflammatory capacities exhibited by naïve rAM during subsequent RSV-infection. The increased inflammatory TLR-reactivity exhibited by post-inflammation rAM is a reasonable explanation for the way in which these cells contributed to the generally increased RSV-induced pulmonary inflammation in post-inflammation mice. RSV contains several ligands for a variety of PRRs on innate immune cells. The RSV genomic RNA is recognized by TLR-3, expressed by AECs and rAM [26], while the RSV F-protein has been identified as a TLR-4 ligand [27]. Furthermore, TLR-7 was recently acknowledged as another member of the TLR-family involved in the recognition of RSV [28, 29]. The lack of any direct antiviral activity of post-inflammation rAM was also very opposing to the efficient antiviral activity of naïve rAM. Given their regained capacity to autonomously produce IFN-β, one would expect however that post-inflammation rAM would exhibit improved antiviral capacities. It is likely that an impaired IFN-β response of the post-inflammation AECs [30], the main target cells for

RSV-infection, or active inhibition of the post-inflammation rAM's IFN-β production by the virus [31, 32] are predominant. Furthermore, spore concentrations of TGF-β, which is abundantly secreted during an allergic bronchial inflammation [33, 34], can exert suppressive actions on the post-inflammation rAM's type I IFN responses [35]. Another possibility is that upon RSV-infection, post-inflammation rAM exhibit deficient production of IL-15, a downstream target of type I IFNs [36] which is implicated in innate and acquired antiviral immunity [37, 38]. BAL macrophages of asthma patients already showed an impaired IL-15 response to rhinovirus infection which was inversely related to AHR and virus load [39]. Therefore, and because of the downstream position to IFN-β, a deficient IL-15 production could represent a dominant bottle neck responsible for the poor antiviral activity of post-inflammation rAM. Additionally, as poor IFN-λ induction in AECs and rAM from asthmatics was correlated with the severity of virus-induced asthma exacerbations [40], deficient production of this type III IFN by post-inflammation rAM could also dominantly contribute to the inadequate antiviral activity of this rAM-subset.

Prior depletion of naïve rAM had also negative effects on morbidity parameters, including body weight and serum levels of IL-6, while these parameters were not affected by prior depletion of post-inflammation rAM. However, one should take into account that the increase in morbidity and pulmonary inflammation observed after RSV-infection of naïve rAM-depleted lungs could be the result of the increased alveolar viral load during the early (d3) pulmonary innate responses in these mice. In this case, increases in pulmonary inflammation and morbidity are secondary effects elicited by the deficient initial clearance of the virus due to the absence of naïve rAM rather than the direct result of the lack of anti-inflammatory signals otherwise provided by naïve rAM. This scenario also stands in strong contrast with the events that are taking place in post-inflammation mice. As prior depletion of post-inflammation rAM resulted in decreased pulmonary inflammation without any concurrent changes in alveolar RSV viral load and morbidity, this decrease in pulmonary inflammation is ascribed to diminished pro-inflammatory signals in the lungs otherwise provided by post-inflammation rAM. Thus, it is clear that the preceding allergic inflammation induced a shift in the functionality of rAM during a subsequent RSV-infection. This shift included the transition from a direct and efficient antiviral function of naïve rAM to a purely pro-inflammatory function of post-inflammation rAM.

The initial encounter between post-inflammation rAM and RSV also had a differential outcome on the development of subsequent RSV-specific immune memory responses. Reinfection of post-inflammation mice with RSV resulted in increased LDLN CTL and Th1-cell responses which both decreased when post-inflammation rAM were initially depleted. In contrast, prior depletion of naïve rAM slightly increased RSV-specific CTL-responses and largely increased Th1-memory responses during RSV-reinfection. These data can reflect differences in the interaction between post-inflammation rAM and airway DCs. Although lung interstitial macrophages are now appointed as the eminent pulmonary suppressors of DC-function during diseases, like allergic asthma [41], previous research reported that naïve rAM also suppress airway DC-maturation, -function and -trafficking to mediastinal LNs [42, 43]. Thus, depletion of naïve rAM prior to primary RSV-infection could increase the APC-function of airway DCs, explaining the increased CTL- and Th1-responses during RSV-reinfection. In contrast, by exhibiting a more pro-inflammatory character, post-inflammation rAM probably exerted immune stimulatory rather than immunosuppressive effects on airway DC-maturation, -function and -trafficking during the initial encounter with RSV. This could explain why CTL- and Th1-responses in post-inflammation rAM^+ mice increased during RSV-reinfection and decreased when post-inflammation rAM were initially depleted. However, these data may be biased by alterations in composition of the resident airway DC-population. The preceding allergic bronchial inflammation can alter the ratio between immunosuppressive pDCs and immunogenic mDCs in the airways which can largely influence the outcome of subsequent LDLN T-cell responses. Despite the differences in LDLN memory responses, no differences were found at the level of local alveolar inflammation during RSV-reinfection between all RSV-reinfected mouse groups. Additionally, no virus was detected in the alveoli of these mice as well. Thus, it remains unclear what the actual (patho)physiological significance is of these observed differences in memory T-cell responses. One possibility is that increased memory CTL- and Th1-responses lead to increased levels of pulmonary IFN-γ which in turn can augment general lung tissue pathology and AHR and can induce acute exacerbation reactions [44]. At the level of humoral memory responses, lower levels of RSV-specific IgG1- and IgG2a-antibodies were observed in post-inflammation mice. However, only depletion of naïve rAM resulted in slightly decreased IgG1 levels. IgG-molecules are pivotal in the neutralization of RSV, inhibiting viral attachment to target cells [45]. However, sera from post-inflammation mice did not show impaired *in vitro* neutralization of RSV. In addition, RSV-rechallenge of post-asthma mice did not yield any productive infection which is possibly due to the immediate antibody-

mediated neutralization of incoming RSV-particles. In this context however, the presence of lung mucosal IgA is also an important parameter. Thus, the actual physiological relevance of the observed differences in RSV-specific IgG1- and IgG2a-levels is not defined yet. One possibility is that lower IgG1- and IgG2a-levels in RSV-infected post-inflammation mice is reflected in lower amounts of opsonising IgG1- and IgG2a-molecules in these mice. Impaired opsonisation of incoming RSV-particles would eventually result in decreased rAM-mediated uptake and killing of the virus.

Investigation of the role of post-inflammation rAM during a subsequent acute *C. muridarum* lung infection showed similar results. The *C. muridarum* infection dose used in this study did not elicit any pulmonary inflammation in naïve mice while a mild inflammation was observed in the lungs of post-inflammation mice. The induction of this mild pulmonary inflammatory response was mainly due to the presence of post-inflammation rAM as prior depletion of this rAM-population resulted in only a minor recruitment of inflammatory leukocytes to the alveoli. As *C. muridarum* contains a number of TLR-ligands, including LPS [46], the TLR-hyperreactivity of post-inflammation rAM can partially underlie the increased inflammatory responses seen in infected post-inflammation lungs. In addition to the increased pulmonary inflammatory response, post-inflammation mice showed also an increased alveolar bacterial burden. Since rAM are the target cells for *C. muridarum* infection [46], increased alveolar *C. muridarum* titers in post-inflammation mice might be mainly due to increased susceptibility of post-inflammation rAM for infection. An important argument that supports this hypothesis is that post-inflammation rAM displayed critical alterations in their regulation of IFN-β production. This type I IFN enhances susceptibility to *C. muridarum* lung infection by promoting bacterial dissemination through enhanced apoptosis of local macrophages [47]. Prior depletion of naïve and post-inflammation rAM both resulted in elevated alveolar *C. muridarum* titers. However, this result may be biased by the fact that the target cells of the bacteria were depleted before the infection. Nevertheless, in both naïve and post-inflammation rAM^- mouse groups, *C. muridarum* lung infection lead to the recruitment of monocytes to alveoli. These monocytes, most likely recruited shortly after the *C. muridarum* challenge, can serve as the new host cells for the bacteria. Strikingly, post-inflammation rAM^- mice exhibited elevated bacterial loads as compared to infected naïve rAM^- mice. These results clearly indicate that additional alterations in post-inflammation lung cells, like AECs, were beneficial for *C. muridarum* survival and/or replication. Another important consideration is the fact that during its life cycle, *C. muridarum* alternates between a non-replicating,

infectious elementary body (EB), and a replicating, non-infectious reticulate body (RB) [48]. As the bacterial titer detection method used in this study was only a read-out for free alveolar EBs of *C. muridarum*, intracellular loads of *C. muridarum* RBs are not known. This is important because the presence of this intracellular chlamydial life-form can influence the actual total amount of bacteria in the lungs. Furthermore, differences in the amount of intracellular RBs can have a differential outcome on the induction of subsequent dormant latent infections [48]. Together, these results highlight the increased susceptibility of post-inflammation mice to subsequent *C. muridarum* lung infection in which a pronounced pro-inflammatory role is played by post-inflammation rAM.

An overview of the most important experimental observations is depicted in figure 1.

Mechanisms underlying allergic inflammation-induced innate imprinting of rAM

The functionality exhibited by cells is often a reflection of the local microenvironment in which they reside. Interactions with soluble proteins and/or other cellular components of the tissue determine the reactivity of these cells in response to pathogens or other foreign substances. In initial mechanistic experiments, we observed a rapid clearance of naïve rAM from the bronchoalveolar lumen during the course of the allergic bronchial inflammation. Thus, post-inflammation rAM originated from monocytes that were freshly recruited during or after the bronchial inflammation. The subsequent differentiation of these recruited monocytes to post-inflammation rAM was then largely orchestrated by the prevailing conditions of the lung environment.

In the case of naïve rAM, it was found that the unusual microenvironment of the alveoli provides this macrophage population with a unique phenotype. For example, rAM are exposed to high oxygen tension and are also bathed in high concentrations of granulocyt-macrophage colony stimulating factor (GM-CSF) [49] and surfactant proteins (SP), like SP-A and SP-D [50]. Especially the pulmonary pool of **GM-CSF** has been shown to be the most critical factor in the induction of the rAM-phenotype. For instance, rAM from GM-CSF$^{-/-}$ mice showed decreased phagocytosis of latex beads, Gram-negative and Gram-positive bacteria [51], and adenoviral particles [52]. Independent of the phagocytic abnormality, intracellular killing of both Gram-positive and Gram-negative bacteria was also reduced in the rAM of these mice [51]. Thus, insufficient levels of pulmonary and/or alveolar GM-CSF in post-

inflammation lungs may lead to the inadequate innate functional design of post-inflammation rAM, as reflected by their poor basal *in vitro* phagocytic activity and their deficient participation to *in vivo* RSV-clearance. Furthermore, insufficient alveolar GM-CSF may impair the bactericidal activity of post-inflammation rAM, leading to increased *C. muridarum* levels in the alveoli of post-inflammation lungs. Furthermore, exaggerated inflammatory responses to bacterial [53] or viral [52] lung infection were observed in GM-CSF$^{-/-}$ mice while transgenic pulmonary expression of this growth factor in these mice rescued deficient pathogen clearance and inhibited exuberant pulmonary inflammatory responses in both disease models [53]. These findings are in line with our results that demonstrate increased pulmonary inflammatory responses in post-inflammation lungs after RSV- and *C. muridarum* infection. However, there are also discrepancies between the phenotype of GM-CSF$^{-/-}$ rAM and post-inflammation rAM. Despite overt pro-inflammatory cytokine production following microbial infection of the lungs, rAM from GM-CSF$^{-/-}$ mice failed to release TNF-α upon LPS-stimulation [51]. Consistent with that observation, reduced expression of multiple components of the TLR-signalling pathway, including TLR-4, TLR-2 and CD14, was observed in rAM from GM-CSF$^{-/-}$ mice [51]. These data are inconsistent with our finding that post-inflammation rAM displayed hyperinflammatory reactivity to *in vitro* TLR-engagement and exhibited no changes in TLR-expression. In addition, GM-CSF is supposed to be the main inducer of CD11c-expression by recruited rAM precursor cells [21]. Post-inflammation rAM expressed CD11c levels comparable to those of naïve rAM, suggesting 'normal' GM-CSF activity in the alveoli of post-inflammation lungs.

Therefore, it is likely that other factors contribute to the increased pro-inflammatory TLR-reactivity of post-inflammation rAM. For instance, the collectins, **SP-A** and **SP-D** are known for their immunomodulatory properties on pulmonary immune responses in order to protect the host from overzealous inflammation that could potentially damage the lung and impair gas exchange [54]. Moreover, SP-A is known to dampen TLR-2 and TLR-4 signalling in human monocyte-derived macrophages [55] while SP-D was found to inhibit CD14/TLR-4 signalling in murine rAM [56]. As post-inflammation rAM exhibited hyperinflammatory responsiveness to *in vitro* engagement of TLR-3, TLR-4 and TLR-7, these results can be indicative for low concentrations of SP-A and/or SP-D in the alveoli of post-inflammation lungs. Furthermore, SP-A and SP-D are important pulmonary pattern recognition molecules which opsonise respiratory pathogens for uptake by pulmonary phagocytes, like rAM. Therefore, insufficient alveolar SP-A and/or SP-D can contribute to the inappropriate antiviral and antibacterial

immune responses of the post-inflammation lungs by decreasing viral and bacterial uptake and killing by post-inflammation rAM and increasing overall pulmonary inflammation.

Next to the presence of immunosuppressive soluble factors in the alveoli of naïve lungs, immunomodulatory interactions between rAM and AECs are also vital to allow the specialized modulation of the rAM-function. For instance, naïve rAM closely adhere to the AECs and this interaction induces the expression of **$\alpha_v\beta_6$-integrin** on the surface of AECs. The importance of $\alpha_v\beta_6$-integrin for keeping the rAM in a quiescent state under steady-state conditions was shown by the fact that $\alpha_v\beta_6$-integrin$^{-/-}$ mice have constitutively activated rAM [57]. Furthermore, recent research identified other novel homeostatic loops which are maintained by the close interaction between rAM and AECs. These loops require the naïve rAM-AEC **CD200R-CD200 axis** [58] and **MUC1** expression on AECs [59, 60]. In the assumption that a preceding allergic bronchial inflammation induces critical alterations in the AEC-phenotype, it is likely that these regulatory circuits are abolished in post-inflammation lungs, thereby abrogating the immunosuppressive activities on post-inflammation rAM. Thus, investigation of the interplay between post-inflammation rAM and post-inflammation AECs could provide further insight into the functional development of post-inflammation rAM.

In this study, we evidenced that the preceding allergic bronchial inflammation is necessary to give rise to the phenotype of post-inflammation rAM. In addition, we showed that the type of preceding bronchial inflammation has a pivotal impact on the way in which post-inflammation rAM are imprinted. For instance, the sustained TLR-desensitization of rAM residing in the alveolar cavity after resolution of respiratory influenza infection [14] stands in strong contrast to the persistent TLR-hyperreactivity observed in post-inflammation rAM from our study. Therefore, the establishment of the post-inflammation rAM's functional phenotype is probably largely influenced by a prolonged exposure to factors associated with the preceding allergic bronchial inflammation. One of the hallmark cytokines secreted during a Th2-mediated eosinophilic allergic bronchial inflammation is IL-4 [61]. This cytokine skews macrophages towards an alternatively activated differentiation status [49]. It has been found that IL-4 mediated alternative activation of macrophages impairs phagocytosis but potentiates microbial-induced signalling and cytokine secretion [62]. The antimicrobial features of these IL-4 induced M2-macrophages show striking similarities with the decreased basal *in vitro* phagocytic activity and increased *in vitro* TLR-reactivity exhibited by post-inflammation rAM. Moreover, we demonstrated that post-inflammation rAM displayed a basal M2-

differentiation status as evidenced by the increased *arg-1* to *inos* ratio [49] we observed in these cells. Nevertheless, a similar post-inflammation rAM-functional phenotype was observed in a recently established mouse model of Th1- and Th17-biased non-eosinophilic severe refractory asthma. In this model, IFN-γ and IL-17 rather than IL-4 are the dominant hallmark cytokines secreted in the lungs [18]. Therefore, the nature of the inflammatory trigger is probably playing a more dominant role in the outcome of the innate imprinting of rAM.

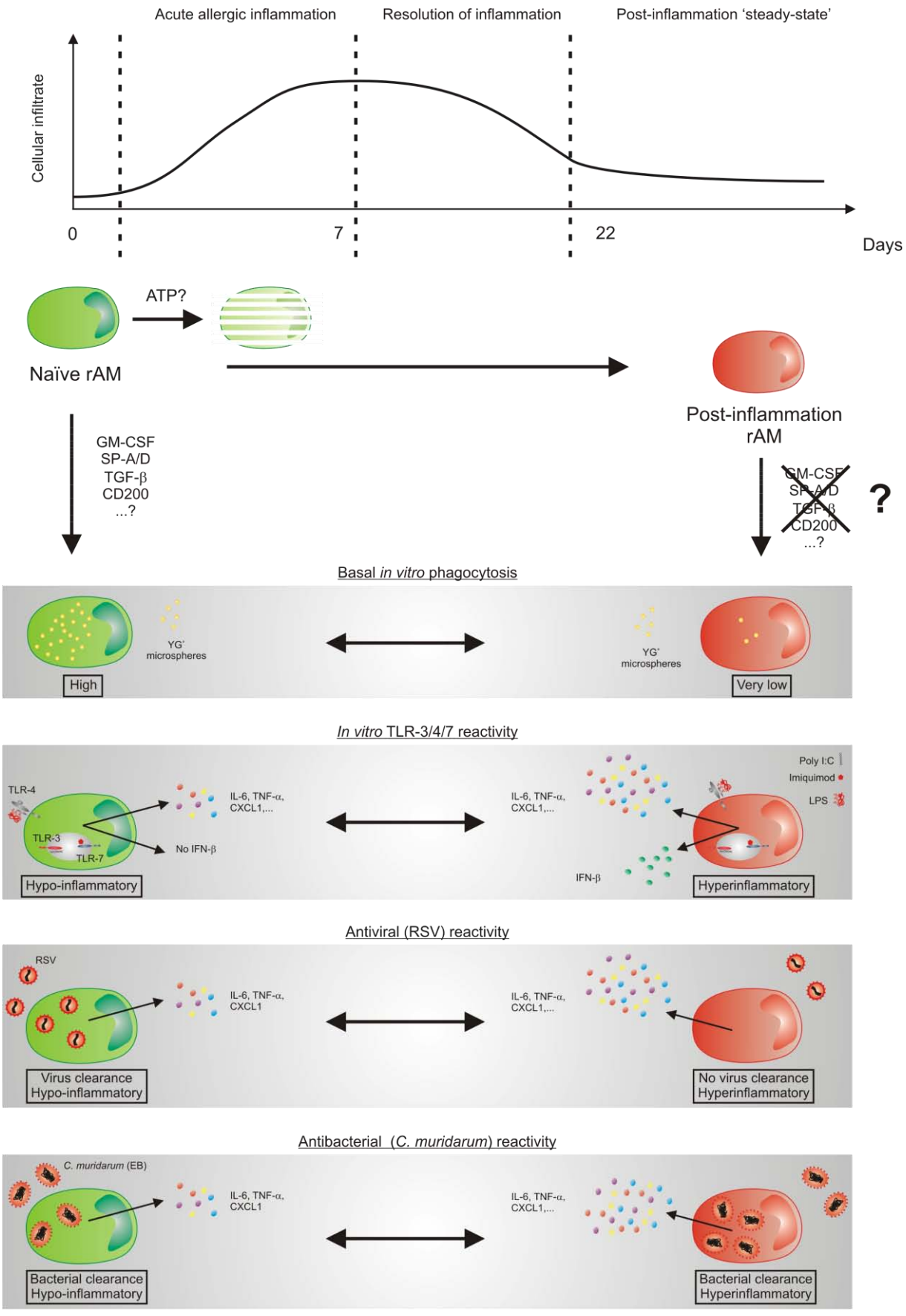
Another molecule that is elevated in patients suffering from asthma [63] and affects TLR-signalling in macrophages is macrophage-colony stimulating factor (**M-CSF**). Although their names suggest many shared functional characteristics, GM-CSF and M-CSF exert very different influences on the functional development of innate leukocytes. For instance, the balance between GM-CSF and M-CSF can have a decisive role in the regulation of TLR-signalling in macrophages. While *in vitro* LPS-stimulation of GM-CSF primed bone-marrow derived macrophages (BMDM) enhances the expression of genes induced by the MyD88-dependent NF-κB pathway like *tnf-α*, *il-12p40* and *il-23p19*, M-CSF-primed BMDM express increased levels of especially MyD88-independent IRF-3 induced genes like *ifn-β* and *ccl5* [64]. Overt M-CSF production during allergic bronchial inflammation could therefore contribute to the enhanced MyD88-independent TLR- and IFN-β-signalling seen in post-inflammation rAM. In addition, the presence of significant levels of the M-CSF receptor, CD115, at the surface of post-inflammation rAM would render these cells more susceptible to the actions of this growth factor.

By depleting the local pool of pulmonary ATP, we were able to prevent the inflammation-induced naïve rAM-turnover. ATP is abundantly produced during the course of an allergic bronchial inflammation and serves as a co-factor for the induction of several signalling cascades in different cell types [65]. One signalling pathway that can be of interest for our observations is the ATP-dependent induction of inflammasome/caspase-1 mediated apoptosis, referred to as pyroptosis [66]. This atypical pathway of apoptosis is found to be a common process during inflammation [67] and represents a plausible mechanism underlying the accelerated allergic inflammation induced rAM-turnover. Importantly, as inhibition of the naïve rAM-turnover during the course of the allergic inflammation results in the persistence of this rAM-population after the resolution of the inflammation, this might affect the outcome of the innate imprinting. In addition, it might provide more insight into the interplay between

the local microenvironment and the intrinsic characteristics of post-inflammation rAM in establishing the phenotype of innate imprinting.

A schematic overview of the possible mechanisms responsible for the naïve rAM-turnover and determining the functional phenotype of post-inflammation rAM is depicted in figure 1.

Figure 1: rAM are the predominant cell type in the alveoli of naïve mice. Their functional phenotype is shaped by the presence of local alveolar mediators, like TGF-β, SP-A/D and GM-CSF, and interactions with neighbouring AECs via for instance the CD200R-CD200 axis. The functional characteristics of naïve rAM include a relatively high basal *in vitro* phagocytic activity together with an *in vitro* TLR-hyporesponsiveness. Furthermore, these cells efficiently clear antimicrobial pathogens from the alveoli without inducing overt subsequent inflammatory responses. During the course of an allergic eosinophilic inflammation, this naïve rAM-population disappears from the alveoli by a mechanism driven by ATP. After the resolution of the allergic inflammation, a new post-inflammation rAM-population resides in the alveoli. In contrast to naïve rAM, post-inflammation rAM exhibit very low basal *in vitro* phagocytic activity. This was however combined with a strong increase in *in vitro* TLR-reactivity. Importantly, this rAM-population also exhibit increased proinflammatory reactivity during an acute RSV and *C. muridarum* infection. In the case of RSV, this increased proinflammatory reactivity was not accompanied by any direct antiviral reactivity. The altered innate functionality of post-inflammation rAM can be induced by the absence or the insufficient levels of immune regulatory factors in the alveoli and/or altered interactions with AECs, both the result of the preceding allergic bronchial inflammation.


Acute allergic inflammation
Resolution of inflammation
Post-inflammation 'steady-state'
Cellular infiltrate
0
7
22
Days
ATP?
Naïve rAM
Post-inflammation rAM
GM-CSF
SP-A/D
TGF-β
CD200
...?
GM-CSF
SP-A/D
TGF-β
CD200
...?
?
Basal in vitro phagocytosis
YG microspheres
High
YG microspheres
Very low
In vitro TLR-3/4/7 reactivity
TLR-4
TLR-3
TLR-7
IL-6, TNF-α, CXCL1,...
No IFN-β
Hypo-inflammatory
IL-6, TNF-α, CXCL1,...
Poly I:C
Imiquimod
LPS
IFN-β
Hyperinflammatory
Antiviral (RSV) reactivity
RSV
IL-6, TNF-α, CXCL1
Virus clearance
Hypo-inflammatory
IL-6, TNF-α, CXCL1,...
No virus clearance
Hyperinflammatory
Antibacterial (C. muridarum) reactivity
C. muridarum (EB)
IL-6, TNF-α, CXCL1
Bacterial clearance
Hypo-inflammatory
IL-6, TNF-α, CXCL1,...
Bacterial clearance
Hyperinflammatory

General conclusions, clinical implications and perspectives for future research

During the last decade, imprinting of resident innate leukocyte populations by inflammation or infection has caught the attention of an increasing number of researchers. The discovery of this immunological phenomenon revealed new insights into the field of heterologous immunity during multiple, often unrelated, inflammatory events. For instance, innate imprinting of rAM by a preceding influenza infection is thought to deliver a significant contribution to the enhanced susceptibility of influenza patients to secondary bacterial pneumonia [14, 15]. Our study provides evidence that rAM are imprinted by a preceding allergic bronchial inflammation. This type of innate imprinting is characterized by the increased pro-inflammatory reactivity of post-inflammation rAM to TLR-engagement and, more importantly, also to subsequent RSV and *C. muridarum* infection. In addition, post-inflammation rAM exhibited decreased basal *in vitro* phagocytic activity and displayed no direct antiviral activity. Impaired antimicrobial innate functionality of post-inflammation rAM affected also later stages of the viral lung infection and the immune memory responses. These data strongly suggest that innate imprinting of rAM by a preceding allergic bronchial inflammation contributes to the onset of respiratory pathogen induced asthma exacerbations of which asthma patients often suffer. Overt inflammatory pulmonary responses, partially induced by post-inflammation rAM, to subsequent RSV or *C. muridarum* lung infections combined with the already present airway structural changes and AHR in these subjects, can eventually result in the collapse of the airways. Therefore, the function of post-inflammation rAM during RSV- and *C. muridarum* infections needs to be further elucidated. As type I IFNs are important mediators in both infectious diseases, the significance of the altered IFN-β responses exhibited by post-inflammation rAM should be evaluated during RSV- and *C. muridarum* infections. In addition, the importance of both the alterations in TLR-reactivity and phagocytic activity displayed by post-inflammation rAM should be determined in RSV- and *C. muridarum* infections as well. Furthermore, an inadequate innate immune reactivity of post-inflammation rAM in mild asthmatics may contribute to the transition towards a more severe asthmatic disease status. We all become infected with respiratory pathogens, which normally induce only in the most severe cases airway remodelling and AHR in non-asthmatic individuals. However, in allergic asthmatics, respiratory viral or bacterial infections can induce more pronounced increases in airway remodelling and AHR compared to non-asthmatic subjects. Therefore, the role of post-inflammation rAM in the development of airway structural changes and AHR after subsequent RSV and *C. muridarum* lung infection is

an important topic for future investigation. In addition, further mechanistic insights in the origin and development of rAM innate imprinting by a preceding allergic bronchial inflammation can deliver crucial information for managing secondary acute respiratory viral and bacterial infections in asthmatic patients. By specifically restraining the pro-inflammatory reactivity and/or enhancing direct antimicrobial activities of post-inflammation rAM in asthmatic patients, respiratory viral and bacterial induced exacerbations could be prevented or treated more efficiently.

The more pronounced pro-inflammatory TLR-responsiveness of post-inflammation rAM also highlights the fact that these cells could also be involved in the sensitization of allergic asthmatics to new allergens. Several airborne allergens are often contaminated with spore concentrations of LPS, exhibit structural homology with TLR-4 ligands or contain proteolytic activity [68]. Compared to rAM from naïve individuals, the encounter between these allergens and post-inflammation rAM may elicit a more pronounced pulmonary inflammation. This pulmonary inflammation may in turn provide the necessary danger signals for promoting DC-maturation and -migration to the lymph nodes after allergen uptake.

However, there are some important issues that make it rather hard to translate the obtained results from this study to the human case of allergic asthma. Firstly, it is vital to consider the limited translational value of the OVA/alum and OVA/CFA based mouse models of allergic asthma used in this study. Allergic asthma has a very complex aetiology and intertwining genetic and environmental aspects significantly contribute to the heterogeneous phenotype of the disease at later age. Clearly, the models of allergic asthma used in this thesis fail to recapitulate all the aspects and features of human allergic asthma but only replicate the Th2-mediated eosinophilia or the Th1- and Th17- mediated neutrophilia observed in the airways of mild or severe asthma patients respectively. The way in which genetic and environmental factors affect the functional status of post-inflammation rAM remains unknown. In addition, the surrounding lung microenvironment can show footprints of many other infectious and non-infectious inflammatory insults which can in turn all affect the functionality of the existing rAM-population. These concerns may challenge the clear-cut phenotype of post-inflammation rAM observed in this study.

Secondly, the proposed model of innate imprinting of post-inflammation rAM might not be applicable to patients suffering from severe non-eosinophilic allergic asthma. Although we found that the representation of the post-inflammation rAM phenotype was generally

independent of the type of allergic inflammation, humans suffering from severe allergic asthma are unlikely to achieve the post-inflammation lung status as pulmonary inflammation is persistently present. In mild asthmatic individuals however, disruption of allergen exposure leads to the resolution of the pulmonary allergic inflammation, a feature which was also mimicked in our OVA/alum based mouse model. Thus, our findings that a preceding Th2-mediated eosinophilic inflammation can affect the rAM's functional phenotype can be of interest for especially this subpopulation of asthma patients.

Collectively, it is clear that the concept of innate imprinting, including its origin and its immunological consequences, is an important piece of the puzzle in the search for the Holy Grail to find proper therapeutic approaches for treating the many pathological facets of allergic asthma.

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SUMMARY

Resident alveolar macrophages (rAM) exist in an environment high in antigenic material of which the majority must be ignored except when the antigen represents an infectious threat. When the activation threshold of rAM is exceeded, the development of a pulmonary inflammation is inevitable. In the case of allergic asthma, pro-inflammatory activation of rAM is induced upon abundant exposure to airborne allergen. Subsequent cytokine and chemokine secretion by activated rAM contributes to monocyte recruitment to the lungs and these cells are in turn involved in the further propagation of the allergic inflammation. Thus, both rAM and recruited monocytes/macrophages contribute to different stages of an allergic bronchial inflammation. However, the dynamics of both macrophage populations during the different stages of the allergic inflammation are still poorly characterized. Therefore, the initial goal of this thesis was to analyze the dynamics of rAM during the course of an allergic bronchial inflammation. By using an OVA/alum-based mouse model of asthma, featuring a Th2-biased sensitization and an eosinophilic airway inflammation reminiscent of the immunopathology of mild to moderate asthma, we found that during the acute stages of the inflammation, the rAM-subset disappeared from the alveoli through an ATP-dependent mechanism. These results implied that a new post-inflammation rAM resided in the airways after the clearance of the allergic bronchial inflammation.

Research through the last decade demonstrated that the rAM-functionality can be influenced or imprinted by a preceding bronchial inflammation. We therefore performed a phenotypical and functional comparison between naïve and post-inflammation rAM to determine to what extent post-inflammation rAM were subjected to innate imprinting by the preceding allergic bronchial inflammation. Although they showed a surface marker profile similar to that of naïve rAM, post-inflammation rAM exhibited a strongly reduced basal phagocytic capacity accompanied by a markedly increased inflammatory reactivity to TLR-3 (poly I:C), TLR-4 (LPS) and TLR-7 (imiquimod) stimulation. Importantly, post-inflammation rAM also exhibited a switch from an IFN-β defective to an IFN-β competent phenotype, thus indicating the occurrence of a new, 'inflammatory-released' rAM-population in the post-allergic lung. Furthermore, the inflammation-induced rAM-turnover was critical for the development of the post-inflammation rAM-phenotype. In contrast, the main characteristics of the post-inflammation rAM-phenotype were not affected by the type of preceding allergic inflammation.

As rAM fulfill a pivotal role in the host's defence against invading respiratory viral pathogens, observed alterations in the post-inflammation rAM's functional status may have detrimental effects on the lung tissue integrity during subsequent respiratory syncytial virus (RSV) infections. Today, this respiratory pathogenic microorganism is considered as the principal cause of the most severe exacerbations in asthmatic individuals. Thus, as a final aim of the thesis, we determined the antiviral immune responses of post-inflammation rAM during subsequent RSV-infection, and addressed their potential contribution to the immunopathology exhibited by the post-inflammation lung during the infection. In contrast to naïve rAM, post-inflammation rAM exhibited strong pro-inflammatory reactivity during RSV-infection. Moreover, while naïve rAM showed a significant contribution to the clearance of the RSV-virus from the lung, post-inflammation rAM clearly displayed no direct antiviral activity and did not contribute to the clearance of the virus. Furthermore, in contrast to naïve rAM which were found to suppress morbidity during later stages of the RSV-infection, post-inflammation rAM did not affect this systemic immunopathology parameter. Finally, the initial encounter between post-inflammation rAM and RSV also had a differential outcome on the development of subsequent RSV-specific immune memory responses. The presence of post-inflammation rAM during primary RSV-encounter resulted in increased lung draining lymph node $CD8^+$ and $CD4^+$ memory T-cell responses during a secondary RSV-challenge. Thus, it is clear that the preceding allergic inflammation induced a shift in the functionality of rAM during a subsequent RSV-infection. This shift included the transition from an efficient antiviral and anti-inflammatory function of naïve rAM to a purely pro-inflammatory function of post-inflammation rAM.

Collectively, the functionality of rAM is imprinted by a preceding allergic bronchial inflammation and these alterations in (innate) rAM-functions can contribute to the onset of respiratory pathogen induced exacerbation reactions in allergic asthma patients.

SAMENVATTING

Residente alveolaire macrofagen (rAM) worden permanent blootgesteld aan een breed spectrum van ingeademde antigenen. Intrinsieke eigenschappen van deze celpopulatie zorgt ervoor dat de meerderheid van deze antigenen geklaard wordt zonder inductie van een pulmonair inflammatoir antwoord. Wanneer de activeringsdrempel van rAM overschreden wordt, is de inductie van een inflammatoire reactie in de longen onvermijdbaar. Overvloedige blootstelling aan ingeademd allergeen leidt in het geval van allergische astma tot de pro-inflammatoire activering van rAM. Verschillende cytokines en chemokines, gesecreteerd door geactiveerde rAM, geven vervolgens aanleiding tot de rekrutering van bloedmonocyten naar de longen. Deze gerekruteerde monocyt/macrofaagpopulaties zijn op hun beurt betrokken bij de verdere propagatie van de allergische inflammatie. Dus zowel rAM als gerekruteerde monocyten/macrofagen dragen bij tot verschillende stadia van een allergische bronchiale inflammatie. De dynamiek die beide macrofaagpopulaties vertonen gedurende de verschillende fasen van een allergische bronchiale inflammatie is echter nog steeds onvoldoende gekarakteriseerd. Het in kaart brengen van de rAM-dynamiek gedurende het verloop van een allergische bronchiale inflammatie was dan ook het initiële doel van deze thesis. Gebruik makend van een muismodel voor eosinofiele allergische astma, werd aangetoond dat deze rAM-populatie verdween uit de alveoli gedurende de acute fase van de allergische inflammatie. Bovendien bleek de geobserveerde verdwijning van deze cellen een ATP-afhankelijk proces te zijn. Deze resultaten leidden tot de conclusie dat, na de klaring van de allergische bronchiale inflammatie, een nieuwe populatie van post-inflammatie rAM zich in de alveoli bevonden.

Recent onderzoek toonde aan dat de functionaliteit van rAM kan beïnvloed worden door een voorafgaande bronchiale inflammatie. Daarom werden vervolgens de fenotypische en functionele karakteristieken van post-inflammatie rAM geanalyseerd en vergeleken met naïeve rAM. Ondanks het feit dat post-inflammatie en naïeve rAM een gelijkaardig oppervlaktemerker profiel deelden, vertoonden post-inflammatie rAM een sterk afwijkende innate functionaliteit. Enerzijds was de basale fagocytose capaciteit sterk gereduceerd in post-inflammatie rAM. Anderzijds vertoonde deze rAM-populatie een sterk toegenomen inflammatoire reactiviteit na TLR-3 (poly I:C), TLR-4 (LPS) en TLR-7 (imiquimod) stimulatie. Bovendien vertoonden post-inflammatie rAM een omschakeling van een IFN-β defectief naar een IFN-β competent fenotype. Verder bleek de inflammatie-geïnduceerde

verdwijning van rAM noodzakelijk te zijn voor de ontwikkeling van het post-inflammatie rAM fenotype. Het type allergische inflammatie had echter geen invloed op de karakteristieken van post-inflammatie rAM.

rAM vervullen een belangrijke functie in de afweer van de gastheer tegen binnendringende respiratoire virale pathogenen. Wijzigingen in de functionele status van post-inflammatie rAM zouden bijgevolg schadelijke gevolgen kunnen hebben voor de integriteit van het longweefsel wanneer er een longinfectie met het respiratory syncytial virus (RSV) optreedt. Daarom werden tenslotte de inflammatoire en antivirale eigenschappen van post-inflammatie rAM gedurende een RSV-infectie onderzocht. In tegenstelling tot naïeve rAM vertoonden post-inflammatie rAM tijdens de infectie een sterk pro-inflammatoir karakter. De efficiënte antivirale eigenschappen van naïeve rAM, die een significante bijdrage leverden tot de klaring van het virus, stonden eveneens in schril contrast met de afwezigheid van enige rechtstreekse antivirale activiteit in post-inflammatie rAM. Deze rAM-populatie droeg bijgevolg niet bij tot klaring van het virus uit longen. De suppressieve werking van naïeve rAM op de morbiditeit gedurende de latere fasen van de infectie was eveneens niet terug te vinden in post-inflammatie rAM. Tot slot leidde de initiële blootstelling van post-inflammatie rAM aan RSV tot de ontwikkeling van een gewijzigd RSV-specifiek immuun geheugenantwoord. De aanwezigheid van post-inflammatie rAM tijdens de primaire RSV-infectie resulteerde in een stijging in $CD8^+$ and $CD4^+$ geheugen T-celantwoorden tijdens een secundaire RSV-infectie. Het is dus duidelijk dat de voorafgaande allergische bronchiale inflammatie aanleiding gaf tot een wijziging in de antivirale eigenschappen van de aanwezige rAM-populatie. Deze wijziging omvatte de overgang van een naïef rAM-fenotype, gekenmerkt door efficiënte antivirale en anti-inflammatoire eigenschappen, naar een post-inflammatie rAM-fenotype, gekenmerkt door een puur pro-inflammatoire rol tijdens RSV-infecties.

Samenvattend kan dus gesteld worden dat de functionaliteit van rAM in sterke mate beïnvloed wordt door een voorafgaande allergische bronchiale inflammatie. Deze wijziging in (innate) rAM-functie kan bijgevolg bijdragen tot de opwekking van pathogeen-geïnduceerde exacerbatiereacties in astma patiënten.

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2007: Course on laboratory animal science I and II: FELASA C certificate for laboratory animal experiments, Ghent University, Belgium

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Publications in Scientific Journals:

Pieter Bogaert, Kurt G. Tournoy, **Thomas Naessens** and Johan Grooten

Where Asthma and Hypersensitivity Pneumonitis Meet and Differ: Noneosinophilic Severe Asthma

American Journal of Pathology (2009): 174: 3-13

Stefaan De Koker, Bruno G. De Geest, Satwinder K. Singh, Riet De Rycke, **Thomas Naessens**, Yvette Van Kooyk, Jo Demeester, Stefaan C. De Smedt and Johan Grooten

Polyelectrolyte Microcapsules as Antigen Delivery Vehicles To Dendritic Cells: Uptake, Processing, and Cross-presentation of Encapsulated Antigens

Angewandte Chemie (2009): 48: 8485-8489

Stefaan De Koker, **Thomas Naessens**, Bruno G. De Geest, Pieter Bogaert, Jo Demeester, Stefaan De Smedt and Johan Grooten

Biodegradable Polyelectrolyte Microcapsules: Antigen Delivery Tools with Th17 Skewing Activity after Pulmonary Delivery

Journal of Immunology (2010): 184: 203-211

Seppe Vander Beken*, Juma'a R. Al Dulayymi*, **Thomas Naessens**, Gani Koza, Max Maza-Iglesias, Richard Rowles, Cornelia Theunissen, Jelle De Medts, Ellen Lanckaker, Mark S. Baird and Johan Grooten

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Molecular structure of the *Mycobacterium tuberculosis* virulence factor, mycolic acid, determines the elicited inflammatory pattern

European Journal of Immunology (2011): 41: 450-460

Thomas Naessens*, Pieter Bogaert*, Stefaan De Koker, Benoit Hennuy, Jonathan Hacha, Muriel Smet, Didier Cataldo, Emmanuel Di Valentin, Jacques Piette, Kurt G. Tournoy and Johan Grooten

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Inflammatory signatures for eosinophilic vs. neutrophilic allergic pulmonary inflammation reveal critical regulatory checkpoints

American Journal of Physiology Lung Cellular and Molecular Physiology (2011): 300: 679-690

Thomas Naessens, Seppe Vander Beken, Pieter Bogaert, Nico Van Rooijen, Stefan Lienenklaus, Siegfried Weiss, Stefaan De Koker and Johan Grooten

Innate Imprinting of Murine Resident Alveolar Macrophages by Allergic Bronchial Inflammation Causes a Switch from Hypo- to Hyperinflammatory Reactivity

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97th Annual meeting of the American Association of Immunology

Baltimore, USA, May 2010

98th Annual meeting of the American Association of Immunology

San Francisco, USA, May 2011

'**Summer Frontiers' Symposium 2012: Training the innate immunity: immunological memory in innate host defense**

Nijmegen, The Netherlands, June 2012

APPENDICES

ADDITIONAL PUBLICATIONS

Where Asthma and Hypersensitivity Pneumonitis Meet and Differ: Noneosinophilic Severe Asthma

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Abstract

Asthma is a type-I allergic airway disease characterized by Th(2) cells and IgE. Episodes of bronchial inflammation, eosinophilic in nature and promoting bronchoconstriction, may become chronic and lead to persistent respiratory symptoms and irreversible structural airway changes. Representative mostly of mild to moderate asthma, this clinical definition fails to account for the atypical and often more severe phenotype found in a considerable proportion of asthmatics who have increased neutrophil cell counts in the airways as a distinguishing trait. Neutrophilic inflammation is a hallmark of another type of allergic airway pathology, hypersensitivity pneumonitis. Considered as an immune counterpart of asthma, hypersensitivity pneumonitis is a prototypical type-III allergic inflammatory reaction involving the alveoli and lung interstitium, steered by Th(1) cells and IgG and, in its chronic form, accompanied by fibrosis. Although pathologically very different and commonly approached as separate disorders, as discussed in this review, clinical studies as well as data from animal models reveal undeniable parallels between both airway diseases. Danger signaling elicited by the allergenic agent or by accompanying microbial patterns emerges as critical in enabling immune sensitization and in determining the type of sensitization and ensuing allergic disease. On this basis, we propose that asthma allergens cause severe noneosinophilic asthma because of sensitization in the presence of hypersensitivity pneumonitis-promoting danger signaling.

Published in *American Journal of Pathology* (2009): 174: 3-13

Polyelectrolyte Microcapsules as Antigen Delivery Vehicles To Dendritic Cells: Uptake, Processing, and Cross-presentation of Encapsulated Antigens

Stefaan De Koker[1], Bruno G. De Geest[2], Satwinder K. Singh[3], Riet De Rycke[1], **Thomas Naessens**[1], Yvette Van Kooyk[3], Jo Demeester[4], Stefaan C. De Smedt[4] and Johan Grooten[1]

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No Abstract available

Published in *Angewandte Chemie* (2009): 48: 8485-8489

Biodegradable Polyelectrolyte Microcapsules: Antigen Delivery Tools with Th17 Skewing Activity after Pulmonary Delivery

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Abstract

Because of their large surface area, the lungs appear an attractive route for noninvasive vaccine delivery, harboring the potential to induce local mucosal immune responses in addition to systemic immunity. To evoke adaptive immunity, Ags require the addition of adjuvants that not only enhance the strength of the immune response but also determine the type of response elicited. In this study, we evaluate the adjuvant characteristics of polyelectrolyte microcapsules (PEMs) consisting of the biopolymers dextran-sulfate and poly-L-arginine. PEMs form an entirely new class of microcapsules that are generated by the sequential adsorption of oppositely charged polymers (polyelectrolytes) onto a sacrificial colloidal template, which is subsequently dissolved leaving a hollow microcapsule surrounded by a thin shell. Following intratracheal instillation, PEMs were not only efficiently taken up by APCs but also enhanced their activation status. Pulmonary adaptive immune responses were characterized by the induction of a strongly Th17-polarized response. When compared with a mixture of soluble Ag with empty microcapsules, Ag encapsulation significantly enhanced the strength of this local mucosal response. Given their unique property to selectively generate Th17-polarized immune responses, PEMs may become of significant interest in the development of effective vaccines against fungal and bacterial species.

Published in *Journal of Immunology* (2010): 184: 203-211

Molecular structure of the *Mycobacterium tuberculosis* virulence factor, mycolic acid, determines the elicited inflammatory pattern

Seppe Vander Beken[1*], Juma'a R. Al Dulayymi[2*], **Thomas Naessens**[1], Gani Koza[2], Max Maza-Iglesias[2], Richard Rowles[2], Cornelia Theunissen[2], Jelle De Medts[1], Ellen Lanckaker[3], Mark S. Baird[2] and Johan Grooten[1]

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Abstract

Mycolic acids (MAs) occur in the cell wall of Mycobacterium tuberculosis as variable mixtures of different classes and chain lengths. Here, we address the relationship between the structure and its inflammatory function of this virulence factor using single synthetic MA isomers, differing in oxygenation class and cis- versus α-methyl-trans proximal cyclopropane orientation. Analysis of bronchoalveolar inflammation, lung histopathology and alveolar macrophage transcription revealed a strong dependence on these meromycolic chemistries of mouse pulmonary inflammation in response to intratracheal treatments with MAs. Whereas α-MA was inert, oxygenated methoxy- and keto-MA with cis-cyclopropane stereochemistry elicited solid to mild inflammatory responses respectively. In trans-cyclopropane orientation, methoxy-MA partially lost its inflammatory activity and keto-MA exerted anti-inflammatory alternative activation of alveolar macrophages and counteracted cis-methoxy-MA induced airway inflammation. The differential innate immune activities of MAs demonstrated here, dependent on oxygenation class and cis versus α-methyl-trans cyclopropane chemistry, identify a novel means for M. tuberculosis to steer host immune responses during infection.

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Inflammatory signatures for eosinophilic vs. neutrophilic allergic pulmonary inflammation reveal critical regulatory checkpoints

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Abstract

Resident alveolar macrophages (rAMs) residing in the bronchoalveolar lumen of the airways play an important role in limiting excessive inflammatory responses in the respiratory tract. High phagocytic activity along with hyporesponsiveness to inflammatory insults and lack of autonomous IFN-β production are crucial assets in this regulatory function. Using a mouse model of asthma, we analyzed the fate of rAMs both during and after allergic bronchial inflammation. Although nearly indistinguishable phenotypically from naïve rAMs, postinflammation rAMs exhibited a strongly reduced basal phagocytic capacity, accompanied by a markedly increased inflammatory reactivity to Toll-like receptors TLR-3 (poly I:C), TLR-4 [lipopolysaccharide (LPS)], and TLR-7 (imiquimod). Importantly, after inflammation, rAMs exhibited a switch from an IFN-β-defective to an IFN-β-competent phenotype, thus indicating the occurrence of a new, inflammatory-released rAM population in the postallergic lung. Analysis of rAM turnover revealed a rapid disappearance of naïve rAMs after the onset of inflammation. This inflammation-induced rAM turnover is critical for the development of the hyperinflammatory rAM phenotype observed after clearance of bronchial inflammation. These data document a novel mechanism of innate imprinting in which noninfectious bronchial inflammation causes alveolar macrophages to acquire a highly modified innate reactivity. The resulting increase in secretion of inflammatory mediators on TLR stimulation implies a role for this phenomenon of innate imprinting in the increased sensitivity of postallergic lungs to inflammatory insults.

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