Modulation of allergic airway inflammation by *Staphylococcus aureus* enterotoxin B in a mouse model

Dissertation

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Preamble

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Contents

List of publications

Jorde I., Hildebrand C. B., Kershaw O., Lücke E., Stegemann-Koniszewski S., Schreiber J. „Modulation of Allergic Sensitization and Allergic Inflammation by *Staphylococcus aureus* Enterotoxin B in an Ovalbumin Mouse Model “
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Microorganisms, 11/2020; 8/1878; doi:10.3390/microorganisms8121878
*contributed equally

List of Publications - others

Trübe P., Hertlein T., Mrochen D. M., Schulz D., **Jorde I.**, Krause B., Zeun J., Fischer S., Wolf S. A., Walther B., Semmler T., Bröker B. M., Ulrich R. G., Ohlsen K., Holtfreter S.
"Bringing together what belongs together: Optimizing murine infection models by using mouse-adapted *Staphylococcus aureus* strains"
International Journal of Medical Microbiology, 10/2018; 309(1): 26-38; doi: 10.1016/j.ijmm.2018.10.007

Hahnet A., Kny M., Pablo C., Todiras M., Willenbrock M., Schmidt S., Schmoeckel K., **Jorde I.**, Bröker B. M., Felix S. B., Scheidereit C., Weber-Carstens S., Butter C., Luft F. C., Fielitz J.
„Serum Amyloid A1 mediates myotube atrophy *via* Toll-like receptor"
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Frontiers in Immunology, 04/2021 15; 12: 642802; doi:10.3389/fimmu.2021.642802

Jorde I.*, Stegemann-Koniszewski S.*, Papra K., Föllner S., Lux A., Schreiber J., Lücke E.
„Association of serum vitamin D levels with disease severity, systemic inflammation, prior lung function loss and exacerbations in a cohort of patients with chronic obstructive pulmonary disease (COPD)"
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*contributed equally

Conference contributions

Ilka Jorde, Christina Hildebrand, Jens Schreiber, Sabine Stegemann-Koniszewski. „The impact of nasal carriage with *Staphylococcus aureus* and its enterotoxin B on allergic asthma." Poster presentation by S. Stegemann-Koniszewski. 49th Annual Meeting German Society for Immunology (DGfI), München, 2019

Ilka Jorde, Christina Hildebrand, Jens Schreiber, Sabine Stegemann-Koniszewski. „Intranasal *Staphylococcal* Enterotoxin B acts immune modulatory and affects the phenotype of allergic asthma in a mouse model." Poster presentation. 19th Fraunhofer-Seminar Models of lung disease, 2020

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Sabine Stegemann-Koniszewski, **Ilka Jorde**, Christina Hildebrand, Olivia Kershaw, Eva Lücke, Jens Schreiber. „Intranasal *Staphylococcus aureus* enterotoxin B modulates allergic airway inflammation in a dose- and time-dependent manner in a mouse model" Oral presentation, presented by Sabine Stegemann-Koniszewski. 33. Mainzer Allergie Workshop Frühjahrstagung der DGAKI. Online Veranstaltung, 2021

Supervision and contribution of student projects

Master theses

Christina Hildebrand

“Auswirkung von *Staphylococcus aureus* Enterotoxin B auf die Entstehung und Ausprägung von allergischem Asthma im Mausmodell“ Masterstudiengang Immunologie, Otto-von-Guericke-Universität Magdeburg, 2019

Parts of the data published in this thesis are shown in **section 4.2**

Irina Han

„Etablierung eines *in vitro* Systems zur Analyse der spezifischen Immunantwort gegen das Modellantigen Ovalbumin und Analyse des Einflusses von intranasal verabreichtem *Staphylococcus aureus* Enterotoxin B auf die allergische Sensibilisierung gegen Ovalbumin im Mausmodell“ Masterstudiengang Immunologie, Otto-von-Guericke-Universität Magdeburg, 2020

Parts of the data published in this thesis are shown in **section 4.3**

Abstract

Approximately 60 % of adult asthmatics and the majority of asthmatic children suffer from allergic asthma. Allergic asthma is typically characterized by a T_h2-dominated immune response towards an aeroallergen. Over the last decade, nasal colonization with *S. aureus* has been recognized as a risk factor for allergic asthma. In this context, amongst others, especially staphylococcal enterotoxin B (SEB) came into focus. SEB is usually associated with a pro inflammatory type 1 immune response. However, recently it was shown that the production of SEB is associated with several atopic diseases, including allergic asthma. The underlying mechanisms remain elusive.

In this thesis, in order to comprehensively investigate the effects of intranasal (*i.n.*) SEB on allergic airway inflammation (AAI), a suited ovalbumin (OVA)-mediated mouse model of AAI was established. The first aim was then to examine the influence of *i.n.* SEB on AAI in a time and dose-dependent manner in this model. For this,

i) 50 ng or 500 ng SEB were *i.n* .administered during the allergic OVA-challenge

ii) 50 ng or 500 ng SEB were *i.n.* administered prior to allergic sensitization. Treatment effects on AAI were analyzed with respect to hallmark features such as IgE production, respiratory cells and cytokines. The *i.n.* administration of 50 ng SEB during the allergic challenge significantly increased absolute numbers of dendritic cells, M2-monocytes/macrophages, T cells and activated B cells in the lung as compared to AAI alone. Strikingly, the administration of 500 ng SEB during the allergic challenge ameliorated allergic features by significantly decreasing airway hyperreactivity and reducing eosinophil counts in the lung, while significantly increasing airway concentrations of pro-inflammatory cytokines (IFN-γ, IL-6, TNF-α). Surprisingly, *i.n.* administration of 50 ng SEB prior to allergic sensitization increased OVA-specific IgE-production, while administration of 500 ng SEB before allergic sensitization ameliorated AAI by significantly decreasing eosinophil counts in the lung and bronchoalveolar lavage (BAL), as well as significantly decreasing concentrations of IL-4, IL-5 and IL-6 in the BAL.

Based on these results, the aim of the second part of this thesis was to elucidate the kinetics and long-term effects of *i.n.* administration of 50 ng or 500 ng SEB on cellularity, lymphocyte activation and cytokine production in the respiratory tract. Administration of 50 ng SEB resulted in significantly increased absolute numbers of granulocytes and activated lymphocytes up to seven days after treatment. Concentrations of pro-inflammatory and type 2 cytokines were significantly increased after seven days in the BAL. The *i.n.* administration of 500 ng SEB showed similar effects. However, total cell numbers, as well as absolute numbers of eosinophils and IL-13 concentrations were significantly increased in the BAL up to 14 days after the last *i.n.* SEB treatment.

Taken together, these results indicate a strong and exceptionally versatile potential of SEB to modulate AAI in a time and dose-dependent manner. Furthermore, *i.n.* administration of SEB alone showed long-lasting effects on the respiratory immune milieu. These data display a basis for future research towards SEB-targeted strategies of prevention and therapy, especially in the light of the large number of *S. aureus* carriers and patients suffering from allergic asthma in the human population.

Zusammenfassung

Allergisches Asthma betrifft ca. 60 % der erwachsenen Asthmatiker und die Mehrheit der an Asthma erkrankten Kinder. Es ist charakterisiert durch eine Th2-dominierte Immunantwort gegen ein Aeroallergen und auch die nasale Besiedelung mit *S. aureus* wird heute zu den Risikofaktoren gezählt. Unter anderem das *S. aureus*-Protein *Staphylococcus* Enterotoxin B (SEB) rückte diesbezüglich in den Fokus. SEB wird typischerweise mit einer proinflammatorischen Typ-1-Immunantwort assoziiert. Allerdings wird die Produktion von SEB mit atopischen Erkrankungen, auch dem allergischen Asthma, in Verbindung gebracht. Die zugrundeliegenden Mechanismen sind bisher nicht bekannt.

In dieser Arbeit wurde zunächst, um Auswirkungen von intranasal (*i.n.*)-verabreichtem SEB auf eine allergische Atemwegsentzündung (*allergic airway inflammation*, AAI) zu untersuchen, ein geeignetes Ovalbumin (OVA)-vermitteltes Mausmodell für AAI etabliert. Erstes Ziel dieser Arbeit war es dann, den zeit- und dosisabhängigen Einfluss von SEB in diesem Modell zu untersuchen. Hierzu wurden

i) 50 ng oder 500 ng SEB während der allergischen OVA-Provokation *i.n.* verabreicht

ii) 50 ng oder 500 ng SEB vor der Sensibilisierung *i.n.* verabreicht. SEB-vermittelte Effekte wurden anhand verschiedener Parameter wie IgE-Produktion, Zellrekrutierung und Zytokinen untersucht.

Verglichen mit AAI erhöhte die Verabreichung von 50 ng SEB während der Provokation die absolute Zellzahl von dendritischen Zellen, M2-Monozyten/Makrophagen, T-Zellen und aktivierten B-Zellen in der Lunge signifikant. Die Verabreichung von 500 ng SEB während der allergischen Provokation hingegen schwächte verschiedene Charakteristika der AAI signifikant ab (Hyperreaktivität der Atemwege, Eosinophilenzahl), während die Konzentrationen von proinflammatorischen Zytokinen (IFN-γ, IL-6, TNF-α) signifikant erhöht waren. Unerwarteterweise erhöhte die *i.n.* Gabe von 50 ng SEB vor der Sensibilisierung die OVA-spezifische IgE-Produktion, während die Verabreichung von 500 ng SEB vor der Sensibilisierung die Eosinophilenzahl in Lunge und bronchoalveolärer Lavage (BAL) sowie die Konzentrationen von IL-4, -5 und -6 in der BAL signifikant verringerte.

Basierend auf diesen Ergebnissen war das Ziel des zweiten Teils dieser Arbeit, die Kinetik und langfristige Auswirkungen der *i.n.* Gabe von 50 ng oder 500 ng SEB auf Zellrekrutierung, -aktivierung und Zytokinproduktion in den Atemwegen zu untersuchen. Die Gabe von 50 ng SEB führte bis 7 Tage nach der letzten Behandlung zu signifikant erhöhten Zahlen von Granulozyten und aktivierten Lymphozyten in der Lunge. Auch die Konzentrationen von proinflammatorischen Zytokinen und Typ-2-Zytokinen waren nach 7 Tagen signifikant erhöht. Die Gabe von 500 ng SEB zeigte ähnliche Effekte. Allerdings waren die Gesamtzellzahl, die Eosinophilenzahl und die IL-13-Konzentration in der BAL bis zu 14 Tage nach der SEB-Behandlung signifikant erhöht.

Die Ergebnisse dieser Arbeit zeigen ein außergewöhnlich vielseitiges Potenzial für SEB, das respiratorische Immunmilieu langanhaltend zu beeinflussen sowie AAI zeit- und dosisabhängig zu modulieren. Diese Daten können eine Grundlage für zukünftige Untersuchungen bezüglich gezielter Strategien der Prävention und Therapie des allergischen Asthmas bieten, insbesondere angesichts der hohen *S. aureus* Besiedlungsrate und der großen Zahl an Patienten mit allergischem Asthma.

List of Abbreviations

%	Percent
/°	Per well
× g	Multiple of the gravitational acceleration
°	Well
°C	Degree Celsius
µg	Micrograms
µl	Microliter
µm	Micrometer
ACK	Ammonium chloride potassium
AD	Atopic dermatitis
AF700	Alexa Fluor™ 700
Ag	Antigen
alum	Aluminum hydroxide
a.o.	Alum only
APC	Antigen-presenting cell
APC	Allophycocyanin
APC/Cy7	Allophycocyanin-cyanine 7
Avidin-HRP	Avidin-Horseradish peroxidase
BAL	Bronchoalveolar lavage
BV421/510/711	Brilliant Violet 421/510/711
c	Concentration
CD	Cluster of differentiation
cm H_2O	Centimeter of water column
CRS	Chronic Rhinosinusitis
d	Day(s)
d	Density
DC	Dendritic cell
DNAse	Deoxyribonuclease
e.g.	*exempli gratia* (for example)

List of Abbreviations

EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
et al.	*et alii* (and others)
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FCS	Forward scatter
FcεRI	Fc epsilon receptor I
fig.	Figure
FMO	Fluorescence minus one
h	Hour(s)
H&E	Hematoxylin and eosin stain
HRP	Horseradish peroxidase
i.n.	Intranasal/intranasally
i.p.	Intraperitoneal/intraperitoneally
i.t.	Intratracheal/intratracheally
IFN	Interferon
Ig	Immunoglobulin
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IL	Interleukin
IMDM	Iscove's modified dulbecco's medium
IU	International unit
IVC	Individually ventilated cage
kDa	Kilodalton
kg	Kilograms
$KHCO_3$	Potassium hydrogen carbonate
LPS	Lipopolysaccharide
M	Molar
MCh	Methacholine
MFI	Mean fluorescence intensity
mg	Milligrams
MHC	Major histocompatibility complex

List of Abbreviations

MilliQ water	Ultrapure water
min	Minute(s)
ml	Milliliter
mM	Millimolar
n	Sample size
Na_2EDTA	Ethylenediamine tetra acetic acid disodium salt
NaCl	Sodium chloride
NH_4CL	Ammonium chloride
ns	non-significant
O_2	Oxygen
OVA	Ovalbumin
p	Probability
PBS	Phosphate-buffered saline
PE	Phycoerythrin
PE/Cy7	Phycoerythrin/Cyanin 7
PerCP/Cy5.5	Peridinin-chlorophyll proteins cyanine 5.5
PFA	Paraformaldehyde
pg	Picogram
pH	Hydrogen ion exponent
PMA	Phorbol 12-myristate 13 acetate
rpm	Rounds per minute
RT	Room temperature
S. aureus	*Staphylococcus aureus*
Sag	Superantigen
SA-PE	Streptavidin-phycoerythrin
SEB	Staphylococcal enterotoxin B
SSC	Side scatter
TCR	T cell receptor
TGF-β	Transforming growth factor β
Th cell	T helper cell
Th1 cell	T helper cell 1
Th17 cell	T helper cell 17

List of Abbreviations

Th2 cell	T helper cell 2
TMB	3,3', 5,5'-Tetramethylbenzidine
TSLP	Thymic stromal lymphopoietin
UV	Ultraviolet
β-Me	2-Mercaptothanol

List of figures

List of tables

1 Introduction

1.1 Bronchial asthma

Bronchial asthma is a chronic inflammatory condition of the airways that affects more than 300 million patients worldwide (Lambrecht & Hammad, 2015). Main characteristics are airway hyperreactivity, increased mucus production as well as structural changes of the airways (Global Initiative for Asthma, GINA; 2020) (see figure 1, p. 2). Airway hyperreactivity displays a characteristic functional abnormality of asthma that is partly reversible with therapy. In patients with severe asthma, a progressive, persistent airflow limitation can develop (Ying *et al.*, 1995; Sumi *et al.*, 2007). The amplified mucus production occurs due to an increase in the number of goblet cells and the size of submucosal glands in the airway epithelium and can display another cause for airflow obstruction (Kuperman *et al.*, 2002). Structural changes include epithelial detachment, which is, among other findings, characterized by the fact that bronchial epithelial cells in patients with asthma experience more apoptosis in comparison to epithelial cells in control persons (Buchhieri *et al.*, 2002; Sumi *et al.*, 2007). Subepithelial fibrosis and an increase in airway smooth muscle mass, which is in part comprised by hyperplasia, are also common observations in asthmatic patients (Sumi *et al.*, 2007; Bentley *et al.*, 2008). During an asthmatic attack or exacerbation, severe shortness of breath, chest tightness and coughing and wheezing can occur due to airflow limitations (Soroksky *et al.*, 2003) (see figure 1, p. 2).

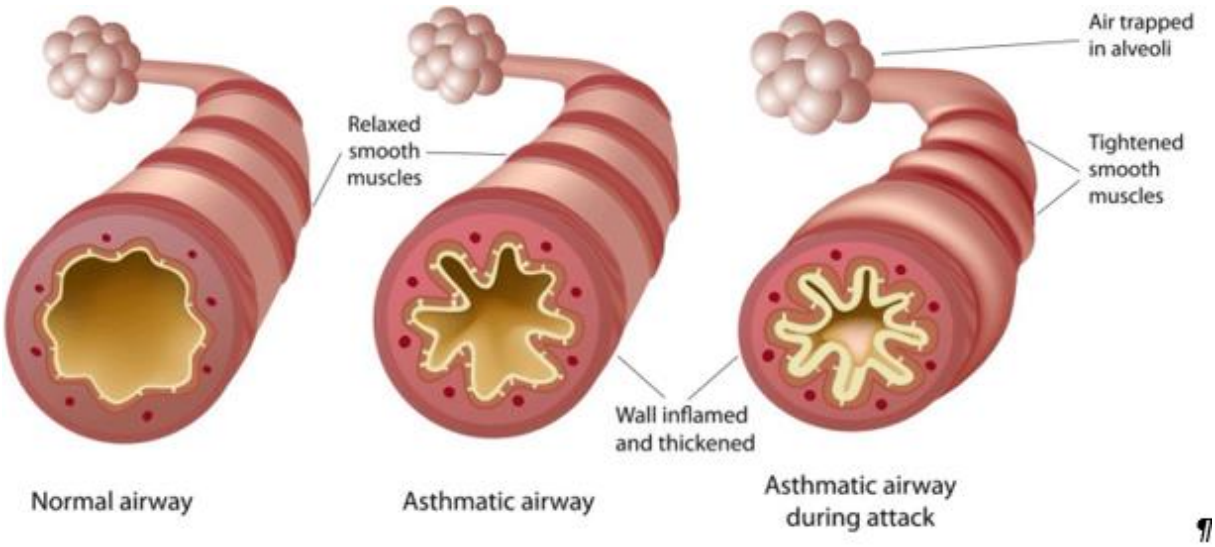


figure 1: Schematic representation of a normal airway, an asthmatic airway and an asthmatic airway during attack. (taken from De la Plaza *et al.*, 2017)

Historically, bronchial asthma was discriminated into two subtypes: The non-allergic and the allergic type. About 10-33 % of asthmatic patients suffer from nonallergic or intrinsic asthma. This form of asthma can be diagnosed by a lack of specific IgE or a negative skin prick test to a variety of seasonal and perennial allergens. Intrinsic asthma is characterized by a female predominance, a more rapid and severe progression - in comparison to allergic asthma - and a later onset in life (Romanet-Manet *et al.*, 2003; Peters 2014). In the some patients suffering from intrinsic asthma a prevalence of neutrophils in the respiratory tract is observed (Baos *et al.*, 2019; Peters *et al.*, 2014; Raedler *et al.*, 2015). Until today, there is no directed therapy to be effective against this endotype (Baos *et al.*, 2019; Muraro *et al.*, 2016).

The majority of children and about 60 % of adult patients suffer from allergic asthma (Vogelmeier *et al.*, 2017). Allergic airway reactions are typically characterized by a T helper type 2 (Th2) cell dominated immune response towards aeroallergens. These responses include the production of allergen-specific IgE antibodies, the release of Th2-inflammatory mediators such as interleukin-4 (IL-4), IL-5 and IL-13 as well as the recruitment and activation of mast cells, eosinophils and basophils (Foster *et al.*, 2017; Caminati *et al.*, 2018).

Nowadays it is known that asthma must be divided into far more than two subtypes: there is e.g. type 2-high or ultra-high and type 2-low asthma, which can again be subdivided into different phenotypes, depending on e.g. inflammatory mediators or the age of the onset of the disease.

While type 2-high asthma is characterized by Th2-associated cytokines, like IL-4, IL-5 and IL-13 and type 2-ultra high asthma reflects a more severe form of the disease, the definition of the type 2-low endotypes is more complex, since no biomarkers have been identified so far. Underlying pathophysiological mechanisms are used to define the different endotypes, leading to direct differences in responsiveness to common therapies. Consequently, all asthmatic patients with no type 2-high inflammation are included in this type-2 low endotype (Hammad & Lambrecht, 2021; Fahy, 2015; Lötvall *et al.*, 2015).

The current understandings of type 2-high and -low asthma are summarized in the following tables: (tables 1 and 2, modified from: Hammad & Lambrecht, 2021).

table 1: The current understanding of type 2-high asthma (modified from Hammad & Lambrecht, 2021)

	Endotype	Severity	Mediators	Further characteristics
Type 2-high asthma	early onset allergic asthma	mild	IL-4, Il-13, IgE	
	early onset allergic asthma	moderate to severe	IL-4, IL-5, Il-13	
	late onset allergic asthma	moderate to severe	IL-4, IL-5, Il-13	nasal polyposis
	complex T2 (ultra) high asthma	very severe	IL-4, IL-5, Il-13, IFN-γ	signs of ultra-high T2-genes T cell dominant disease

table 2: The current understanding of type 2-low asthma (modified from Hammad & Lambrecht, 2021)

	Endotype	Risk factors	Obstruction?	Mediators
Type 2-low asthma	(often) stable, mild disease		intermittent obstruction	
	Late onset airway disease	obesity	minimal obstruction	IL-1β, IL-6
	Early onset disease	high dose corticosteroids	moderate obstruction	
	Late onset disease	Infection/smoking	moderate obstruction	IL-1β, neutrophils

1.1.1 Pathophysiology of allergic asthma

While Th1 cells play an important role in the protection against intracellular pathogens and are responsible for the induction of phagocytosis by e.g. macrophages or dendritic cells (DCs). Additionally, due to their specific cytokine production B cells are activated and produce complement-fixing and opsonizing antibodies. Th2 cells ensure the protection against helminths and promote acute and chronic inflammatory responses against allergens. The clearance of extracellular pathogens and fungi is the main role of Th17 cells (Basu *et al*., 2021). During the allergic immune response in allergic asthma, this balance is shifted towards a type 2 or Th2-dominated response. Due to the large amount of type 2-specific cytokines, Th1 and Th17 cells are suppressed, resulting in a potentiating type 2 immune response which leads to an allergic inflammation in which a large number of immunological processes take place at the same time (Deo *et al.*, 2010). In these, structural cells, cells of the innate and the adaptive immune system as well as a variety of humoral factors are involved (summarized in figure 2, p. 7). It is in many points unclear, what is responsible for the shift towards Th2-responses in atopic individuals.

1.1.1.1 Airway epithelium

According to the American Academy of Allergy, Asthma & Immunology (AAAAI) “an allergy is a chronic condition involving an abnormal reaction to an ordinarily harmless substance called an allergen. Allergens can include aeroallergens such as dust mite, mold and tree weed and grass pollen, as well as food allergens such as milk, egg, soy, wheat, nut or fish proteins” (AAAAI, 2021).

In type 2-high (and ultra-high) asthma, different mediators and cells types are of crucial importance. Airway epithelial cells form the first barrier for inhaled allergens and pathogens. It has been shown that allergens such as house dust mite (HDM) or cockroach antigens can lead to a loss of cell-cell contact due to their enzymatic activities (Papi *et al.*, 2018). Due to the fact that epithelial cells express a myriad of pattern recognition receptors of the innate immune system, like Toll like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors, retinoic acid-inducible gene-(RIG)-I-like receptors, C-type lectin receptors (CLRs) and others, they are able to respond to a variety of external triggers by producing cytokines and chemokines (Hammad & Lambrecht, 2021). In mice, allergen

exposure is able to trigger the production of epithelial derived cytokines, like IL-1α, transforming growth factor β (TGF-β) or granulocyte-macrophage colony-stimulating factor (GM-CFS) (Willart *et al.*, 2012; Denney *et al.*, 2015). The most extensively studied cytokines in this context are IL-33, thymic stromal lymphopoietin (TSLP) and IL-25, which contribute to type 2-high asthma (Hammad *et al.*, 2009; Cayrol *et al.*, 2018). In patients with allergic eosinophilic asthma increased serum levels of IL-33 could be demonstrated compared to non-eosinophilic phenotypes (Gasiuniene *et al*., 2019) and higher levels of IL-25 are associated with a greater airway hyperresponsiveness, increased airway and blood eosinophils as well as higher levels of serum IgE (Cheng *et al.*, 2014).

1.1.1.2 Effector cells and soluble mediators of allergic asthma

After lung epithelial cells, DCs are next in line in allergen-directed immune responses of allergic asthma. During the allergic sensitization in the context of allergic asthma, inhaled allergens are taken up and processed by antigen presenting cells (APCs), in particular DCs. These cells are essential for the sensitization and the induction of allergic airway inflammation (AAI) by migrating to the draining lymph nodes, where the processed allergen is presented to antigen-specific B and T cells in the form of peptide antigens (Holt 2000; Boemen *et al.*, 2007). Most conventional DCs found in the lungs of asthmatic patients express the high-affinity IgE receptor FcεRI, which indicates a role for IgE and DCs in Th2 airway inflammation (Dutertre *et al.*, 2019; Naessens *et al.*, 2020). It has also been shown that DCs furthermore contribute to the allergic reaction in sensitized individuals by amplifying the type 2 T helper cell (Th2 cell) response (Hirose *et al.*, 2017; Plantinga *et al.*, 2013; van Rijt *et al.* 2005).

After antigen-presentation to T and B cells in the local draining lymph nodes, the resulting interaction between APC (i.e. DCs) and lymphocyte induces specific responses. These are influenced and characterized by the cytokine milieu as well as the presence or absence of specific co-stimulatory molecules. Due to peptide-presentation *via* major histocompatibility complex-II (MHC II) molecules on the surface of the DCs and under the influence of epithelial-derived cytokines, allergen-specific Th2 cells are activated (Agrawal *et al.*, 2010). The activation leads to a massive clonal expansion of Th2 cells, which are crucial for the induction of asthmatic symptoms. In an ovalbumin (OVA)-induced mouse model for AAI it has been shown that the depletion of $CD4^+$ T helper cells prevented asthma development, while the

adoptive transfer of *in vitro*-polarized Th2 cells from mice with transgenic expression of an OVA peptide-specific T cell antigen receptor lead to the induction of features of AAI and thus, to a strong production of specific type 2 cytokines, like IL-4, IL-5 and IL-13 (Cohn *et al.*, 1997). IL-4 and IL-13 promote the class switch of activated B cells to IgE-producing plasma cells (Finkelman *et al.*, 1988; Swain *et al.*, 1990; Foster *et al.* 2017). Once synthesized, IgE antibodies circulate in the blood and eventually bind to the high-affinity IgE receptor FcεRI, which is expressed on the surface of, amongst other cells, mast cells. These display another key effector cell type in the pathophysiology of allergic asthma (Reuter *et al.*, 2010). Once the antigen-specific IgE molecules on the surface of mast cells are cross linked due to antigen or allergen binding, mast cells will be activated, degranulate and thereby release a variety of different mediators such as histamine, leukotrienes, proteases and prostaglandins (Bradding *et al.*, 2006; Reuter *et al.*, 2010). Increased concentrations of these mediators lead to vasodilation, contraction of smooth muscle cells and mucus secretion and promote inflammation (Roquet *et al.*, 1997; O'Byrne, 2000). Next to B cell class switch, there are additional effects of increased IL-4 production, such as promoting Th2 cell polarization, mediating the expression of vascular cell adhesion molecule-1 (VCAM-1) and promoting of mucus production as well as the transmigration of eosinophils across the endothelium (Steinke & Borish, 2001; Henderson *et al*., 2000). Also, IL-13 has a broad spectrum of action in asthma. Amongst other effects, IL-13 promotes the migration of eosinophils into the lung by increasing the synthesis of eotaxin, induces goblet cell hyperplasia and thereby increased mucus production, leads to the proliferation of smooth muscle cells and stimulates airway hyperresponsiveness (Corren, 2013; Horie *et al*., 1997; Kondo *et al.*, 2006; Bossé *et al.*, 2008; Chiba *et al.*, 2009).

IL-5 supports the recruitment and survival of one of the most important key effector cell types in AAI: eosinophilic granulocytes or eosinophils (Matucci *et al.*, 2018). Eosinophils are derived from the bone marrow and circulate in the bloodstream. Direct correlations between eosinophil counts in the respiratory tract and asthma exacerbations have been demonstrated (Bousquet *et al.*, 1990; Garcia *et al.*, 2013). In addition to IL-5, other cytokines affect eosinophils. IL-3 as well as GM-CSF activate and enhance eosinophil functions, such as cytotoxic killing, superoxide and leukotriene production (Nagat *et al.*, 1999; Matucci *et al.*, 2018). Eosinophils only release their granules with cytotoxic, immune-modulating and remodeling-promoting properties when they reach a target organ, in case of bronchial asthma

the lung, where they contribute to inflammation (Malm-Erjefält *et al.,* 2005). The released leukotrienes e.g. are potent bronchoconstrictors (Lemanske & Busse; 2010). Furthermore, eosinophils regulate the immune response through direct effects on T cell functions, e.g. they influence both Th1 and Th2 cytokine production (Possa *et al.*, 2013; Jacobsen *et al.*, 2012; Esnault *et al.*, 2012). In humans it also has been confirmed that eosinophils are able to present antigens to T cells (Farhan *et al.*, 2016).

In conclusion, it can be summarized that the inflammatory pathways and mechanisms in patients with allergic asthma are very heterogeneous, which leads to varying symptoms, different severity of the disease and a high demand for individual treatment regimen.

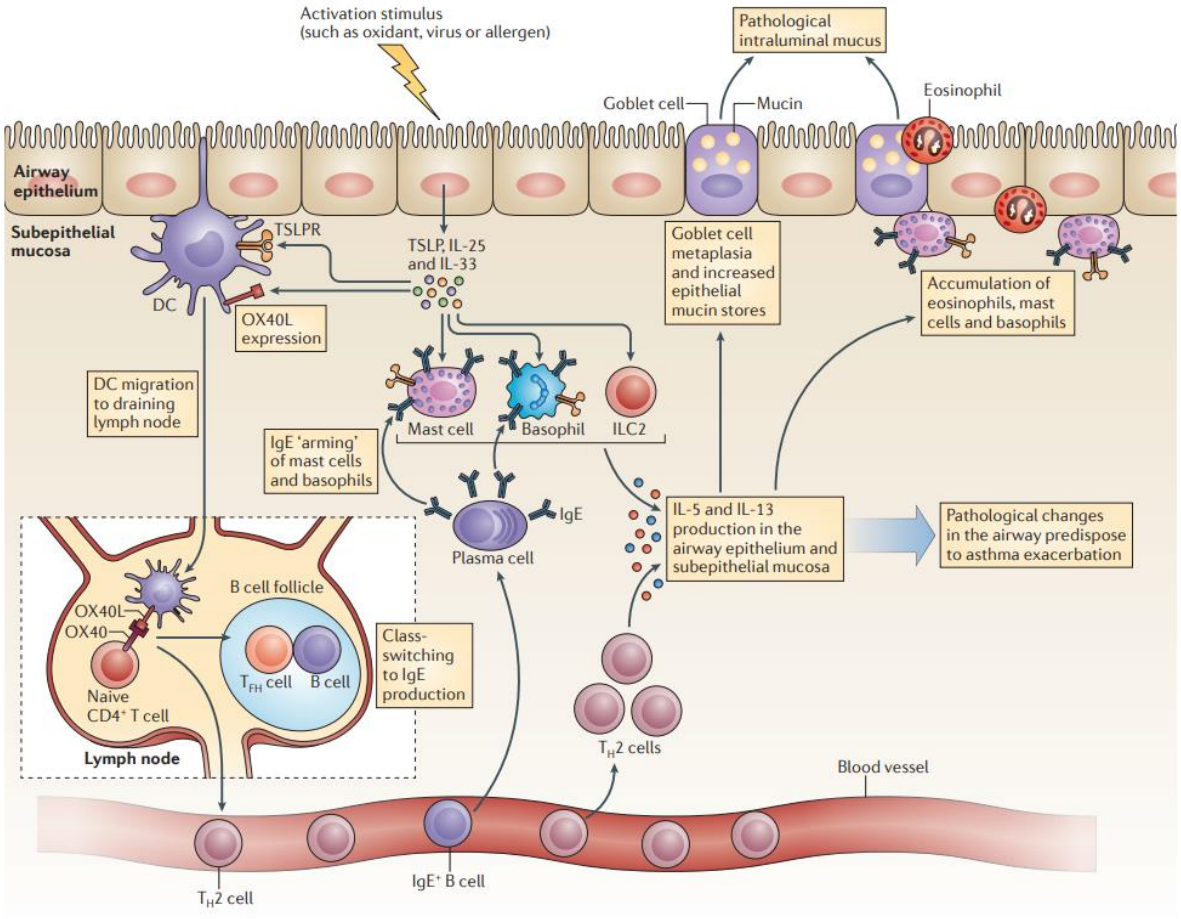


figure 2: Type 2 immune response in asthma. (taken from Fahy, 2015)
After an activating stimulus like an allergen, the epithelium releases different cytokines like IL-33 or TSLP, which induce the expression of OX40L on DCs to promote their mobilization to local draining lymph nodes. In the draining lymph nodes, the activation of naïve $CD4^+$ T cells takes place, which then migrate to the B cell zones and differentiate to T follicular helper cells (T_{FH} cells), move into the circulation and complete their maturation to T helper cells type 2 (Th2 cells). The produced IL-4 from T_{FH} cells mediate an IgE class-switch in B cells, while the produced IL-5 and IL-13 from Th2 cells mediate remodeling and inflammatory changes in the airway mucosa.

1.1.1.3 Airway hyperresponsiveness

Next to the cellular and humoral Th2 immune response in allergic asthma, the impairment of lung functional parameters is one of the most important symptoms and also an important diagnostic parameter of this disease.

Airway hyperresponsiveness (AHR) is a defining and consistent feature of bronchial asthma (Cockcroft *et al.*, 2006). Already over three decades ago, it has been shown that there are two semi-independent components to AHR:

i) Persistent AHR is present in the majority of asthma patients. This component likely relates to physiological and structural changes of the airways, also known as airway remodeling, and reflects chronicity of the disease.

ii) Variable or episodic AHR is inducible by certain exposures or triggers and improved by others (Dolovich *et al.*, 1981; Hargreave *et al.*, 1986). Different studies show that the variable component of AHR is associated with asthma activity and severity and at the same time reflects airway inflammation. To this day however, the mechanisms underlying variable AHR remain largely elusive (Cockcroft *et al.*, 1992; Cockcroft *et al.*, 2006).

One of several parameters for the diagnosis of asthma is the measurement of AHR towards unspecific triggers with a e.g. methacholine provocation test. After the acquisition of the baseline lung function, patients are challenged with increasing doses of nebulized methacholine *via* inhalation until the forced expiratory pressure in one second (FEV_1) falls by more than 20 % of its baseline value (Kraemer *et al.*, 2016; Seo *et al.*, 2018). The FEV_1 is defined as “the volume of air that an individual can exhale during a forced breath in one second” (Ponce & Sharma, 2020). There are some factors which affect the average FEV_1, like age, gender, height, body weight as well as ethnicity (Zakaria *et al.*, 2019). If the FEV_1 value is greater than 80 % of the predicted average value it is considered as normal (Shapira *et al.*, 2019).

If it is already known, which specific allergen could trigger the allergic asthma symptomatology, e.g. through a pervious skin prick test or the analysis of serum-specific IgE levels, also an allergen provocation test can be performed using this specific allergen. (Agache *et al*., 2015).

1.1.1.4 Current therapies for allergic asthma

Carrying out effective therapies for asthma is accompanied by a number of difficulties, including the disease spectrum and the complexity the molecular mechanisms. Medications currently used for the treatment of allergic asthma relieve symptoms only temporarily by bronchodilatation or suppressing inflammation. E.g. leukotriene receptor antagonists, antihistamines and glucocorticoids are used for this purpose (Bousquet *et al.*, 2009; Akdis, 2012).

One of the most recent approaches of treating chronic inflammation in allergic asthma is targeting the cytokine network coordinating the inflammation. To date, several cytokine- and immunoglobulin-specific therapies are already approved (Kusumoto & Mathis, 2021). The monoclonal antibody omalizumab neutralizes IgE and downregulates the IgE receptor on basophils. By the downregulation, the sensitization of the immune system, the chronic activation of Th2 responses is prevented (Godse *et al.*, 2015; Froidure *et al.*, 2016). It has been shown that omalizumab is an effective treatment in patients with moderate to severe, poorly controlled asthma or allergic rhinitis (Lin *et al.*, 2004; Busse *et al.*, 2011). Another monoclonal antibody targeting not IgE, but IL-5 is mepolizumab. By neutralizing the binding capacity to IL-5 receptor alpha, eosinophil maturation is disabled in the bone marrow resulting in decreased eosinophil levels in blood and bronchial mucus (Menzella *et al.*, 2016; Faverio *et al.*, 2018). The IL-5 receptor alpha is also targeted by the monoclonal antibody reslizumab (Nixon *et al.*, 2017). Unlike mepolizumab and reslizumab, benralizumab is able to deplete eosinophils through antibody-dependent cell-mediated cytotoxicity (Menzella *et al.*, 2016). Another ability of benralizumab is to bind to natural killer cells, macrophages and neutrophils with its heavy chain Fcγ-receptor (González *et al.*, 2019). Targeting the IL-4/IL-13 co-receptor, dupilumab prevents the signal transduction *via* IL-4R and IL-13. Through inhibiting key inflammatory pathways, the IgM to IgE class switch, triggered by IL-4, as well as increased mucus production (triggered by IL-13) mediating airway constriction is reduced (Barranco *et al.*, 2017; Matsunaga *et al.*, 2020).

Through the use of allergen-specific immunotherapy (SIT) however, a long-term improvement as well as reduction of the risk of progression for allergic diseases can be achieved. In SIT, immune tolerance to allergens is induced through multiple cellular and molecular mechanisms by repeatedly administering, increasing doses of the causative allergens (Akdis & Akdis, 2009; Durham *et al.*, 2010). One possible effect of SIT is the rapid desensitization of mast cells and

basophils. Although a high presence of allergen-specific IgE in serum of patients was detected, a clear decrease in the susceptibility of mast cells and basophils has been observed (Romano *et al.*, 2011). Another effect of SIT is the induction of peripheral regulatory T cells (T_{reg} cells). During SIT, peripheral tolerance is induced by the cytokines IL-10 and TGF-β (Jutel *et al*., 2003). It has been demonstrated that SIT leads to increased numbers of T_{reg} cells in the nasal mucosa (Radulovic *et al.*, 2008). An increase in T_{reg} cell numbers leads to increased concentrations of IL-10, which suppresses IL-5 production. This results in the suppression of mast cell, basophil and eosinophil function and activity (Marshall *et al.*, 1996).

In summary, there are therapeutic approaches which focus on reducing or preventing the allergic inflammation. For a specific and effective treatment of allergic asthma and the associated chronic inflammation, there is a high need for a detailed knowledge of the inflammatory phenotype and ideally the underlying pathophysiology at play in individual patients. In addition to these therapeutic examples, current research is focusing on relevant factors favoring the development of allergic asthma, such as microbiome dysbiosis or the colonization with different pathogens like *S. aureus*, as such knowledge in the future ideally will allow targeted prophylactic measures.

1.1.1.5 Risk factors

As heterogeneous as the disease bronchial asthma itself, the triggers and risk factors for developing this disease are just as variable. The most important inhalation allergens that trigger IgE-mediated allergies include grass-, tree- and herb-pollen, as well as house dust mites, animal dander and mold spores (LGL, 2022).

Nowadays, multiple environmental and genetic risk factors that further promote the development of asthma are known which are addressed in the following section.

One commonly known environmental risk factor for developing asthma is tobacco. The association of active smoking and a higher risk of suffering from asthma has been demonstrated in a number of studies (Flodin *et al.*, 1995; Torén *et al.*, 1999; Piipari *et al.*, 2004). Additionally, it has been shown that the prenatal or postnatal exposure to passive smoking increases the risk of developing asthma significantly in children (Toskala & Kennedy, 2015; Burke *et al.*, 2012).

The increasing air pollution, especially in bigger cities is another significant risk factor for the development of asthma, as well as for triggering asthma symptoms. A substantial difference in asthma prevalence in morbidity among urban, compared to non-urban children has been documented (Togias *et al.*, 2010).

Another important environmental risk factor for the development of asthma are microbes. Bacterial and viral infections can be important factors in asthma pathogenesis. Commonly identified viruses which are causing respiratory tract infections are, amongst others, human rhinoviruses, the respiratory syncytial virus, influenza and parainfluenza virus as well as coronaviruses (Sly *et al*., 2008; Xepapadaki *et al*., 2012). The underlying mechanisms remain elusive, but several studies could demonstrate a significant association between early viral lower respiratory tract infections and the development of childhood asthma (Holt & Sly, 2012; Holtzman, 2012). In addition to viral infections, the colonization with bacteria like *Streptococcus pneumoniae, Haemophilus influenzae* or *Moraxella catarrhalis* in early life is associated with a persistent wheezing phenotype and asthma diagnosis (Anderson & Jackson, 2017). Over the last decades especially the colonization with *Staphylococcus aureus* (*S. aureus*) came into focus as a risk factor for the development of asthma, not only early in life but also in adulthood. Different studies show a significant association between *S. aureus* colonization and asthma prevalence (Kim *et al.*, 2019; Davis *et al*., 2015). A special role is ascribed to *staphylococcal* enterotoxins*.* Several studies showed enterotoxin-specific IgE sensitization in asthmatic patients (Bachert *et al.*, 2012; Tomassen *et al.*, 2013; Tanaka *et al*., 2015; Bachert *et al*., 2020). Even patients diagnosed as nonatopic frequently showed IgE-sensitization towards *S. aureus* enterotoxins (Schreiber *et al.* 2019).

In contrary to microbes considered as risk factors for the development of asthma, there is the hygiene hypothesis, which states that childhood infections and the exposure to microbial antigens early in life seem to present a strong negative correlation with allergies. Due to the increased use of antibiotics, improved hygiene and urbanization, the prevalence of allergic diseases is on the rise in western industrial countries (Bufford & Gern, 2005; Chu *et al*., 2003). Amongst environmental factors, physical and psychological conditions like obesity or stress can enhance the risk to develop asthma. Epidemiological studies could show that asthma is more likely to occur in obese patients and obese asthmatics tend to experience more symptoms and increased asthma severity (Weiss, 2005; Sutherland, 2014). Due to a potential mechanism which includes changes in the methylation and expression of genes that regulate

autonomic, behavioral and immunologic responses to stress, an association between chronic psychosocial stress and asthma was suggested by Rosenberg *et al.* (Rosenberg *et al.*, 2014).
In addition to environmental risk factors, genetic risk factor can play an important role in the development of asthma. Since 1989 more than 600 candidate genes have been described in relation with asthma (Toskala & Kennedy, 2015). Promising candidates, that have been extensively replicated include genes involving cytokines, signaling proteins and transcription factors in Th1 and Th2 differentiation, e.g. *IL4, STAT6, GATA3* or *TBX21* (Haller *et al.*, 2009; Pykalainen *et al.*, 2005; Suttner *et al.*, 2009).

1.2 *Staphylococcus aureus* (*S. aureus*)

The first description of *S. aureus* was in 1880 by Alexander Ogston who was able to detect this pathogen in purulent abscesses. This bacterium owes its name to its characteristic cluster-like aggregation (*staphyle*, Greek for “grape”) and the golden yellow pigmentation of the colonies (*aurum*, Latin for “gold”) (Lowy, 1998). *S. aureus* is a gram-positive facultative bacterial pathogen, which can be distinguished from other, less pathogenic *staphylococci* by its ability for β-hemolysis and positive results of mannitol-, fermentation- and desoxyribonuclease-tests (Hof, 2005; Taylor & Unakal, 2021). As a commensal, *S. aureus* constantly colonizes about 30 % of the adult population (Wertheim *et al.*, 2005a; Mertz *et al.*, 2009; Kolata *et al.*, 2011). Preferred sites of colonization are the skin as well as mucous membranes of the nasopharynx (Wertheim *et al.*, 2005a; Grumann *et al.*, 2014; Chua *et al.*, 2014; Ryu *et al.* 2014). Newborns tend to have a higher colonization rate with *S. aureus*: more than 70 % show colonization. However, the colonization rate declines again with increasing age. Only 21 % of six-month-old babies are tested positive for *S. aureus* (Wertheim *et al.*, 2005a). Some population groups tend to have a higher colonization rate of *S. aureus* (up to 80%), e.g. healthcare workers, patients with type 1 diabetes, people who use intravenous drugs, hemodialysis patients, surgical patients and patients with acquired immune deficiencies and atopic diseases (Lowy, 1998; Taylor & Unakal, 2021). Since fairly recently, it is accepted that the airways are constantly colonized by microorganisms, including *S. aureus*, and that these interact with the immune system in multiple ways in health and disease (Calson *et al.*, 2011; Budden *et al.*, 2019).

Colonization with *S. aureus* can be constant or intermittent, but not all studies on *S. aureus* colonization take this into account (van Belkum *et al.*, 2009).

Besides its role as a commensal, *S. aureus* can induce several diseases, which can range from deep skin infections to life threatening conditions like pneumonia or sepsis (Wertheim *et al.*, 2005a; Wertheim *et al.*, 2005b). The treatment of *S. aureus* infections is complicated by antibiotic resistance. About 60 % of all *S. aureus* isolates express a β-lactamase and are therefore resistant to β-lactam antibiotics, e.g. ampicillin. Nowadays already 20 % of all hospital isolates and also 0.3 % of colonizing isolates show a resistance to β-lactam antibiotics of the 2nd generation, e.g. methicillin (Aghadassi et al., 2016; Ippolito et al., 2010). One reason for the rapid development of resistance is the highly variable genome of *S. aureus*, which is about 2.8 Mb in size and the genome difference between two strains can be up to 20 % (Lindsay *et al.*, 2006; Goerke *et al.*, 2009). The enormous variability of the genomes between different *S. aureus* strains is based on a large number of mobile genetic elements (MGE), which are used for horizontal transfer between two strains. MGEs contain pathogenicity islands, plasmids, transposons, prophages and chromosomal cassettes (Lindsay & Holden; 2004; Plata *et al*., 2009). As a result, MGEs contribute significantly to the spread of antibiotic resistance and decisively determine the virulence of an *S. aureus* strain (Deurenberg & Stobberingh, 2008). The most important virulence factors include superantigens (SAgs), pore-forming units and exfoliative toxins. *S. aureus* SAgs include for example staphylococcal enterotoxins (SEs), enterotoxin-like toxins and the toxic-shock syndrome toxin 1 (TSST-1) (Shettigar & Murali, 2020).

Despite intensive efforts over the past decades, it has failed to develop a *S. aureus* vaccine for humans, due to the fact that no protective immunity could be generated (Proctor, 2012; Fowler & Proctor, 2014; Bagnoli *et al.*, 2012; Giersing *et al.*, 2016).

1.2.1 *S. aureus* and atopy

As already mentioned above, patients with atopic diseases like atopic dermatitis (AD), allergic rhinitis (AR) and allergic asthma, are more likely to be colonized with *S. aureus*. In addition, through the induction of type 2 inflammation *S. aureus* is capable of escaping the immune system (see figure 3, p. 15).

AD is a chronic and also relapsing inflammatory disorder of the skin affecting mainly infants and young children, but also occurring during adulthood (Ogonowska *et al.,* 2021). It is characterized by an impaired epidermal barrier function, a dysbiosis of the cutaneous microbiota and resulting recurring infections, especially with *S. aureus* (Weidinger *et al.,* 2018; Di Domenico *et al.*, 2019; Kobayashi *et al.*, 2015; Kim *et al.*, 2019; Blicharz *et al.*, 2019; Iwamoto *et al.*, 2019). The skin of up to 100 % of patients with AD is colonized with *S. aureus* of which up to 65 % were found to be able to produce enterotoxins with superantigenic properties, for example the *staphylococcal* enterotoxin B (SEB) (Breuer *et al.*, 2002; Tomi *et al.*, 2005).

AR is described as an inflammatory condition, caused by an IgE-mediated type 1 hypersensitivity response to a variety of environmental allergens (Papadopoulos & Guibas, 2016; Liva *et al.*, 2021). It is characterized by nasal congestion, anterior and posterior rhinorrhea as well as itching of the nose and sneezing for more than one hour on two or more consecutive days (Bousquet *et al.*, 2009). As well as in patients with AD, the colonization rate in patients with AR is described to be significantly higher than in healthy individuals (Sollid *et al.,* 2014; Muluk *et al.*, 2018; Vickery *et al.*, 2019; Abdurrahman *et al.,* 2020).

Allergic asthma is one of the common comorbidities associated with AR and it could be shown that 21-27 % of the patients suffering from allergic asthma in combination with AR were IgE sensitized against staphylococcal toxins like staphylococcal enterotoxin A (SEA), SEB or TSST-1 (Liu *et al.*, 2014). For allergic asthma, a significant relationship between *S. aureus* nasal carriage and the disease has been recognized. Here, especially an allergic sensitization against *staphylococcal* enterotoxins, mainly SEB, could be observed (Tomassen *et al.*, 2013; Song *et al.*, 2014; Song *et al.*, 2016; Flora *et al.*, 2019; Kim *et al.*, 2019). Next to SEB, other *S. aureus* derived proteins such as the serine protease-like protein D (SplD) have been shown to support Th2-biased immune responses after intratracheal (*i.t.*) exposure (Stentzel *et al.*, 2017; Teufelsberger *et al.*, 2018).

These findings indicate that *S. aureus*, besides its primary role as a pathogen, could also play an important role in inducing allergic diseases. However, to date is not clear by which mechanisms *S. aureus* acts on atopy and how atopy in turn possibly supports colonization.

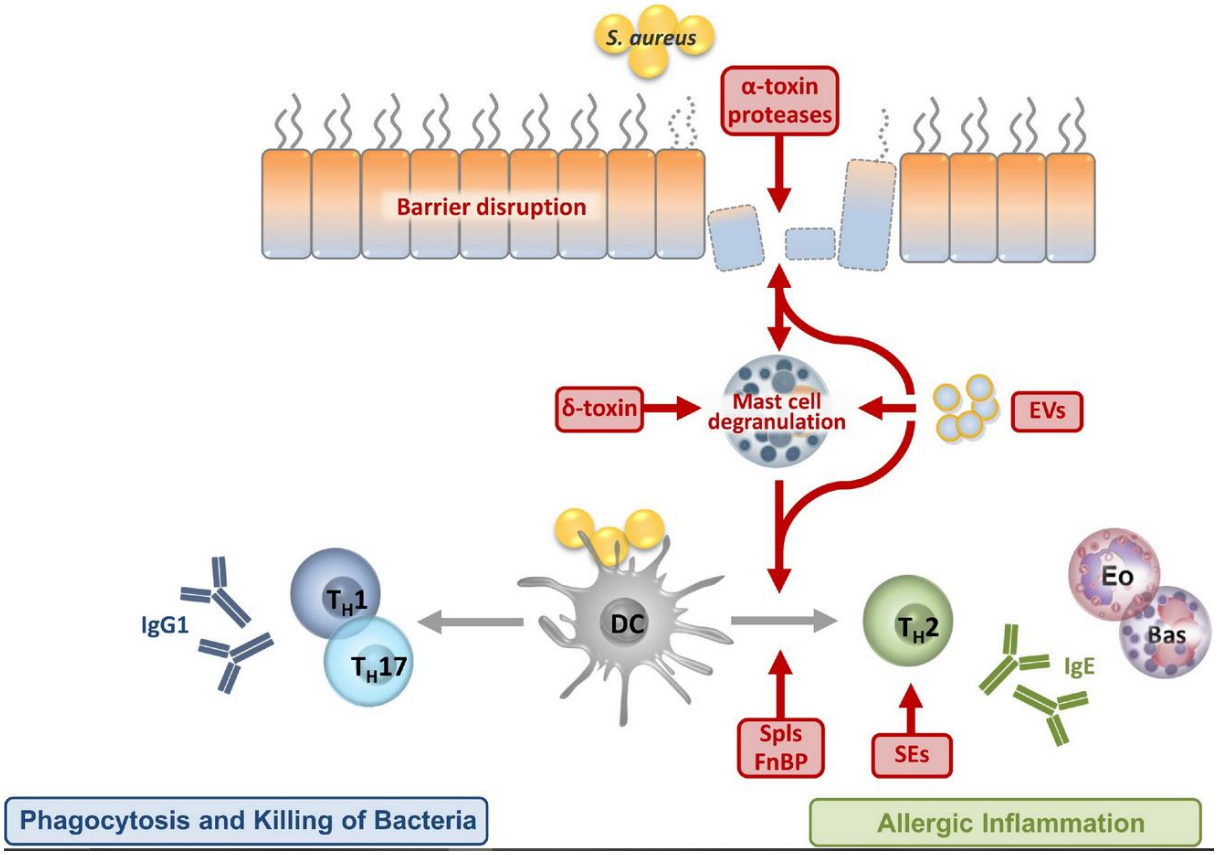


figure 3: *S. aureus* can induce type 2 inflammation in order to escape the immune system. (taken from Nordengrün *et al.,* 2018).
After the disruption of the epithelial barrier through human leukocyte antigen (Hla) and bacterial proteases, *S. aureus* facilitates the entry of other bacterial factors and aeroallergens. Due to *S. aureus* δ-toxin and extracellular vesicles (EVs) mast cell degranulation is induced and a pro-allergic microenvironment created. Other secreted proteins, especially serine-protease like proteases (Spls) and staphylococcal enterotoxin (SEs) trigger an allergic inflammation and elicitin a specific IgE response, resulting in activation of e.g. basophils (Bas) and eosinophils (Eo). As a result, *S. aureus* is able to shift the host immune response away from a Th1/Th17 profile, towards a type 2 inflammation.

1.2.2 Staphylococcal enterotoxin B (SEB)

Up to 80 % of isolated *S. aureus* strains are capable of producing enterotoxins (Flora *et al.*, 2019; Varshney *et al.*, 2009; Lawrynowicz-Paciorek *et al.* 2007; Mrabet-Dahbi *et al.*, 2005). Staphylococcal enterotoxins are a family of 22 structurally related toxins discovered until today (Yang *et al.*, 2011). SEB is one of the best characterized toxins in this family and is listed as a category B select agent by the U.S. Centers for Disease Control and Prevention (Ulrich *et al.*, 2007). It is resistant to heat, acids and also to inactivation by gastrointestinal proteases like papain, trypsin or pepsin (Lawrynowicz-Paciorek *et al.*, 2007; Pinchuk *et al.*, 2010; Gill, 1982; Le Loir, Gautier, 2003).

SEB belongs to the superantigen family of *S. aureus*, which are potent immune activators (Liu *et al.*, 2006; Rossi *et al.*, 2004; Schlievert *et al.*, 2008). SAgs mediate a direct interaction between peptide-MHC class II and the CDR2 loop of the variable chain of the T cell receptor (Xu *et al*., 2009) (see figure 4, p. 17). The SAg-mediated T cell activation is therefore independent of antigen-presentation on the MHC II molecule of APCs. Already at picomolar concentrations, SEB leads to the activation of a very large number (5-30 %) of the exposed T cell population (Choi *et al.*, 1989; Fraser & Proft, 2008; Spaulding *et al.*, 2013). Conventional antigens administered in a much higher concentration, only activate around <0.01 % of the exposed T cells (Krakauer, 2019). Therefore, superantigens can lead to a cytokine storm which can result in a toxic shock syndrome, multi organ failure and even death (Xu et al., 2015).

In humans, the ingestion of less than 1 µg SEB can lead to food poisoning, which is characterized by symptoms like nausea, vomiting, abdominal pain, cramps and diarrhea (Dinges *et al.*, 2000; Pinchuk *et al.*, 2010). In patients with AD the severity of the disease correlates with the amount of colonizing *S. aureus* strains which are able to produce enterotoxins. Here, SEB induces the maturation of DC *via* TLR-2, which then favors the polarization of naïve T cells to Th2 cells (Jinho *et al*., 2012).

In mice, the intraperitoneal (*i.p.*) administration of SEB leads to toxic shock which manifests in increased levels of various proinflammatory cytokines like IL-1, IL-3, TNF-α and IFN-γ (Faulkner *et al.*, 2005; Krakauer *et al.*, 2010a). When SEB is administered *via* the intranasal (*i.n.*) route or inhalation it can trigger acute lung injury, characterized by excessive cytokine production, immune cell infiltration, necrosis in endothelial cells as well as pulmonary edema (Neumann *et al.*, 1997; Saeed *et al.*, 2012). Because of the lower toxin affinity to murine MHC II molecules as compared to the human version, it is necessary to use non-physiological doses of SEB, or use potentiating agents like D-galactosamine or lipopolysaccharide (LPS), to investigate SEB-mediated effects in mice (Krakauer, 2019).

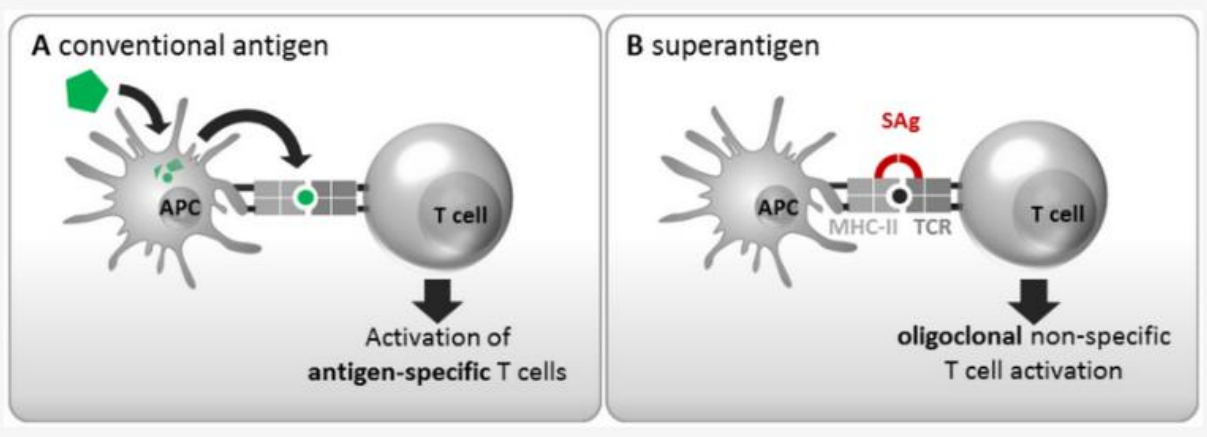


figure 4: Superantigens unspecifically activate T cells. (taken from Abdurraham *et al*., 2020)
Conventional antigens are taken up by antigen-presenting cells, processed and presented on MHC-II molecules to $CD4^+$ T cells with the complementary T cell receptor. Superantigens on the other hand circumvent this highly specific interaction by directly cross-linking the T cell receptor and MHC-II molecule outside their peptide binding site. This unspecific binding results in an oligoclonal T cell activation.

1.2.2.1 SEB and allergic airway inflammations (AAI)

Regarding effects of *S. aureus* and its toxins on AAI, especially SEB has come into focus (Hellings *et al.*, 2006; Bachert *et al.*, 2007; Huvenne *et al.*, 2010a). So far, only few studies have experimentally addressed short-term effects of *S. aureus* and SEB in the respiratory tract and on allergic asthma (see figure 5, p. 19).

It has been demonstrated, that in serum of severe asthmatic patients more often SE-specific IgE antibodies could be detected as compared to patients suffering from less severe asthma (Perez-Novo *et al.*, 2004; Bachert *et al.*, 2007). Furthermore, Schreiber *et al.* could show that the enterotoxin-specific IgE sensitization in asthma patients is mainly directed against SEB (Schreiber *et al.*, 2019).

Apart from SEB-specific IgE sensitization, SEB potentially also affects sensitization and allergic reactions to common allergens through its activities as a toxin and superantigen. In a mouse model, it has been shown that *i.n.* application of SEB alone leads to increased numbers of lymphocytes, neutrophils as well as eosinophils in BAL in a dose dependent manner (Herz *et al.*, 1999). Combined with *i.n*. OVA-treatment, SEB facilitated the OVA-specific allergic sensitization and inflammation and its activity was described as “adjuvant-like” (Krysko *et al.*, 2013). Also, a repeated *i.n.* SEB-treatment immediately before the allergic challenge has been described to lead to enhanced AAI in previously sensitized mice, characterized by increased immigration of eosinophils to the respiratory tract, as well as an increased mRNA-levels of

IL-4, IL-5 and eotaxin-1 (Hellings *et al.*, 2006). Huvenne *et al.* combined the *i.n.* OVA-treatment with the additional treatment with different *S. aureus* toxins (SEA, SEB, TSST-1) and LPS. Only the combination of *i.n.* application of OVA and SEB led to an increased OVA-specific IgE response, an increased influx of eosinophils and lymphocytes to the respiratory tract and increased production of typical type 2 cytokines (Huvenne *et al.*, 2010b). Other studies have shown that even an SEB sensitization *via* the skin injury prior to *i.n.* OVA-challenge, led to an increase in airway inflammation (Yu *et al.*, 2012). In HLA-DR3 transgenic mice, which express the human MHC molecule DR3 molecule on all APCs as well as $CD4^+$ T cells, the *i.n.* application of SEB led to an increased accumulation of macrophages, eosinophils and neutrophils in the BAL, as well as an increased airway resistance after methacholine challenge (Govindarajan *et al.*, 2006)

Taken together, these experimental studies suggest a high immune-modulatory potential of SEB in AAI and a detailed knowledge of the underlying mechanisms will be essential for developing related diagnostic, prophylactic and therapeutic approaches.

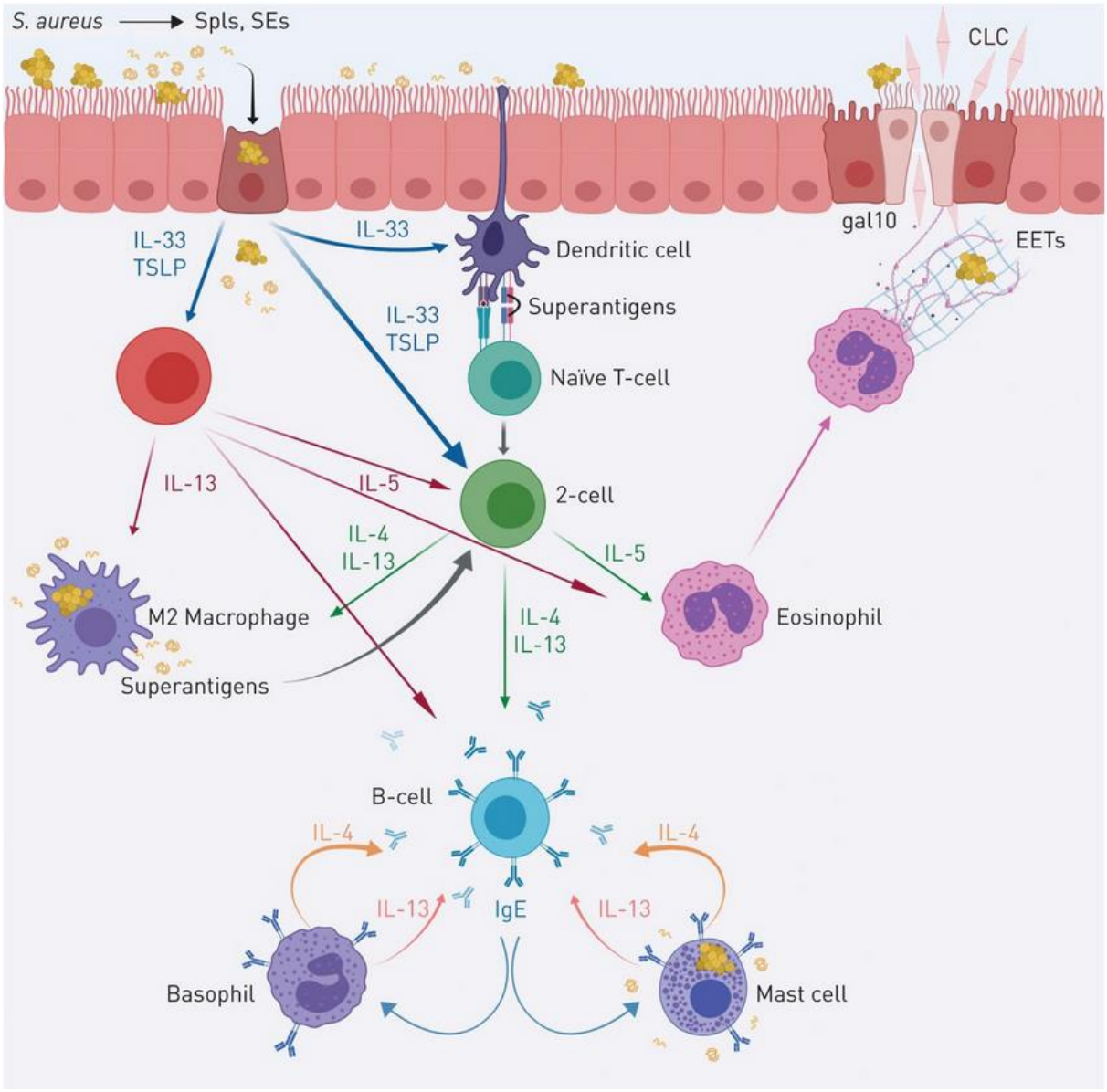


figure 5: Effects of *S. aureus* and its enterotoxins on the human airway immune response. (taken from Bachert *et al*., 2020)

When *S. aureus* colonizes the respiratory epithelium, it induces the release of IL-33 and TSLP. This recruits innate lymphocyte cells 2 (ILC2) and activates them to release IL-5 and IL-13. *S. aureus* can invade activated M2 macrophages and releases proteins such as TLSP or SEB through them, which further trigger T cells. IL-33 additionally activates DCs which may activate naïve T cell *via* superantigens leading to the massive production of IL-4 and IL-13. B cells are activated and differentiate into IgE-producing plasma cells. IL-5 released by ILC2 and Th2 cells activates eosinophils which migrate to the airway epithelium to release extracellular eosinophil trap, consisting of major basic protein, DNA and galectin 10, in turn leading to persistent stimulation of the respiratory epithelium.

Abbreviations: CLC: cholesterine-liquid-crytal; EET: eosinophil extracellular traps; gal10: galectin 10

1.3 Mouse models of allergic airway inflammation

AAI is a heterogeneous condition and different structural cells as well as cells of the innate and the adaptive immune system orchestrate the allergen-specific immune response. Due to this complexity, animal models are of crucial importance for the analysis of the underlying pathomechanisms of allergic diseases as well as the identification of strategies for prophylaxis and intervention.

The pathogenic characteristics of AAI have been observed in several species like the mouse (Nials *et al.*, 2008), rat (Motta *et al.*, 2004), guinea pig (Towards *et al.*, 2004), rabbit (Gascoigne *et al.*, 2003), ferret (Motonori *et al.*, 200), dog (Barrett *et al.*, 2003), cat (Norris *et al.*, 2003), sheep (Bischof *et al.*, 2003), pig (Fomhem *et al.*, 1996) as well as some primates (Ayanoglu *et al.*, 2010; van Scott *et al.*, 2004) and horses (Turlej *et al.*, 2001). Of note, only a few species spontaneously develop asthmatic symptoms, including cats and horses (Bates *et al.*, 2009)

The use of the mouse as laboratory animal offers many advantages. The housing and breeding of mice do not involve any major challenges and is relatively inexpensive. Furthermore, mice have a short generation time and there are many genetic variants and genetically engineered strains available. Also, research reagents for this species are largely available (Aun *et al.*, 2017). Next to these advantages, IgE is the primary allergic antibody in mice, amongst other aspects, making this species appropriate for the investigation of AAI (Shin *et al.*, 2009). Therefore, one can find multiple different mouse models of AAI in the past and current literature and multiple important contributions to our understanding of the pathophysiology of allergic asthma have been identified in mouse models of AAI (Hammad & Lambrecht, 2021).

To choose a suited mouse model of AAI, different factors have to be taken into consideration for the specific research question. Studies have demonstrated that not all mouse strains show similar responses with respect to AAI (Torres *et al.*, 2005). For example, it has been shown that in comparison to C57BL/6 mice, the sensitization of BALB/c mice leads to a significantly higher production of total, as well as antigen-specific IgE (Herz *et al.*, 1998, Torres *et al.*, 2005). Additionally, BALB/c mice develop stronger bronchial hyperreactivity as compared to C57BL/6 mice (Brewer *et al.*, 1999; Gueders *et al.*, 2009). Further, previous studies have shown that AAI induced in BALB/c mice leads to higher numbers of mast cells in the lung tissue as compared with C57BL/6 mice. In contrast, AAI induced in C57BL/6 mice is characterized by significantly higher numbers of eosinophils as well as neutrophils in the BAL. In terms of smooth muscle cell hyperplasia and the increase of goblet cells, no differences between the

strains could be detected (Gueders *et al.*, 2009, van Hove *et al.* 2009). It has been proposed, that C57BL/6 mice could be suited to study the inflammatory component of severe asthma, while BALB/c mice might be suited for studying mild, or early asthma (Gueders *et al.*, 2009). However, more genetically engineered strains, such as e.g. gene-specific knock-outs, are available on the C57BL/6 genetic background.

In addition to the selection of a suited mouse strain, the optimal allergen must also be identified. Traditionally, the most common (model-)antigen used for the induction of AAI in mice is OVA. It is derived from chicken egg and can easily be produced in high quantity. Typically, AAI induction with OVA consists of a specific sensitization, usually with an adjuvant, followed by an airway challenge. The most common strategy for the sensitization is multiple *i.p.* injections of the antigen OVA in combination with an adjuvant such as aluminum hydroxide (alum) (Kumar *et al.*, 2008; Shin *et al.* 2009; Conrad *et al.*, 2009). However, sensitization can also be carried out subcutaneously (*s.c.*) (Carvalho *et al.*, 1999; Mine *et al.*, 2007), intranasally (*i.n.*) (Kumar et al., 2008; Liang *et al.*, 2016) or *i.t.* (Hanashiro *et al.*, 2019, Reuter *et al.*, 2019) with different adjuvants or with OVA alone. The most common method for the application of the allergen challenge is the *i.n.* route (Sjöber *et al.*, 2017; Khumalo *et al.*, 2019) with the major advantage that the allergen dose can be precisely determined and controlled. Other forms of the airway challenge are the *i.t.* installation (Jung *et al.*, 2016) or the inhalation of nebulized OVA (Wang *et al.*, 2016). The inhalation is less stressful for the animal, because they do not have to be sedated but it is difficult to determine the exact amount of antigen inhaled and at the same time there is oral intake e.g, *via* licking the fur.

While there are numerous advantages for the model antigen OVA for the induction of AAI such as the wide availability of immunological research tools, there are also significant drawbacks to be taken into account. E.g., OVA is not a natural allergen and mice can develop tolerance against this innocuous antigen after repeated exposure (Fröde *et al.*, 2005; Aun *et al.*, 2017).

An alternative antigen often used to induce AAI in mouse models is house dust mite extract (HDM). HDM mainly consists of *dermatophagoides pteronissinus* (*D. pter*), which is one of the most widespread dust mites. Its protein Der p1 has been identified as having a cystein protease activity, capable of inducing inflammatory cytokine release and stimulating pulmonary inflammation as well as Th2 cell and IgE humoral responses (Doras *et al.*, 2018; Kikuchi *et al.*, 2006). Doras *et al.* showed that AAI induced using HDM leads to a more specific

airway inflammation, expressed by a Th1/Th2 imbalance in addition to the more pronounced increase of eosinophils (Doras *et al.*, 2018).

Other extracts, purified from human allergens are derived from cockroaches, ragweed or fungi (Shin *et al.*, 2009).

Domestic cockroach species like *Blattella germanica* and *Periplaneta americana* are known to produce several potent allergens, for example Bla g4 (caycin) or Blag5 (glutathione-S-transferase) (Arruda *et al.*, 2001).

Ragweed or common ragweed (*Ambrosia artemisiifolia* and *Ambrosia elatior*) is the main cause of health problems in the sensitized population. For example, in the USA and Canada more than 15 million people suffer from ragweed pollen allergy, but also in Europe the incidence of people which are sensitized to ragweed is increasing (Wopfner *et al.*, 2005).

Fungi which are often used for the induction of AAI are *Aspergillus fumigatus* or *Alternaria alternata* (Aun *et al.*, 2017). Those are ecological molds which reproduce through the production of airborne spores. Mice sensitized with *Aspergillus* or *Alternaria* develop high levels of eosinophilia, IgE and lung inflammation (Takazona *et al.*, 2016).

In summary, there are tremendous variations in the described animal models of AAI. These differ in host species, allergen and sensitization or challenge protocol. Ultimately the selection of an appropriate model depends on the hypothesis to be investigated. Using the mouse as the experimental animal to model for human AAI is obviously practical in many points but also there are very few pathological features not shared in mouse models and human asthma. Nonetheless, mouse models of AAI, like every other species and model, have several limitations, such as the fact that they do not spontaneously develop AAI or that there is no long-lasting bronchoconstriction as it is typical in human asthmatics (Shin *et al.*, 2009). In addition, the majority of experimental models require a non-physiologically high concentration of the allergen to induce AAI (Torres *et al.*, 2005). Nevertheless, for several reasons the mouse displays a valuable experimental model for AAI: Key immunological and histological processes of AAI can be reproduced with increasing accuracy in the mouse, it is possible to investigate pathomechanisms with a wider perspective, in greater detail and in more controlled settings as it would be possible in human asthmatics. Furthermore, there are several experimental advantages over other species, e.g. due to the wide range of the available genetically manipulated strains, like transgenic and knockout mice.

1.4 Aims of the study

It has been proposed that allergic sensitization and inflammation in asthma can be affected by *S. aureus*. These associations are of high clinical relevance, but are not well understood to date. *S. aureus* is a gram-positive, facultative pathogen, colonizing around 30 % of the adult population and capable of producing a wide range of toxins including the SAg SEB, a potent activator of the immune system. In recent years, SEB has increasingly moved into focus as a potential modulator of AAI.

Most scientific work published so far on this topic describes a proinflammatory effect of SEB when administered to the respiratory system and an enhancement of the induced AAI in different mouse models. However, most of these studies are limited to the cellular components of the local immune response and only consider the simultaneous treatment with an allergen and SEB. To extend the existing knowledge on the immunomodulatory effects of SEB on *in vivo* induced AAI, on a cellular and humoral level, as well as the temporal aspect of the *i.n.* SEB treatment, this study aimed at establishing an *in vivo* OVA-induced mouse model of AAI that could be combined with *i.n.* SEB administration at different time-points. The hypothesis followed in this model was that SEB would have diverging effects on AAI depending in whether it is encountered at a low or a high dose as well as before allergic sensitization or during allergic challenge.

To follow this aim and to address this hypothesis, in a first step a suited OVA-mediated model of AAI was to be established. Read-out parameters for the characterization of AAI were histological changes as well as the influx and numbers of different immune cells such as eosinophils, DCs, activated lymphocytes and Th2 cells in the BAL, lungs and spleen. In addition to the cellular response in AAI, the humoral immune response was to be examined. Here, concentrations of typical type 2 cytokines such as IL-4, IL-5 and IL-13, as well as proinflammatory cytokines (IL-6/TNF-α) in the respiratory tract were major read-out parameters next to the serum OVA-specific IgE response. An additional major AAI-associated parameter to be taken into account was the unspecific bronchial hyperreactivity in response to methacholine. The mouse model of AAI to be established was to be characterized by robustly detectable inflammation of the airways in response to the model antigen OVA, a specific IgE response as well as significant airway hyperreactivity. Ideally, these parameters

were to be of medium severity to allow assessing both aggravating as wells as possibly ameliorating effects of SEB.

Employing the established OVA-mediated mouse model of AAI, the effects of SEB on AAI in a time- and dose-dependent manner were to be assessed. Here, aspects regarding the dose and the timing of SEB encounter were to be addressed by combining allergic sensitization, allergic challenge and *i.n.* SEB administration in different experimental regimen. In the course of these experimental approaches, also acute as well as long-term effects of SEB alone were to be assessed in order to elucidate SEB-mediated effects on the respiratory immune milieu independent of allergic sensitization and challenge.

In summary, the two major objectives of this thesis were:

Aim 1: Establishing a suited OVA-mediated model of AAI and comprehensively assessing the time- and dose-dependent effects of intranasal SEB administration on allergic sensitization and allergic responses in the respiratory tract with respect to hallmark parameters of AAI.

Aim 2: Elucidating the short- and long-term impact of intranasal SEB-treatment on the local immune environment of the respiratory tract

2 Material

2.1 Consumables

table 3: Consumables

Product	Cat.-number	Manufacturer
cover film ROTILABO®	0954.1	Carl Roth, Karlsruhe
cannula Sterican (26G) ®	260091	B. Braun Melsungen AG, Melsungen
cell strainer		Greiner Bio-One International
70 µm	542070	GmbH, Austria
100 µm	542000	
centrifuge tubes		
15 ml	188271	Greiner Bio-One International GmbH, Austria
50 ml	227261	
FACS™ tubes	1010270	Corning, New York, USA
glass capillary (20µl)	1041816	Sarstedt, Nümbrecht
Immuno 96-well-plate	439454	Fisher Scientific GmbH, Schwerte
pasteur pipettes	8.611.171.010	Sarstedt, Nümbrecht
petri dish	1020586	Paul Boettger GmbH & Co. KG, Bodenmais
pipette tips	70.1116	Sarstedt, Nümbrecht
	70.760.012	
	70.762.010	
reaction vessels		
0.5 ml	1029140	
1.5 ml	1012830	Eppendorf AG, Hamburg
2 ml	1025401	
5 ml	1037782	
serological pipettes		
5 ml	4487	Corning, New York, USA
10 ml	4100	Corning, New York, USA
25 ml	94024	TPP Techno Plastic Products AG, Trasadingen, Switzerland
syringes		
Omnifix ® F Solo (1 ml)	1020427	B. Braun Melsungen AG, Melsungen
Omnican ® 100 (1 ml)	9151133S	B. Braun Melsungen AG,

		Melsungen
BD Discardit II (10 ml)	03626823	
BD Discardit II (20 ml)	07358756	
		Bioscience, Heidelberg
		Bioscience, Heidelberg
UltraComp eBeads compensation beads	01-2222-42	Thermo Fisher Scientific Inc., Massachusetts, USA
well plates		
6-well	3516	Corning, New York, USA
24-well	3526	Corning, New York, USA
96-well (round bottom)	821.582.001	Sarstedt, Nümbrecht

2.2 Chemicals

table 4: Chemicals

Product	Cat.-number	Manufacturer
Acetyl-3-methylcholine chloride (Methacholinchloride)	A2251-25G	Sigma-Aldrich, St. Louis, Missouri, USA
Albumin from chicken egg white Grade III	A5378-1G	Sigma Aldrich, Missouri, USA
Albumin from chicken egg white Grade V	A5503-1G	Sigma Aldrich, Missouri, USA
Ammonium chloride (NH_4Cl)	P726.1	Carl Roth, Karlsruhe
Attune Focusing Fluid	A24904	Thermo Scientific, Massachusetts, USA
Attune Shutdown Solution	A24975	Thermo Scientific, Massachusetts, USA
Attune Wash Solution	A24974	Thermo Scientific, Massachusetts, USA
Brefeldin A	420601	Biolegend, San Diego, USA
Collagenase D	COLLD-RO	Roche Diagnostics, Mannheim
Desoxyribunuclease (DNAse)	D4527-10KU	QIAGEN GmbH, Hilden
DMSO	A994.1	Carl Roth, Karlsruhe
Ethylenediamine tetraacetic acid disodium salt (Na_2EDTA)	8043.1	Carl Roth, Karlsruhe
fetal bovine serum (FBS)	P40-47500	PAN-Biotech GmbH, Aidenbach
Imject™ Alum	77161	Thermo Scientific, Massachusetts, USA
Ionomycin	I-700	Sigma-Aldrich, St. Louis, Missouri, USA
Iscove's Modified Dulbecco's Medium (IMDM)	21980065	Thermo Scientific, Massachusetts, USA

Isoflurane CP 1ml/ml	1214	CP-Pharma Handelsgesellschaft mbH, Burgdorf
Ketamine (10 %)	3048734	MEDISTAR Arzneimittelvertrieb GmbH, Ascheberg
Monensin	420701	Biolegend, San Diego, USA
Paraformaldehyde (PFA)	0335.1	Carl Roth, Karlsruhe
Penicillin/Streptomycin (5000 U/ml) (Pen/Strep)	15070063	Thermo Scientific, Massachusetts, USA
PercoII™	17-0897-02	GE Healthcare, Illinois, USA
Potassium hydrogen carbonate	P748.1	Carl Roth, Karlsruhe
Phorbol 12-myristate 13-acetate (PMA)	P1585	Sigma-Aldrich, St. Louis, Missouri, USA
Phosphate-buffered saline (PBS) tablets	18912014	Thermo Scientific, Massachusetts, USA
Precision Count Beads ™	424902	Biolegend, San Diego, USA
Sodium chloride	3957.1	Carl Roth, Karlsruhe
***Staphylococcus* Enterotoxin B (SEB)**	S4881-1MG	Sigma-Aldrich, St. Louis, Missouri, USA
Sulfuric acid (2N)	X873.1	Carl Roth, Karlsruhe
Tetramethylbenzidine (TMB)	555214	BD Bioschience, Heidelberg
Tween20	37470.1	SERVA Electrophoresis GmbG, Heidelberg
Xylazine (Rompun® 2 %)	QN05CM92	Bayer AG, Leverkusen
β-Mercaptoethanol	4227.1	Carl Roth, Karlsruhe

2.3 Kits

table 5: Kits

Product	Cat-.number	Manufacturer
LEGEND MAX™ Mouse OVA Specific IgE ELISA Kit	439807	Biolegend, San Diego, USA
LEGENDplex™ Mouse TH Cytokine Panel (13-plex)	740741	Biolegend, San Diego, USA

2.4 Technical devices

table 6: Technical devices

Device	Model	Manufacturer
autoclave	Systec V-150	Systec GmbH, Linde
centrifuge	Multifuge® 3 S-R/3 L-R, Legend® RT	Kendro Laboratory Products GmbH, Hessen
	Heraeus Fresco 17 Centrifuge	Fisher Scientific GmbH, Schwerte
	Centrifuge 5910R	Eppendorf AG, Hamburg
clean bench/bio-safety cabinet	Thermo Scientific™ Safe 2020	Fisher Scientific GmbH, Schwerte
	Baker SterilGARD® III Advance	The Baker Company, Sanford, Maine, USA
counting chamber	Neubauer-improved, 0.1 mm	Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen
dissecting tools		W.O. Schmidt GmbH, Braunschweig
flow cytometer	Attune NxT Flow	Thermo Fisher Scientific Inc., Massachusettes, USA
microscope	Inversmicroskop TCM400	Labomed, Regensburg
photometer	Infinite® M. Plex	Rothacher Medical GmbH, Schwitzerland
pipettes	PIPETMAN Classic	Gilson, Villiers-le-Bel, France
pipetting aid	PIPETBOY acu 2	INTEGRA Biosciences AG, Schwitzerland
plethysmograph	Buxco FinePoint RC	Data Sciences International, Minnesota, USA
precision scale	EMB	KERN & SOHN GmbH, Balingen-Frommern
shaker	neoLab Multi Shaker	neolab Migge GmbH, Heidelberg
suction pump	VACUSAFE	INTEGRA Biosciences AG, Schwitzerland
thermomixer	ThermoMixer® C	Eppendorf AG, Hamburg
veterinary anesthesia machine	combi-vet® Base Anesthesia System	Rothacher Medical GmbH, Schwitzerland
vortexer	neoVortex® Schüttler	neolab Migge GmbH, Heidelberg
water bath	WNB 7	Memmert GmbH + Co.. KG, Schwabach

2.5 Antibodies

2.5.1 Flow cytometry panels

All antibodies listed in the following sections were titrated prior to use in order to determine the optimal antibody staining concentration (high signal/background ratio) for the different analyses. The zombie fixable viability stain is a reactive fluorescent dye that is non-permeant to live cells, but permeant to the cells with compromised membranes. Therefore, it can be used to assess live *vs*. dead status of cells.

table 7: Flow cytometry staining panel 1

Detected antigen	Conjugation	Clone	Manufacturer
CD4	FITC	RM4-5	Biolegend, San Diego, USA
CD11b	APC/Cy7	M1/70	Biolegend, San Diego, USA
CD11c	APC	N418	Biolegend, San Diego, USA
CD19	PerCP/Cy5.5	1D2/CD19	Biolegend, San Diego, USA
CD8a	PE/Cy7	53-6.7	Biolegend, San Diego, USA
Ly-6G	AF700	1A8	BD Bioscience, Heidelberg
MHCII	BV711	M5/114.15.2	Biolegend, San Diego, USA
Siglec-F	PE	E50-2440	Biolegend, San Diego, USA
Zombie fixable viability stain	Aqua		Biolegend, San Diego, USA

table 8: Flow cytometry staining panel 2

Detected antigen	Conjugation	Clone	Manufacturer
CD11c	APC	N418	Biolegend, San Diego, USA
B220	PerCP-Cy5.5	RA3-6B2	Biolegend, San Diego, USA
CD4	APC-Cy7	GK1.5	Biolegend, San Diego, USA

CD69	BV421	H1.2F3	Biolegend, San Diego, USA
CD8a	PE-Cy7	53-6.7	Biolegend, San Diego, USA
MHCII	BV711	M5/114.15.2	Biolegend, San Diego, USA
Siglec-F	PE	E50-2440	BD Bioscience, Heidelberg
ST2	FITC	DJ8	MD Bioproducts, Minnesota, USA
Zombie fixable viability stain	Aqua		Biolegend, San Diego, USA

table 9: Flow cytometry staining panel 3

Detected antigen	Conjugation	Clone	Manufacturer
B220	BV510	RA3-6B2	Biolegend, San Diego, USA
CD117 (c-kit)	BV421	2B8	Biolegend, San Diego, USA
CD11b	PerCP-Cy5.5	M1/70	Biolegend, San Diego, USA
CD3	BV510	17A2	Biolegend, San Diego, USA
CD49b	APC	HMα2	Biolegend, San Diego, USA
FcεRI	PE-Cy7	MAR-1	Biolegend, San Diego, USA
Ly-6C	FITC	HK1.4	Biolegend, San Diego, USA
Ly-6G	AF700	1A8	Biolegend, San Diego, USA
NK1.1	BV510	PK136	Biolegend, San Diego, USA
Siglec-F	PE	E50-2440	BD Bioscience, Heidelberg
Zombie fixable viability stain	NIR		Biolegend , San Diego, USA

2.5.2 ELISA

table 10: Secondary antibody for ELISA

Antibody	Cat.-number	Manufacturer
goat-anti-mouse IgG POD	1030-05	Southern Biotech

2.6 Buffers, media and solutions

table 11: PBS-solution

Solution	Components
PBS solution sodium phosphate 10 mM potassium chloride 2.68 mM sodium chloride 140 mM	1 PBS tablet 500 ml MilliQ water

table 12: Ammonium chloride potassium lysis buffer

Buffer	Composition
Ammonium chloride potassium lysis buffer **→ in MilliQ water**	150 mM NH_4Cl 10 mM $KHCO_3$ 0.1 mM Na_2EDTA

table 13: FACS buffer

Buffer	Composition
FACS buffer **→ in PBS**	2 % FCS 2 mM EDTA

table 14: IMDM complete

Medium	Composition
IMDM complete **→ in IMDM**	1 % Penicillin/Streptomycin 10 % FCS 0.25 mM β-Mercaptoethanol

table 15: Anesthesia

Medium	Composition
Animal anesthesia	1 ml ketamine (10 %) 0.5 ml xylazine (2 %) 8.5 ml PBS

table 16: PFA (4 %)

Solution	Composition
PFA (4 %)	4 g PFA 100 ml PBS

table 17: PFA (1 %)

Solutions	Composition
PFA (1 %)	3 ml PFA (4 %) 9 ml PBS

table 18: Percoll solution

Solution	Composition
Percoll solution **→ in MilliQ water**	1.5 M NaCl Percoll (stock solution)
final c NaCl	0.15 M
final density (d) Percoll solution	d = 1.041g/ml

table 19: Lung tissue enzymatic digestion medium

Medium	Composition
Lung tissue enzymatic digestion medium **→ in IMDM**	0.2 mg/ml Collagenase D 0.01 mg/ml DNAse 5 % FCS

table 20: Wash buffer IgG ELISA

Buffer	Composition
Wash buffer IgG-ELISA **→ in PBS**	0.05 % Tween20

table 21: Blocking buffer IgG ELISA

Buffer	Composition
Blocking buffer IgG-ELISA **→ in PBS**	10 % FCS

2.7 Software

table 22: Software

Software	Manufacturer
FinePointe v2.7.0	Date Sciences International, Minnesota, USA
FlowJo	Tree Star Inc., Ashland, USA
GraphPad Prism 8.0	GraphPad Software Inc., San Diego, USA
i-controlTM 2.0 (TECAN)	Tecan Group Ltd., Switzerland
Attune^{TM} NxT Software v3.1.1	Thermo Fisher Scientific, Massachusetts, USA

3 Methods

3.1 Mice

Experiments were performed in specific-pathogen free (SPF) female mice, 7-8 weeks of age upon the first treatment. C57BL/6JRj mice (Janvier Labs, Saint-Berthevin, France) were used unless otherwise indicated. For the establishment of the OVA-mediated mouse model of AAI, C57BL/6J mice were obtained from Charles River (Sulzfeld, Germany) and C57BL/6JRj mice from Janvier Labs. All mice were housed in individually ventilated cages in groups of 3-5 mice and were fed food and water *ad libitum*. All animal experiments were performed according to regional and institutional guidelines and approved by the Landesverwaltungsamt Sachsen-Anhalt (203.6.3-42502-2-1495).

3.2 Ovalbumin (OVA)-mediated mouse model of allergic airway inflammation (AAI)

3.2.1 Preparation of OVA-stocks

OVA-stocks for grade III OVA (≥ 90 %) and grade V OVA (≥ 98 %) were prepared in PBS. Lyophilized OVA was resuspended in sterile PBS, so that a stock concentration of 10 mg/ml was achieved for grade III OVA. For grade V OVA, a stock concentration of 500 ng/ml was prepared likewise. OVA-stocks were stored at -20 °C until further use.

3.2.2 Intraperitoneal OVA-sensitization

Mice were sensitized with a mixture of OVA (grade V, Sigma-Aldrich) and the adjuvant aluminum hydroxide (Imject^{TM} Alum Adjuvant, Thermo Fisher Scientific). For each sensitization, the OVA/aluminum hydroxide solution was freshly prepared. For each mouse 3.6 µl OVA-stock (10 µg OVA; see above) were pipetted into 151.4 µl sterile PBS. After adding 25 µl alum dropwise to the OVA-solution, the OVA/aluminum hydroxide-solution was incubated for 30 minutes on a shaker (100 rpm). Mice were sensitized *i.p.* (180 µl/mouse) either two or three times in weekly intervals (d 0 and 7 or 0, 7 and 14). Control animals were

i.p. treated with 180 µl PBS containing 1 mg aluminum hydroxide only (165 µl PBS + 25 µl aluminum hydroxide).

3.2.3 Intranasal OVA-challenge

One week after the last sensitization (d 14 or d 21), mice were *i.n.* challenged under light isoflurane anesthesia.

For the experiments establishing and characterizing the murine model of OVA-induced AAI, the isoflurane anesthesia was performed in a beaker covered with aluminum foil. A small amount of isoflurane was applied to a paper towel, which was placed in the beaker and covered with another paper towel to avoid direct contact between the animal and the isoflurane. Single mice were placed in the beaker and carefully observed. Upon the induction of the anesthesia, the anesthetized mice were quickly removed from the beaker, held in an upright position and 30 µl PBS containing 10 µg or 250 µg OVA (grade III, Sigma-Aldrich) were pipetted dropwise alternately on both nostrils. The respective solution was freshly prepared before each challenge (10 µg OVA: 1 µl OVA-stock solution (10 mg/ml; 1:10 diluted in PBS) + 29 µl sterile PBS; 250 µg: 2,5 µl OVA-stock solution (10 mg/ml) + 27,5 µl sterile PBS). After the *i.n.* challenge, mice were kept in an upright position for about 30 seconds to ensure inhalation of the entire fluid. Subsequently, mice were returned to their cage and monitored until fully awake. Mice were challenged on two or three consecutive days, depending on the experimental protocol.

For anesthesia induction with the combi-vet® Base Anesthesia System, mice were placed in an anesthesia induction chamber, where they were anesthetized through inhalation of 5 % isoflurane supplemented with oxygen at a flow rate of 1 liter/minute in ambient air. After the induction of anesthesia, the isoflurane concentration was reduced to 1-2 % in order to ensure continued anesthesia. Anesthetized mice were removed from the induction chamber one by one, held in an upright position and 30 µl PBS containing 100 µg (grade III, Sigma-Aldrich) were pipetted dropwise alternately on both nostrils. The solution was freshly prepared before each challenge (10 µl OVA-stock solution (10 mg/ml) + 20 µl sterile PBS). After the *i.n.* challenge, mice were kept in upright position for about 30 seconds to ensure inhalation of the entire fluid. Subsequently, mice were returned to their cage and monitored until fully awake. Mice

were challenged on two or three consecutive days. Control animals were *i.n.* treated with 30 µl PBS.

48 h after the last challenge, mice were sacrificed for analysis.

3.3 Staphylococcal enterotoxin B (SEB)

3.3.1 Preparation of an SEB-stock and treatment dilutions

Before used for animal treatment, an SEB-stock solution was prepared from lyophilized SEB in sterile PBS. The stock solution was prepared at a protein concentration of 244.5 µg/ml and was stored in aliquots of 10 or 25 µl at -20 °C until further use.

Before *i.n.* SEB-treatment, the respective treatment solution was freshly prepared as follows: For *i.n.* challenge with 500 ng SEB in 30 µl PBS, 2.05 µl of the stock solution were diluted in 27.95 µl sterile PBS for each animal to be treated. For the preparation of the 50 ng SEB dose, the stock solution was first diluted 1:10 in sterile PBS and 2.05 µl of the 1:10 diluted stock were further diluted in 27.95 µl sterile PBS.

3.3.2 Intranasal SEB-treatment

For *i.n.* SEB-treatment, mice underwent light isoflurane anesthesia as described above (see 3.2.3, p. 35). Mice were held in an upright position and the SEB-solution (30 µl) was pipetted dropwise alternately onto both nostrils. Mice were kept in upright position for another 30 seconds to ensure the entire volume was inhaled. Subsequently, mice were placed back into their cage and monitored until fully awake.

3.4 Health surveillance

All animals were closely monitored for immediate reactions right after *i.p.* sensitizations as well as after *i.n.* challenge and administration of SEB. During the entire duration of the experiments, the well-being of the animals was regularly assessed with the respect to body weight loss, general condition, spontaneous behavior and clinical findings according to a sore

sheet. The score sheet was designed to prevent any overt suffering of the mice by defining clear termination criteria for all experiments.

3.5 Sample preparation

3.5.1 Preparation of serum from whole blood

Blood was collected *post mortem* through cardiac puncture or punctuation of the retroorbital plexus and was centrifuged (10 minutes, 1500 × g, 4 °C) after incubation at 37 °C for 20 minutes and at 4 °C for 5 minutes. The collected serum was stored in aliquots at -80 °C until further analysis.

3.5.2 Preparation of bronchoalveolar lavage (BAL)

The trachea was dissected and punctured with a 20G indwelling venous cannula (Braun). Using a 1 ml syringe, the lungs were flushed once through the cannula and trachea with 1 ml cold sterile PBS to obtain bronchoalveolar lavage (BAL). After centrifugation of the BAL fluid (10 minutes, 4000 × g, 4 °C) the supernatant was separated from the cell pellet, centrifuged (10 minutes, 10 000 × g, 4 °C) for the removal of debris, aliquoted and stored at -80 °C until further analysis. BAL cells were resuspended in 200 µl ACK-Lysis buffer (see table 12, p. 31) and after a short incubation of 30 – 60 sec, 1 ml PBS was added. After centrifugation of the sample (10 minutes, 2000 × g, 4 °C), BAL cells were resuspended in 200 µl PBS and analyzed using flow cytometry.

3.6 Isolation of leukocytes from the lungs, spleen and lymph nodes

Lavaged lungs were perfused with 10 ml cold sterile PBS through punctuation of the right ventricle, excised and minced using scissors on ice. For enzymatic digestion, 3 ml of Iscove's modified Dulbecco's medium containing 0.2 mg/ml Collagenase D (Sigma-Aldrich), 0.01 mg/ml DNase (Sigma-Aldrich) and 5 % fetal calf serum were added. Samples were incubated for 30 minutes at 37 °C in a water bath and shortly mixed in 5-minute intervals using disposable transfer pipettes. Subsequently, 2 ml fresh digestion medium were added and

samples were likewise incubated for an additional 15 minutes. After the addition of 60 µl 0.5 M ethylenediaminetetraacetic acid (EDTA) (5 mM final concentration) to stop the digestion process, samples rested for 5 minutes. Suspensions were then filtered through a cell strainer (70 µm) and pelleted by centrifugation (10 minutes, 330 × g, 4 °C). For erythrocyte lysis by osmotic shock, the centrifuged cells were resuspended in 1 ml ACK-Lysis buffer (see table 12, p., 31) and incubated for 2 minutes. Lysis was stopped by adding 8 ml FACS-buffer (see table 13, p. 31). After centrifugation (10 minutes, 330 × g, 4 °C) of the samples, leukocyte enrichment was performed using a Percoll solution (GE Healthcare Life Sciences, see table 18, p. 32) and density gradient centrifugation (20 minutes, 360 × g, RT, w/o break). Splenocytes as well as leukocytes from lymph nodes (cervical, bronchial and mesenteric) were isolated by homogenization of spleens/lymph nodes in PBS through a 70 µm cell strainer using a syringe plunger followed by centrifugation of the cell suspension (5 minutes, 330 × g, 4 °C). Splenocytes were resuspended in 3 ml ACK-Lysis buffer and incubated for 2 minutes. After adding 10 ml FACS-buffer the cells were centrifuged (5 min, 330 × g, 4 °C) and resuspended in 3 ml FACS-buffer/spleen. Isolated leukocytes from lymph nodes were resuspended in 1 ml FAC-buffer/sample. Splenocytes as well as isolated lymph node cells were analyzed using flow cytometry.

3.7 Flow cytometry

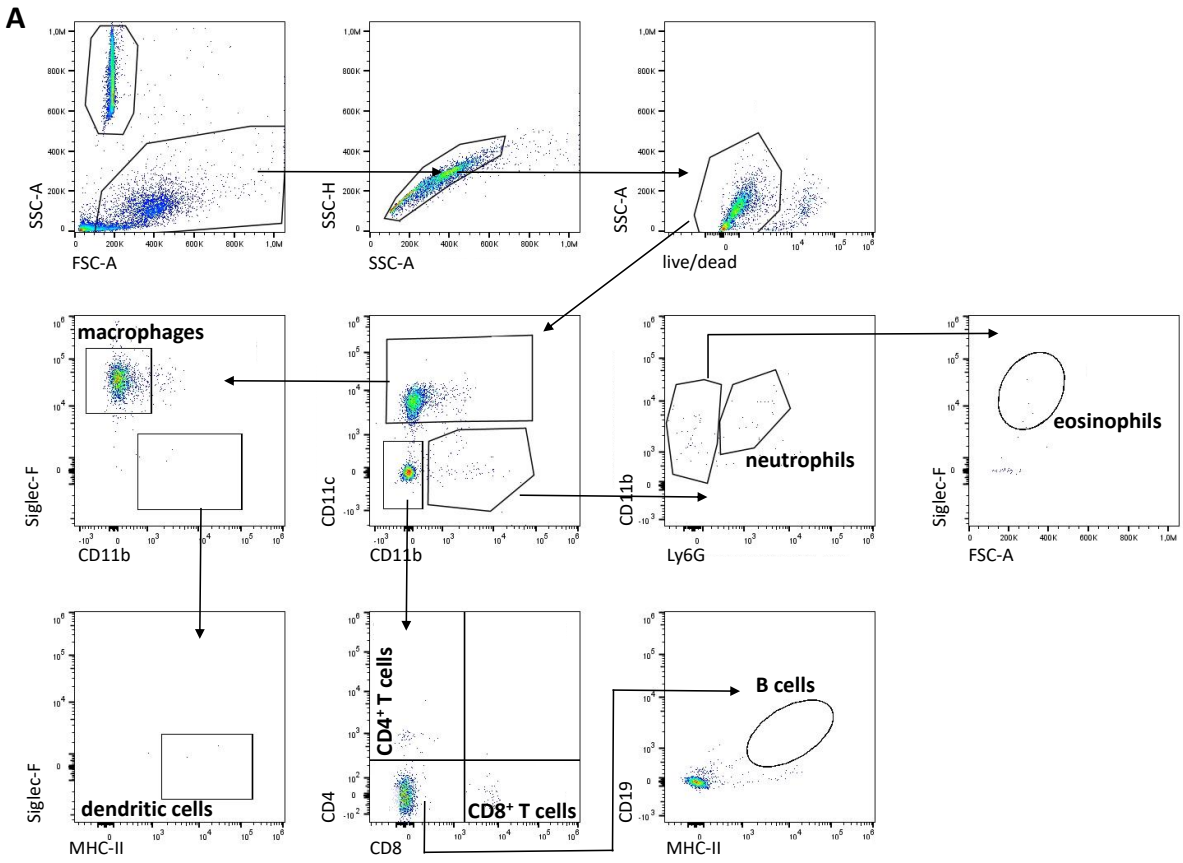
Leukocytes from BAL, lung, spleen and lymph nodes (see 3.6, p. 37) were incubated for blocking of Fc-receptors (anti CD16/CD36) and simultaneously stained with fixable live/dead stain (Biolegend) for 30 minutes at RT in the dark. After washing the cells using 150 µl FACS-buffer (see table 13, p. 31) they were centrifuged (5 minutes, 1200 rpm, 4 °C) and antibody stainings were performed for B220, CD3, CD4, CD8, CD11b, CD11c, CD49b, CD69, CD117, FcεRIα, Ly6C, Ly6G, MHCII, NK1.1, Siglec F (ThermoFisher) and ST2 in different combinations (see table 7, table 8 and table 9 above). All antibodies were purchased from Biolegend unless indicated otherwise. Antibodies were diluted in FACS-buffer and cells were stained in 100 µl of the respective antibody cocktail. After incubation with the antibodies (15 min, RT, in the dark), the cells were washed using 150 µl PBS, centrifuged (5 minutes, 1200 rpm, 4 °C) and fixed by resuspension and incubation in 100 µl paraformaldehyde (1 % in PBS, see table 17,

p. 32, 20 minutes, RT, in the dark). Following another washing step (addition of 150 µl PBS) and centrifugation (5 minutes, 1200 rpm, 4 °C), cells were resuspended in 350 µl PBS for flow cytometric analysis.

Data were acquired using an Attune NxT instrument (ThermoFisher) and analyzed using the FlowJo software (Tree Star). Single stainings were performed for all fluorochromes for compensation using UltraComp eBeads (ThermoFisher) and fluorescence-minus-one stainings were performed on splenocytes for gating. For the calculation of absolute cell numbers from relative frequencies (total cells/measured population), 50.000 fluorescent beads (Precision Count Beads, BioLegend) were added to each sample. Absolute cell numbers were calculated as cells/ml BAL, cells/spleen, cells/lung and cells/lymph nodes. Following singlet-gating using the FSC-laser (FSC-H/FSC-A) and dead cell exclusion using live/dead staining, cell populations were gated as described below for the different staining approaches.

3.7.1 Flow cytometry staining panel 1 (BAL)

Leukocytes from BAL (the entire amount of isolated cells/mouse BAL; see 3.6, p. 37) were stained with flow cytometry staining panel 1 (see table 7, p. 29): Live single cells were gated for $CD11c^+$, $CD11b^+/CD11c^-$ and $CD11b^-/CD11c^-$ cells. $CD11c^+$ cells were further divided into macrophages (gated as $CD11c^+$/Siglec F^+) and Siglec F^- cells from which DCs ($CD11c^+$/Siglec F^-/$MHCII^+$) were gated using MHCII as a marker. $CD11b^+/CD11c^-$ cells were further gated for the Ly6G and Siglec F markers. Neutrophils were gated as $CD11b^+/CD11c^-/Ly6G^+$ cells and eosinophils as $CD11b^+/CD11c^-/Ly6G^-$/Siglec F^+ cells. $CD4^+$ T cells were gated as $CD11b^-/CD11c^-/CD4^+$ and $CD8^+$ T cells as $CD11b^-/CD11c^-/CD8^+$ cells. B cells were gated as $CD11b^-/CD11c^-/CD4^-/CD8^-/CD19^+/MHCII^+$.



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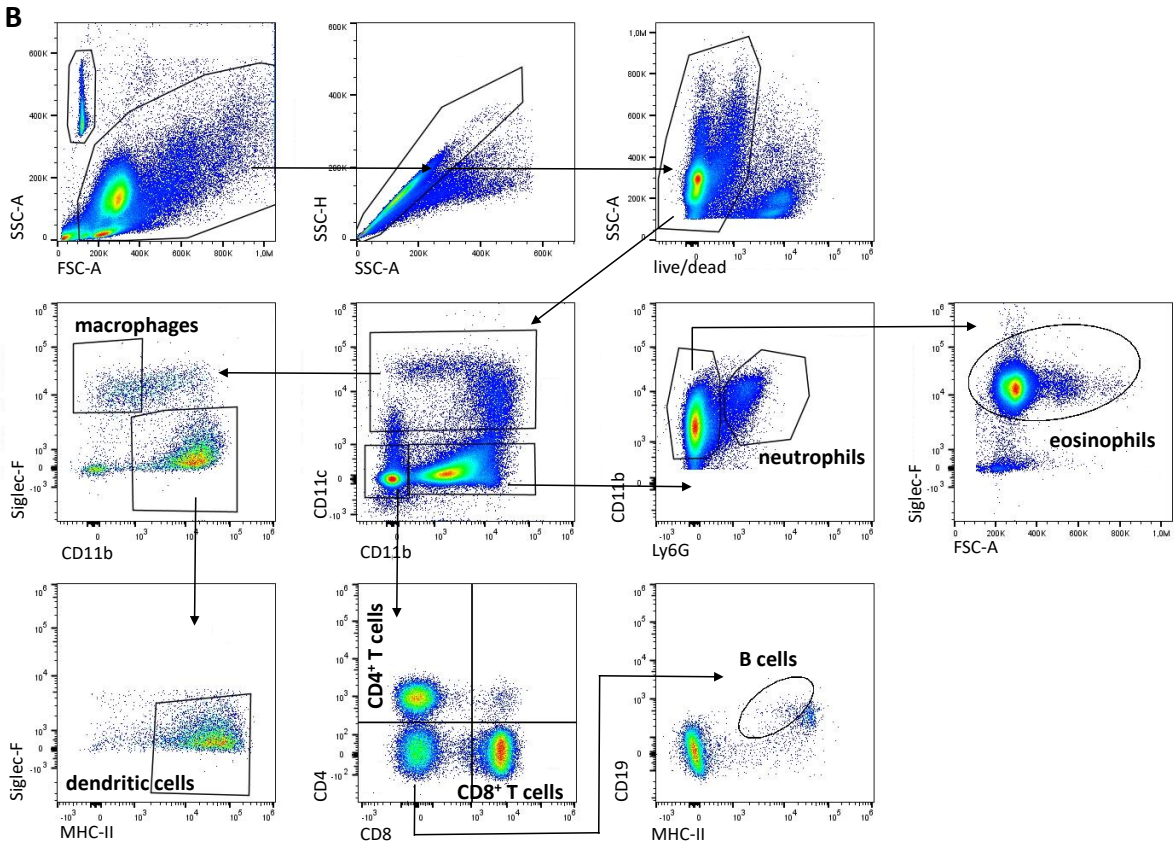
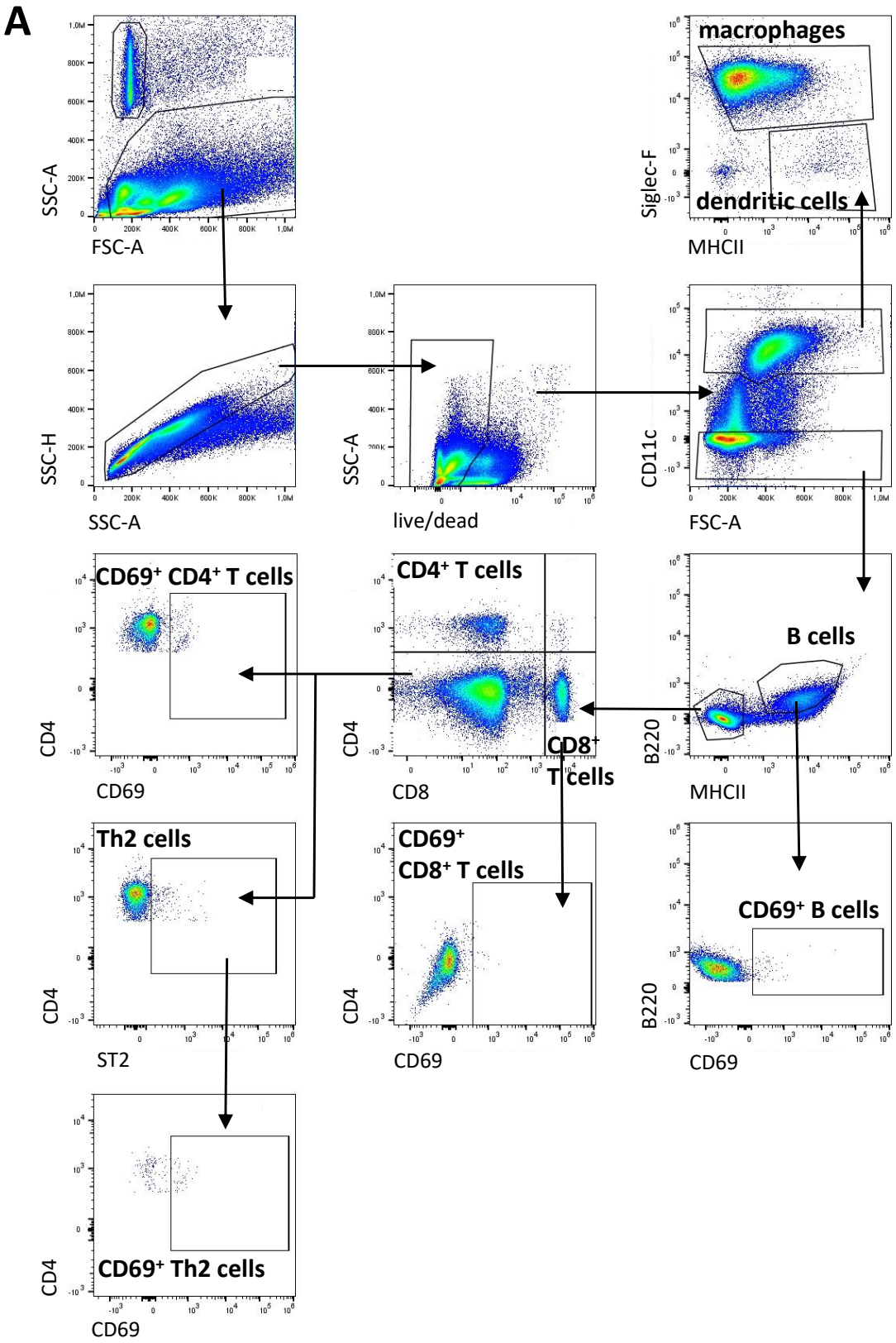


figure 6: Gating strategy panel 1.
After the exclusion of doublets and dead cells, cells were divided into $CD11c^+$, $CD11b^+/CD11c^-$ and $CD11b^-/CD11c^-$ cells. $CD11c^+$ cells were further divided into macrophages (gated as $CD11c^+/Siglec\ F^+$) and $Siglec\ F^-$ cells from which DCs ($CD11c^+/Siglec\ F^-/MHCII^+$) were gated using MHCII as a marker. $CD11b^+/CD11c^-$ cells were further gated for the Ly6G and Siglec F markers. Neutrophils were gated as $CD11b^+/CD11c^-/Ly6G^+$ cells and eosinophils as $CD11b^+/CD11c^-/Ly6G^-/Siglec\ F^+$ cells. $CD4^+$ T cells were gated as $CD11b^-/CD11c^-/CD4^+$ and $CD8^+$ T cells as $CD11b^-/CD11c^-/CD8^+$ cells. B cells were gated as $CD11b^-/CD11c^-/CD4^-/CD8^-/CD19^+/MHCII^+$.
Displayed here are representative examples of an OVA-sensitized and PBS challenged mouse (A) and an OVA-sensitized and OVA-challenged mouse (B).

3.7.2 Flow cytometry staining panel 2

Using the flow cytometry staining panel 2 (see table 8, p. 29), from live single cells from lung leukocytes (half of the isolated leukocytes/mouse lung), splenocytes (1×10^6 cells) and single cell suspensions from lymph nodes (the entire amount of isolated cells/mouse; see 3.6, p. 37) $CD11c^+$ and $CD11c^-$ cells were gated. The latter were further gated by using the B220 and MHCII markers. B cells were gated as $CD11c^-/B220^+/MHCII^+$ cells. The remaining $CD11c^-/B220^-/MHCII^-$ cells were further gated for $CD4^+$ T cells ($CD11c^-/B220^-/MHCII^-/CD4^+$) and $CD8^+$ T cells ($CD11c^-/B220^-/MHCII^-/CD8^+$). Th2 cells were gated as $ST2^+$ $CD4^+$ T cells. The activation status of different cell types was determined using the CD69 marker while gating on $CD69^+$ cells within the respective population.



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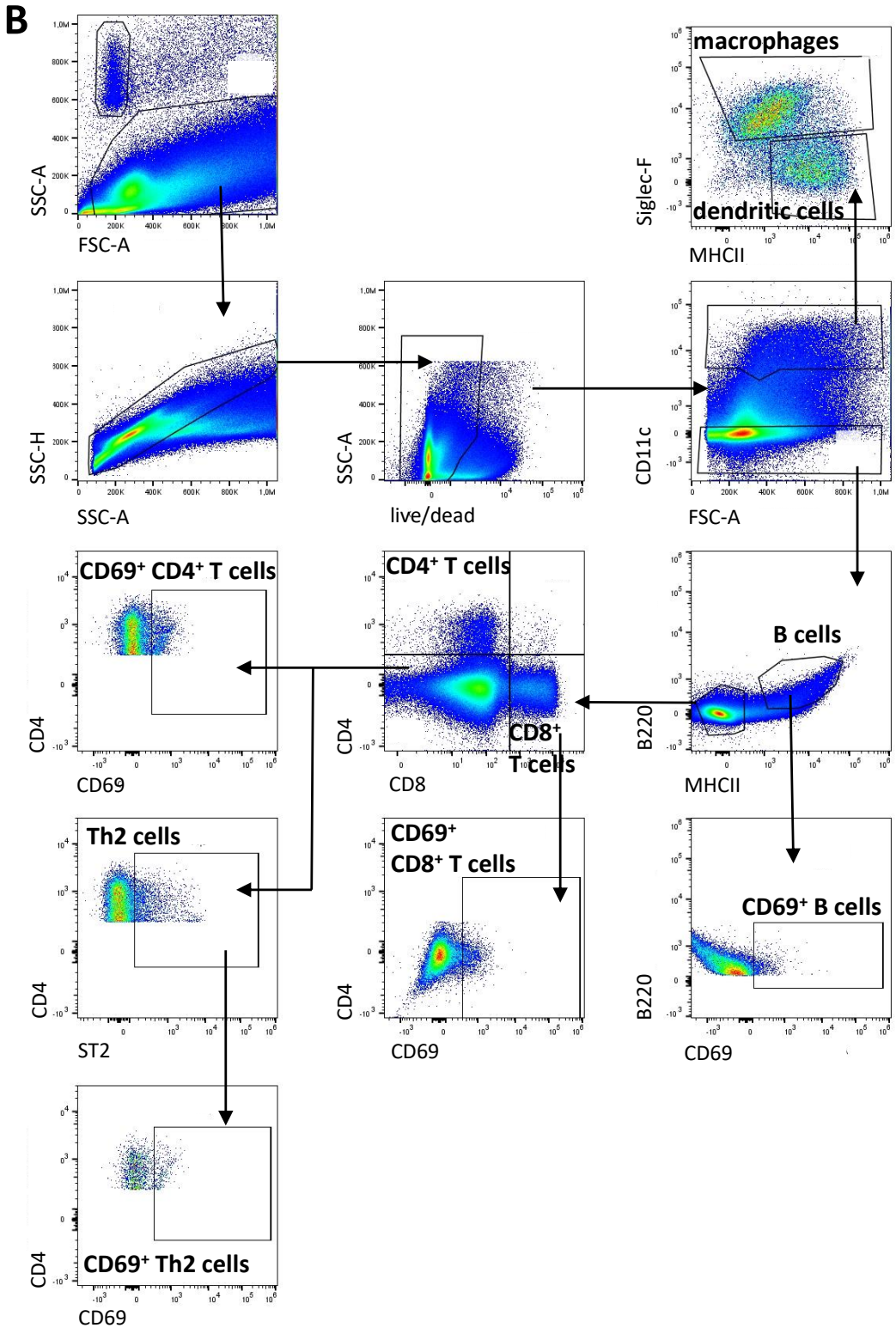
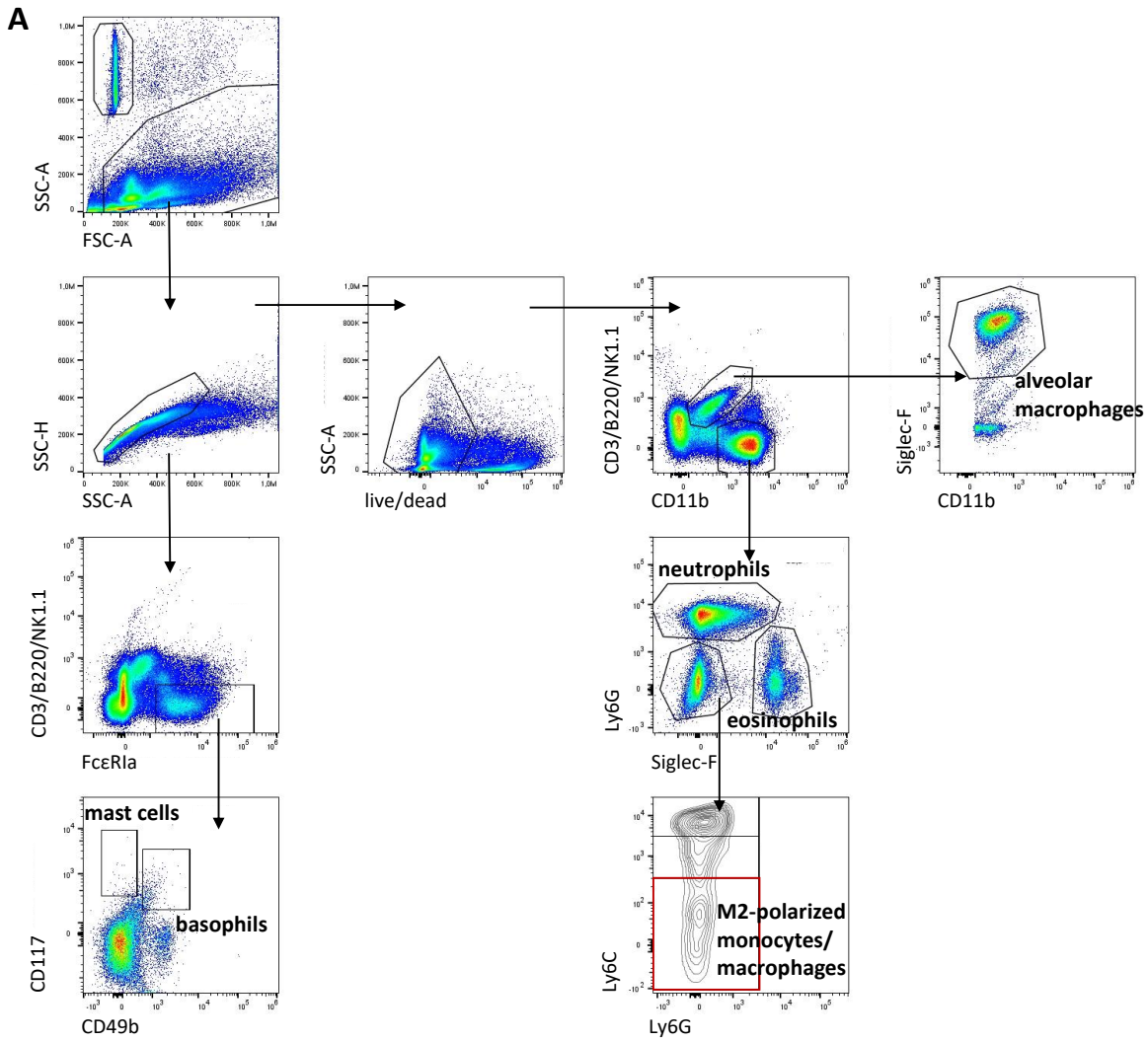


figure 7: Gating strategy panel 2.
After the exclusion of doublets and dead cells, cells were divided into $CD11c^+$ and $CD11c^-$ cells. The latter were further gated for the B220 and MHCII markers. B cells were gated as $CD11c^-/B220^+/MHCII^+$ cells. $CD11c^-/B220^-/MHCII^-$ were further gated for T helper cells ($CD11c^-/B220^-/MHCII^-/CD4^+$) and cytotoxic T cells ($CD11c^-/B220^-/MHCII^-/CD8^+$). Th2 cells were gated as $ST2^+$ T helper cells. The activations status of different cell subsets was determined using the CD69 marker. Displayed here is a representative OVA-sensitized and PBS-challenged control mouse (A) and an OVA-sensitized, OVA-challenged mouse (B).

3.7.3 Flow cytometry staining panel 3

In addition to staining panel 2 (see table 8, p. 29), leukocytes from lung and spleen were stained with staining panel 3 (see table **9**, p. 30) for the identification of (alveolar) macrophages, monocytes/macrophages, DCs, neutrophils, eosinophils basophils and mast cells. In lung leukocytes, alveolar macrophages were gated from live single cells as $CD11b^-$ /Siglec F^+ autofluorescent cells. In lung leukocytes, splenocytes and single cells isolated from lymph nodes (see 3.6, p. 37) $autofluorescence^-/CD3^-/NK1.1^-/B220^-/CD11b^+$ cells were gated from live single cells and further gated for neutrophils ($CD3^-/NK1.1^-/B220^-$ $/CD11b^+/Ly6G^+/Siglec\ F^-$) and eosinophils ($CD3^-/NK1.1^-/B220^-/CD11b^+/Ly6G^-/Siglec\ F^+$). $CD3^-$ $/NK1.1^-/B220^-/CD11b^+/Ly6G^-/Siglec\ F^-$ cells were further gated for inflammatory monocytes/macrophages ($CD3^-/NK1.1^-/B220^-/CD11b^+/Ly6G^-/Siglec\ F^-/Ly6C^{high}$) and M2-polarized monocytes/macrophages ($CD3^-/NK1.1^-/B220^-/CD11b^+/Ly6G^-/Siglec\ F^-/Ly6C^{low}$) by using the Ly6C marker. Mast cells and basophils were gated from singlets without prior dead cell exclusion. Mast cells were gated as $FcεRIa^+/CD117^+/CD49^-$ and basophils as $FcεRIa^+/CD117^+/CD49^+$ cells.



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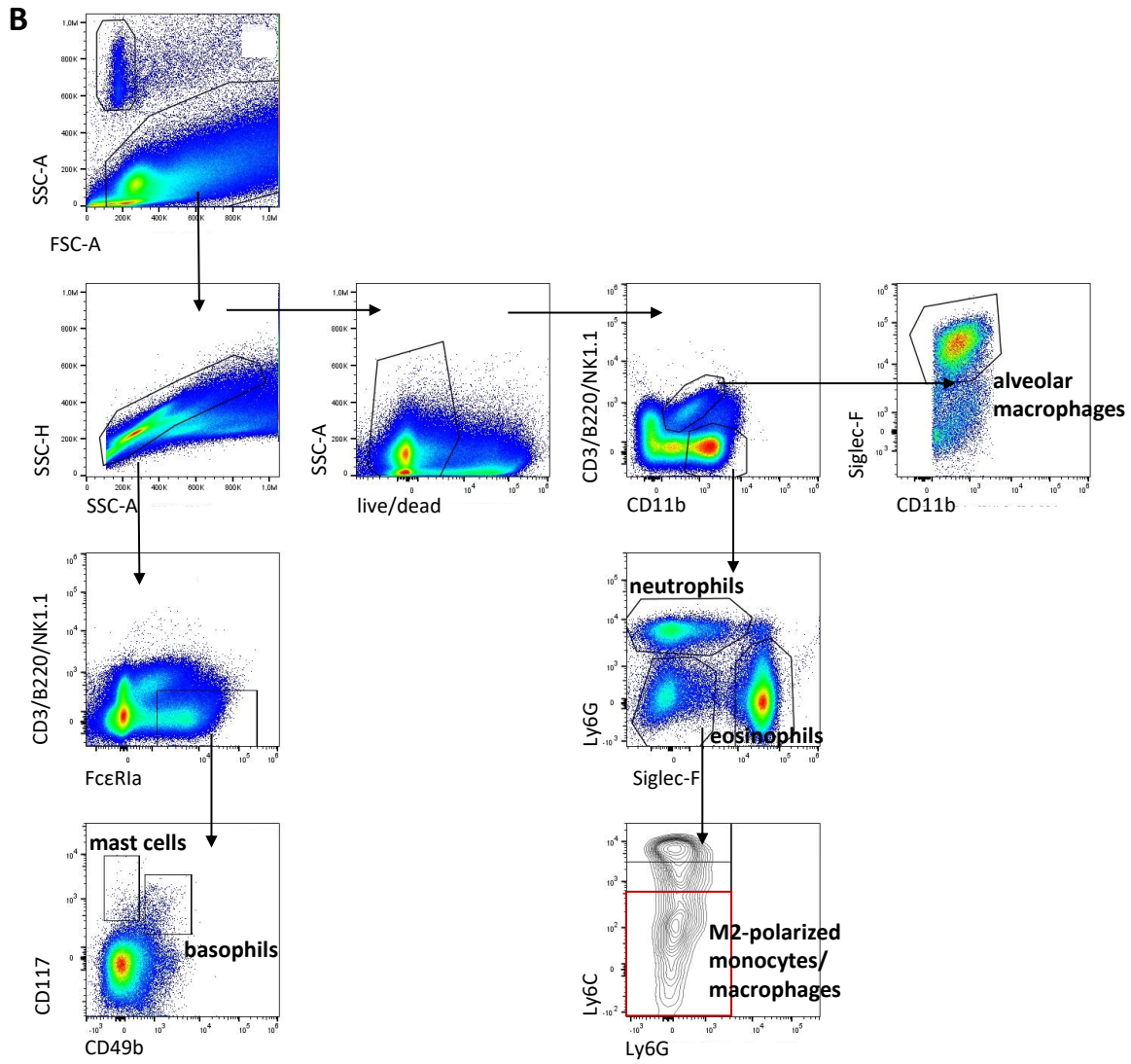

figure 8: Gating strategy panel 3.
After the exclusion of doublets and dead cells, alveolar macrophages were gated as autofluorescence^{+}/CD11b^{-}/Siglec F^{+} cells. In addition, CD3^{-}/NK1.1^{-}/B220^{-}/CD11b^{+} cells were gated form live singlets. These were further gated for into neutrophils (CD3^{-}/NK1.1^{-}/B220^{-}/CD11b^{+}/Ly6G^{+}/Siglec F^{-}) and eosinophils (CD3^{-}/NK1.1^{-}/B220^{-}/CD11b^{+}/Ly6G^{-}/Siglec F^{+}). CD3^{-}/NK1.1^{-}/B220^{-}/CD11b^{+}/Ly6G^{-}/Siglec F^{-} cells were gated for inflammatory monocytes/macrophages (CD3^{-}/NK1.1^{-}/B220^{-}/CD11b^{+}/ Ly6G^{-}/Siglec F^{-}/Ly6C^{high}) and M2-polarized monocytes/macrophages (CD3^{-}/NK1.1^{-}/B220^{-}/CD11b^{+}/Ly6G^{-}/Siglec F^{-}/Ly6C^{low}) by using the Ly6C marker. Mast cells and basophils were gated from single cells without prior dead cell exclusion. Mast cells were gated as FcεRIα^{+}/CD117^{+}/CD49^{-}cells and basophils as FcεRIα^{+}/CD117^{+}/CD49^{+} cells. Displayed here is a representative OVA- sensitized, PBS-challenged control mouse (A) and an OVA-sensitized, OVA-challenged mouse (B).

3.8 Enzyme-linked immunosorbent assay (ELISA)

3.8.1 OVA-IgE ELISA

OVA-specific IgE was detected in serum samples by a specific enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's recommendations (LEGEND MAX™ Mouse OVA Specific IgE ELISA Kit, BioLegend). At first, 50 µl Matrix A were pipetted into standard wells and 50 µl Assay buffer A into sample wells. For generating a standard curve, the reconstituted provided standard (starting with a concentration of 10 ng/ml), was diluted in 1:2 steps down to a concentration of 0.156 ng/ml. Next, 50 µl of 1:2 diluted serum samples (see 3.5.1, p. 37) were added to the sample wells of the pre-coated plate. After a 2 h incubation period (RT, shaking 100 rpm), the plate was washed four times by pipetting and discarding 250 µl wash buffer for every washing step. Afterwards, 100 µl detection antibody was added to the plate and incubated for an additional 1 h at room temperature while shaking (100 rpm). After the plates were washed (four times, 250 µl wash buffer at each step), 100 µl Streptavidin HRP D solution was added to the wells. After incubation (30 min, RT, shaking 100 rpm) and another washing step (five times, 250 µl washing buffer at each step), 100 µl substrate solution F was added to the wells and incubated for 15 minutes at RT in the dark. The substrate reaction was stopped by adding 100 µl Stop Solution to the wells.

The absorbance of the samples was measured at 450 nm using the Tecan Infinite® M Plex Photometer and the data were analyzed using Microsoft excel. For the analysis, a standard curve was generated using the absorbance of the standard wells and their known concentration. The concentration (ng/ml) of OVA-specific IgE in sample wells was determined from the acquired absorbance and the standard curve.

3.8.2 OVA-IgG ELISA

In 96°-plates (Nunc Maxisorb, Thermo Scientific), the wells were coated by incubation with 100 µl OVA (10 µg/ml; grade III, Sigma-Aldrich) diluted in coating buffer (CANDOR) overnight (4 °C). Following coating, wells were washed three times with 250 µl washing buffer (see table 20, p. 32) and blocked with 200 µl blocking buffer (see table 21, p. 32) for 1 h at RT, while shaking (100 rpm). After blocking, the plates were washed three times with 250 µl wash buffer

and diluted serum samples (see 3.5.1, p. 37) were pipetted into the wells in duplicates (serum samples of sensitized animals were diluted 1:500 in blocking buffer, serum samples of control animals were diluted 1:100 in blocking buffer) and incubated (1 h, RT, shaking 100 rpm). After another washing step (three times with 250 µl washing buffer/each step) 50 µl of the diluted detection antibody (1:10 000; goat-anti-mouse IgG POD, Southern Biotech) were added to the wells. Following incubation (1 h, RT, shaking), the wells were washed three times with 250 µl washing buffer and incubated for 15 minutes in the dark with the substrate solution (50 µl/well). To stop the reaction, 20 µl sulfuric acid were pipetted into the wells.

The absorbance was immediately acquired at 450 nm using the Tecan Infinite® M Plex Photometer and the data were analyzed using Microsoft excel. For the analysis, the mean absorbance of duplicates was calculated, the background was subtracted and values were multiplied by the respective dilution factor. Results are presented as absorbance (OD_{450}).

3.9 Multiplex quantification of cytokines in bronchoalveolar lavage (BAL)

Concentrations of different cytokines (IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-17A, IL-17F, IL-21, IL-22, and TNF-α) were simultaneously detected in the supernatant of the BAL (see 3.6, p. 37) using a cytometric bead array according to the manufacturer's recommendations (LEGENDplex™ Th cytokine panel, BioLegend). First, 12.5 µl Assay Buffer were added to the wells of the included 96-well plate. For generating a standard curve, the top standard was diluted six times in 1:4 steps and 12.5 µl of each standard dilution were added to the standard wells. At the same time, 12.5 µl undiluted BAL samples were added in duplicates into sample wells. Next, 12.5 µl of the provided bead-mix were added to each well. The plate was incubated over night at 4 °C while shaking (200 rpm). After centrifugation (5 minutes, 1050 rpm, 4 °C), the supernatant was discarded and the plates were washed twice using 200 µl wash buffer. Next, 12.5 µl of the detection antibody were added to the wells, followed by incubation at RT while shaking (200 rpm) for 1 h. Without washing, 12.5 µl of the provided streptavidin R-Phycoerythrin solution were added to the wells and incubated for 30 minutes at RT, shaking at 200 rpm. The plates were centrifuged (5 minutes, 1050 rpm, 4 °C), the supernatant was discarded and wells were washed using 200 µl washing buffer.

Beads were transferred into acquisition tubes and data were acquired using an Attune NxT flow cytometer and analyzed using the LEGENDplex v8.0 software.

3.10 Measurement of airway hyperreactivity

Airway hyperreactivity was assessed two days following the last OVA-challenge. Airway hyperreactivity or hyperresponsiveness describes the excessive reaction of the smooth muscle cells of the respiratory tract to a certain stimulus, that would produce no or only little effect in healthy individuals (Chapman & Irvin, 2015). To assess airway hyperreactivity, airway resistance, which refers to degree of resistance to the flow of air through the respiratory tract during inspiration and expiration is measured following the administration of increasing doses of methacholine to the airways.

At first, methacholine (Sigma) was dissolved in sterile PBS to obtain a solution with a concentration of 100 mg/ml. This solution was diluted four times in 1:2 dilutions, resulting in 50 mg/ml, 25 mg/ml, 12.5 mg/ml and 6.25 mg/ml methacholine solutions.

The mice were anesthetized and tracheostomized. After intubation, they were mechanically ventilated at a rate of 100 breaths/minute and placed into the acquisition chamber. Methacholine nebulization, challenge and subsequent measurements were performed using the Buxco FinePointe Resistance and Compliance system (DSI™ USA). During the whole procedure, the electrocardiogram (ECG) and heart rate of the animals were monitored. After a five minutes acclimatization period, 10 µl of the lowest concentrated methacholine solution (0 mg/ml; PBS) were administered over the intubation tube over a period of 20 seconds by the use of an integrated ultrasonic nebulizer. The airway resistance in response to the different methacholine doses (6, 25, 12.5, 25, 50, 100 mg/ml) was calculated based on the single compartment lung model, using the lung pressure and air flow values that were continuously measured for three minutes in response to each dose. Thereby, for each individual animal and methacholine concentration, the average resistance over the entire respective three min response time was assessed. Between the administration of each of the increasing concentrations of methacholine, there was a one minute return-to-baseline-period. The data were analyzed using the FinePoint™ software.

3.11 Histopathological analysis

The preparation of sections as well as the histopathological analysis were performed in cooperation with Dr. Olivia Kershaw (Institute of Veterinary Pathology, FU Berlin), a veterinary pathologist certified by the European College of Veterinary Pathologists. Whole lungs were fixed in 4 % paraformaldehyde and routinely embedded in paraffin. Sections (5 µm) were cut, dewaxed and stained with hematoxylin and eosin (H&E) or Azan. Blinded histological evaluation was performed by Dr. Olivia Kershaw (FU Berlin). The grade, extent and pattern of inflammation were classified and scored. The scoring of lung inflammation was based on the number, kind (eosinophils, neutrophils, macrophages, lymphocytes) and location (interstitial, perivascular, intra-alveolar/intrabronchial) of infiltrating cells, as well as goblet cell hyperplasia (grade 0, not detected; grade 1, minimal; grade 2, mild; grade 3, moderate; and grade 4, severe). Additionally, the grade of fibrosis was examined.

3.12 Statistical analysis

All statistical analyses were performed using the Graph Pad Prism software version 8 (Graph Pad Software).

To assess the induction of AAI, comparison of all treatment groups to the control group was performed. To assess the effects of *i.n.* SEB-treatment on AAI, comparisons between all treatment groups were performed. Data for all experimental groups were tested for normality using the Shapiro-Wilk normality test. In case of Gaussian distribution for all groups in a comparison, one-way ANOVA and Bonferroni post-hoc testing was performed. In the case of non-Gaussian distribution in at least one of the groups in a comparison, Kruskal-Wallis testing with Dunn's post- hoc testing was performed.

To assess long-term SEB effects over time, comparison between all treatment groups and different time points were performed. Data for all experimental groups were tested for normality using the Shapiro-Wilk normality test. In case of Gaussian distribution for all groups in a comparison, two-way ANOVA and Tukey's multiple comparison test were performed. In case of non-Gaussian distribution in at least one of the groups in a comparison, mixed-effects analysis with Tukey's multiple comparison test was performed. $P \leq 0.05$ was considered indicative of statistical significance (*$p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.0001$).

4 Results

4.1 Establishment of an OVA-induced mouse model of AAI for the analysis of SEB-mediated effects

The induction of AAI in mice through the model antigen OVA can be achieved through a peripheral sensitization with the antigen OVA in combination with alum as an adjuvant (*i.p.* injection) and the local (*i.n.*) challenge of sensitized animals with the antigen OVA only.

Published models for OVA-mediated AAI in mice vary widely with respect to the frequency, timing and dosing of the allergic sensitization and challenge. To determine the experimental regime best suited for the aim of this thesis, i.e. the analysis of SEB-mediated effects on AAI, six different treatment regimens were tested, based on previously published models (Herz *et al.*, 1999; Huvenne *et al.*, 2010; Huvenne *et al.*, 2013; Kepert *et al.*, 2017; Klassen *et al*., 2017). These varied in the frequency and antigen concentration for the peripheral sensitization and local challenge (see table 23, p. 52). Main criteria for the selection of the final mouse model included the immune cellular influx in the lungs and BAL, the local cytokine response in the BAL and the OVA-specific IgE concentration in the serum.

table 23: Treatment regimen initially selected for from the literature the establishment of a mouse model of OVA-mediated AAI

	sensitization **(10 µg OVA in 1 mg alum; in weekly intervals)**	challenge **(OVA in PBS; in daily intervals; from one week after the last sensitization)**		designation of treatment groups **in the figure legends (figures 9-11)**
		10 µg OVA	250 µg OVA	
1.	2 ×	2 ×		10-10/10-10
2.	2 ×	3 ×		10-10/10-10-10
3.	3 ×	2 ×		10-10-10/10-10
4.	3 ×	3 ×		10-10-10/10-10-10
5.	2 ×		2 ×	10-10/250-250
6.	2 ×		3 ×	10-10/250-250-250

For any experimental setup and animal model used to induce a certain biological response or to examine a specific hypothesis, it is of crucial importance to assess the efficiency and specificity of any treatment condition with appropriate control groups. For establishing a suited mouse model of AAI, five different control groups displaying different combinations of control treatments were included in the experiments (see table 24, p. 53).

table 24: Control groups analyzed for establishing the mouse model of AAI.
Framed in red: control group, chosen for later experiments and typically shown in result figures

	sensitization **(in weekly intervals)**		challenge **(in PBS; in daily intervals; from one week after the last sensitization)**			name of control groups **in the figure legends**
	10 µg OVA in 1 mg Alum	1 mg alum in PBS (alum only; a.o.)	10 µg OVA in PBS	250 µg OVA in PBS	PBS only	
1.	3 x				2 x	10-10-10/sal-sal
2.	3 x				3 x	10-10-10/sal-sal-sal
3.		2 x	2 x			a.o.-a.o./10-10
4.		2 x	3 x			a.o.-a.o./10-10-10
5.		2 x		2 x		a.o.-a.o./250-250

4.1.1 Health burden following OVA allergic sensitization and challenge

In order to establish a reliable and consistent mouse model of AAI, different treatment regimen (see table 23, p. 52) as well as control groups (see table 24, p. 53) were analyzed. Mice were *i.p.* sensitized two or three times (d 0, 7, 14) with 10 µg OVA in 1 mg alum and then *i.n.* challenged two or three times from one week after the last sensitization (d 14 or 21) with either 10 µg or 250 µg OVA in PBS. Control groups were either sensitized with 10 µg OVA/alum and then mock-challenged with PBS or mock-sensitized with alum only and then *i.n.* challenged with 10 µg or 250 µg OVA. Because there were no notable or significant differences between the different control groups (data not shown) and for reasons of clarity, in the following figures only one control group is shown (3x sensitized with OVA in alum, 3x mock-challenged with PBS).

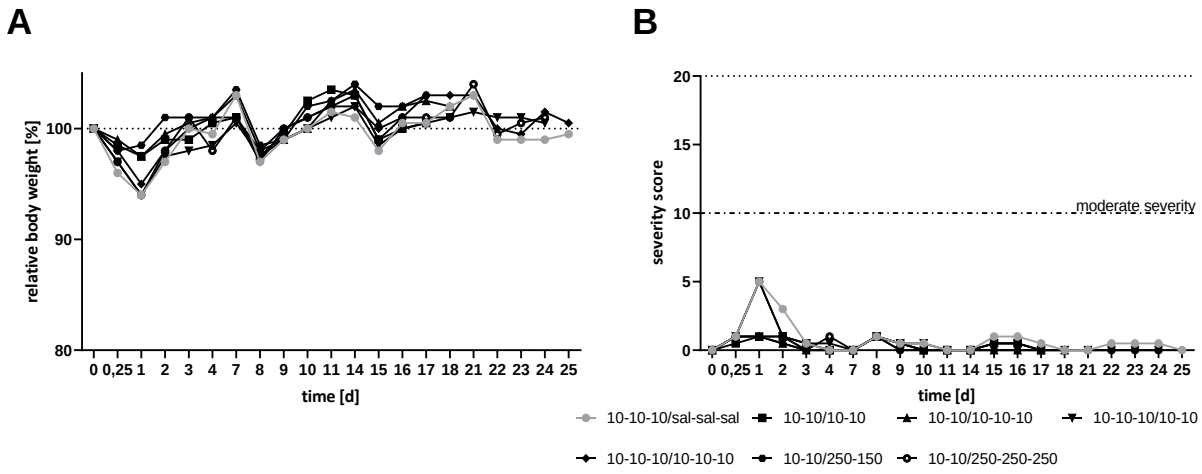

figure 9: Establishment of a murine model of AAI: The *i.p.* sensitization and *i.n.* challenge only marginally affect the relative body weight and severity score.
For the establishment of a murine model of AAI different treatment regimen were tested. All animals were sensitized with 10 µg OVA in 1 mg alum twice or three times in weekly intervals. Mice belonging to group 1 (10-10/10-10; ■) were *i.p.* sensitized two times in weekly intervals and then one week later *i.n.* challenged on two consecutive days with 10 µg OVA in PBS. Group 2 (10-10/10-10-10; ▲): Animals were sensitized *i.p.* twice in weekly intervals and challenge *i.n.* with 10 µg OVA in PBS on three consecutive days from one week after the last sensitization. Mice belonging to group 3 (10-10-10/10-10; ▼) and 4 (10-10-10/10-10-10; ♦) were sensitized three times in weekly intervals Group 3 was challenged with 10 µg OVA in PBS on two consecutive days, group 4 was challenge with 10 µg OVA in PBS on three consecutive days from one week after the last sensitization. Group 5 (10-10/250-250; ●): Mice were *i.p.* sensitized twice and *i.n.* challenged on two consecutive days from one week after the last sensitization with 250 µg OVA in PBS. Animals in group 6 (10-10/250-250-250; ○) were *i.p.* sensitized thrice and challenged on three consecutive days from one week after the last sensitization with 250 µg OVA in PBS. Control mice (●) were sensitized three times in weekly intervals with 10 µg OVA in 1 mg alum and *i.n.* challenged with PBS only on three consecutive days from one week after the last sensitization.
All mice were weighted and scored daily according to an experiment-specific score sheet. For each sensitization, the relative body weight loss (A) following that sensitization was normalized to the body weight of the day prior to that sensitization. For the *i.n.* challenge, the relative body weight was calculated in relation to the body weight prior to the first challenge. The severity score (B) was determined according to the score sheet. Data are compiled from at least two independent experiments and shown as group median only.

To assess how sensitization and induction of OVA-mediated AAI affected the health status of the mice, they were weighted and their general condition was closely observed according to an experiment-specific score sheet (see 3.4, p. 36) on a daily basis. No severe weight loss or increased severity score was observed in any of the treatment groups. One day after each sensitization, a mild weight loss (≤ 6 % of the starting weight) and increase of the cumulative severity score that also takes into account the weight loss (≤ 5 points) was detected. Overall, the health burden caused by the different treatments was rated as mild at all times.

4.1.2 Cellular and humoral inflammatory responses following sensitization and challenge with OVA

To characterize the OVA-induced AAI resulting from the different treatment regimen, the local cellular immune response was analyzed by evaluating the immune cellular influx into the airways using flow cytometric analysis of cells isolated from the BAL and lungs. For the characterization of the humoral immune response, the concentrations of different type 2 cytokines were measured in the BAL *via* cytometric bead array and antigen-specific IgE-antibodies in the serum were measured using an enzyme-linked immunoassay (ELISA). Because there were no notable or significant differences between the different control groups (figure 11, p. 57) and for reasons of clarity, in the following figure only one control group is shown (3x sensitized with OVA in alum, 3x mock-challenged with PBS). To demonstrate the similar background cell numbers in the lungs and BAL of mice of the different control groups, the mean total cell numbers retrieved from the lung and BAL of mice in all control groups are displayed in figure 11.

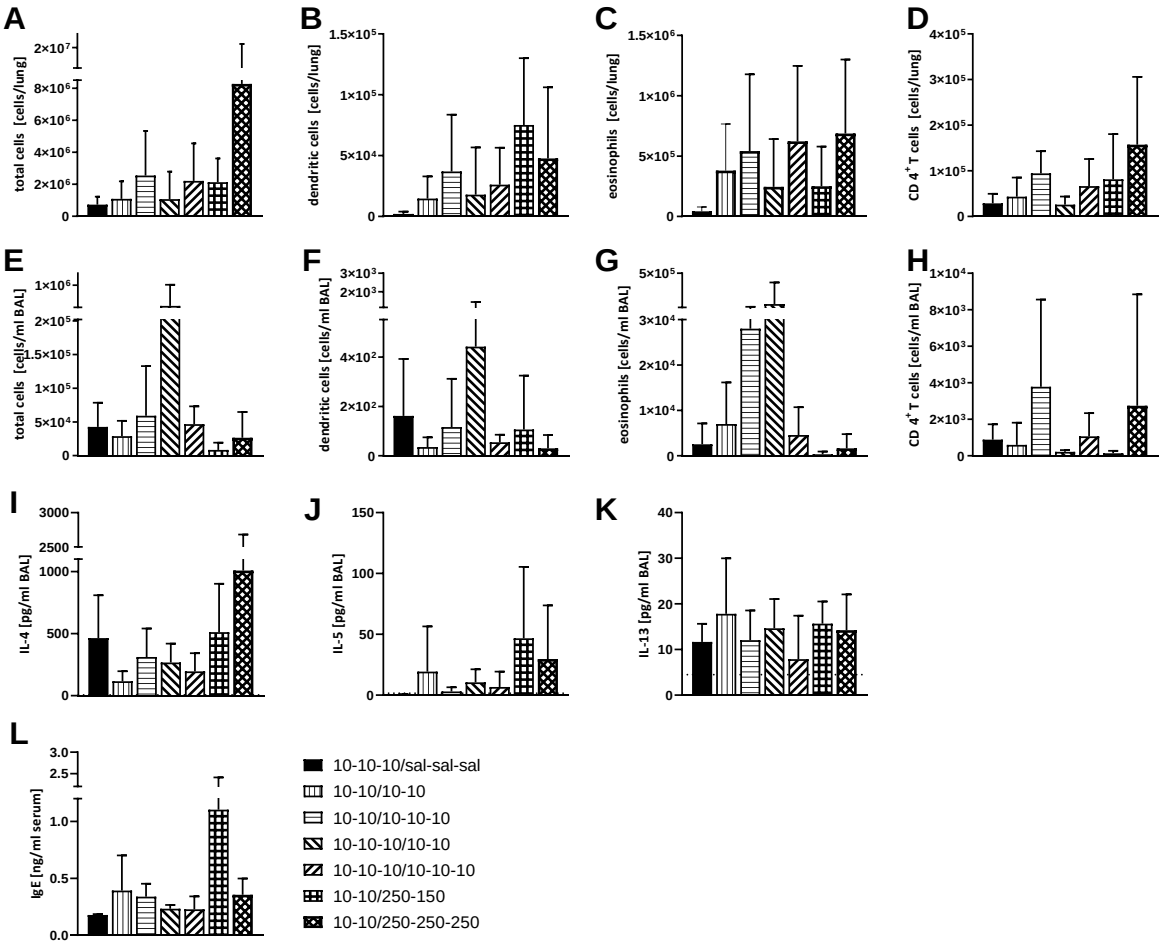

figure 10: Establishment of a murine model of OVA-mediated AAI: Different regimen of *i.p.* OVA sensitizations and *i.n.* OVA challenges lead to distinct changes in cell recruitment and cytokine production in the respiratory tract as wells as antigen-specific IgE levels in the serum.

For establishing a murine model of AAI several treatment regimen of OVA sensitization and challenge were tested for the induction of AAI. All animals were sensitized with 10 µg OVA in 1 mg alum. Mice in group 1 (10-10/10-10) were *i.p.* sensitized two times in weekly intervals and then *i.n.* challenged on two consecutive days from one week after the last sensitization with 10 µg OVA in PBS. Group 2 (10-10/10-10-10): Animals were sensitized *i.p.* twice in weekly intervals and challenged *i.n.* with 10 µg OVA on three consecutive days from one week after the last sensitization. Mice in group 3 (10-10-10/10-10) and 4 (10-10-10/10-10-10) were sensitized three times in weekly intervals. Group 3 was challenged on two consecutive days, group 4 was challenge three times from one week after the last sensitization. Group 5 (10-10/250-250): Mice were *i.p.* sensitized twice and *i.n.* challenged on two consecutive days from one week after the last sensitization with 250 µg OVA in PBS. Animals in group 6 (10-10/250-250-250) were *i.p.* sensitized thrice and challenged on three consecutive days from one week after the last sensitization with 250 µg OVA in PBS. Forty-eight h after the last *i.n.* challenge, lung and BAL leukocytes were analyzed for total cell count (A/E) and total numbers of DCs (B/F), eosinophils (C/G) and $CD4^+$ T cells (D/H). The BAL was analyzed for concentrations of IL-4 (I), IL-5 (J) and IL-13 (K). OVA-specific IgE-levels (L) were assessed in the serum. Treatment groups were compared with each other and with the control group. They were compared by one-way ANOVA with Bonferroni post-hoc test or Kruskal-Wallis test with Dunn's post-hoc test depending on whether data were normally distributed or not (according to Shapiro-Wilk testing). Data are compiled from at least two individual experiments and are shown as mean with SD of $n \geq 6$ mice per group. Due to the large number of groups in the statistical analyses and the explorative character if these experiments, there were no significant differences between the groups.

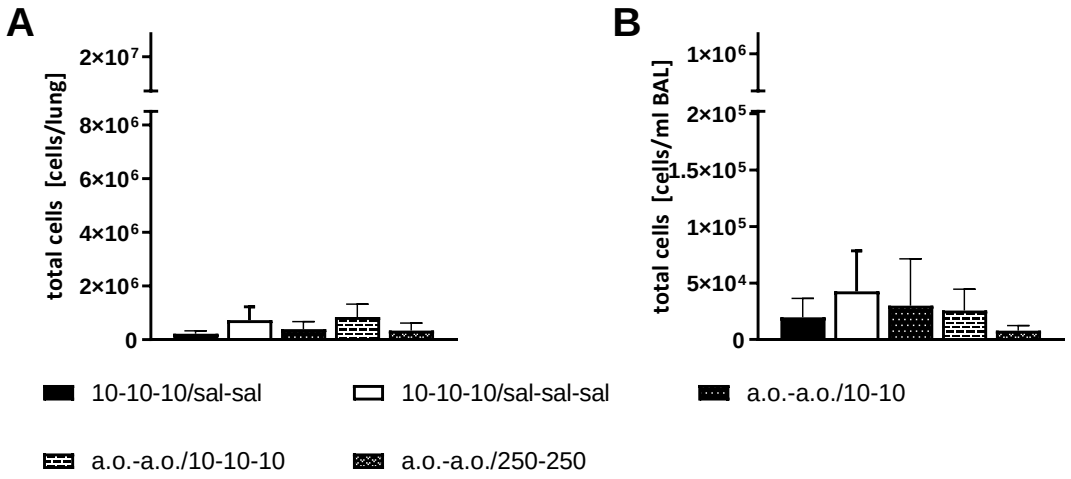

figure 11: Establishment of a murine model of OVA-mediated AAI: Different treatment regimen of *i.p.* OVA-sensitization and mock challenge with PBS or mock-sensitization with alum only and OVA-challenge.
Control groups in the establishment of an OVA-mediated mouse model of AAI were either *i.p.* sensitized with 10 µg OVA and 1 mg alum two or three times in weekly intervals and mock-challenged with PBS on two or three consecutive days or they were mock-sensitized with 1 mg alum only two times in weekly intervals and then challenge with 10 or 250 µg OVA on two or three consecutive days. Forty-eight h after the last *i.n.* challenge, lung and BAL leukocytes were analyzed for total cell counts (A, B). As this figure only serves to demonstrate the similar background cell numbers in the lungs and BAL of mice of the different control groups no statistical analysis was performed.

In the lung, the different treatment regimen led to increased total cell numbers as compared to the control group (figure 10 A). Especially mice sensitized three times with 10 µg OVA and challenged three times with 250 µg OVA showed clearly increased total cell numbers as compared to control mice. Total numbers of DCs were also increased in all treatment groups in comparison to the PBS-challenged control group. When challenged with 10 µg OVA, it seemed that the third challenge led to a slightly more pronounced influx of DCs as compared to mice which were only challenged twice, while challenging sensitized mice twice with 250 µg OVA led to a stronger influx of DCs as compared to sensitized mice, which were challenged three times with 250 µg OVA (figure 10 B). The peripheral sensitization and local challenge with OVA led to a clear increase of absolute numbers of eosinophils in the lungs as compared to control animals. Here, the difference in total numbers was more pronounced in animals which were challenged three times with either 10 µg or 250 µg OVA instead of twice (figure 10 C). Absolute numbers of $CD4^+$ T cells were increased in all treatment groups as compared to the control group with an exception of mice from group 3 (10-10-10/10-10). Again, the administration of three challenge doses was more effective than two challenge doses and led

to a minor more pronounced increase in absolute numbers of $CD4^+$ T cells in the lungs (figure 10 D).

For the BAL, the different regimen of peripheral sensitization and local challenge with OVA led to more heterogenous results. In comparison to the number of total cells in the control group, the sensitization with 10 µg OVA (twice) and the following challenge with 250 µg OVA (twice or thrice) led to reduced numbers of total cells and DCs. The only treatment group which showed a strong increase in total cell and DC numbers compared to the control, was group 3 (10-10/10-10) (figure 10 E, F).

Treatment groups 5 (10-10/250-250) and 6 (10-10/250-250-250) did not show increased eosinophil numbers compared to the control group. There was a minor increase in eosinophil numbers in treatment groups 1 (10-10/10-10) and 4 (10-10-10/10-10-10) as compared to the control group. The most pronounced increase in total numbers of eosinophils in the BAL was observed in treatment group 2 (10-10/10-10-10) and group 3 (10-10-10/10-10) (figure 10 G).

As compared to the control group, total numbers of $CD4^+$ T cells were only increased in treatment groups 2 (10-10/10-10-10) and 6 (10-10-10/250-250-250), the other treatments did not show major effects as compared to the control group (figure 10 H).

Cytokine levels were measured in the BAL. Mice, challenged three times with 250 µg OVA showed increased levels of IL-4 in comparison to the control group. Sensitization and challenge with 10 µg OVA (treatment groups 1-4) did not affect the IL-4 concentration (figure 10 I). IL-5 concentrations were increased in all treatment groups, but the increase was most pronounced in the BAL of mice which were sensitized twice and challenged with 250 µg twice (figure 10 J). IL-13 concentrations were not or only marginally affected by the *i.p.* OVA-sensitization and local OVA-challenge when compared to the control group (figure 10 K).

The peripheral sensitization and local airway challenge with OVA led to clearly increased levels of OVA-specific IgE in the serum in mice which were OVA-sensitized twice and challenged with 250 µg OVA twice. All other treatment groups showed a slight increase in OVA-specific IgE levels as compared to the control group (figure 10 L).

Generally, in these experiments, substantial variation within the individual groups was observed. Statistical analysis was performed (one-way ANOVA or non-parametric test), but presumably also due to the large number of experimental groups no significant differences were yielded in these experiments. Nevertheless, it was shown that the peripheral sensitization with OVA and the subsequent *i.n.* OVA challenge resulted in distinct AAI which

was characterized by cell influx of key asthma cell types (eosinophils, DCs, $CD4^+$ T cells) to the respiratory tract, increased Th2 cytokine concentrations (IL-4, IL-5), as well as increased antigen-specific IgE levels in the serum of treated animals.

From these experiments it was concluded that the OVA-challenge dose of 10 µg was too low and for future experiments mice were sensitized three times in weekly intervals with 10 µg OVA and 1 mg alum and challenged *i.n.* with an intermediate dose of 100 µg OVA on three consecutive days one week after the last sensitization.

4.1.3 Adjustment of sensitization and challenge conditions defining the final OVA-induced model of AAI for subsequent analyses

Based on the results of the previous experiments towards establishing a robust OVA-mediated model of AAI, conditions were adjusted and the model was applied for all further studies. Mice were sensitized three times in weekly intervals with 10 µg OVA (grade V) and 1 mg alum (3.2.2, p. 34) and challenged *i.n.* with 100 µg OVA (grade III) in PBS (3.2.3, p. 35) on three consecutive days one week after the last sensitization (OVA/OVA) (see figure 12, p. 56). Control animals were likewise sensitized *i.p.* with 10 µg OVA and 1 mg alum and *i.n.* challenged with PBS only (OVA/sal).

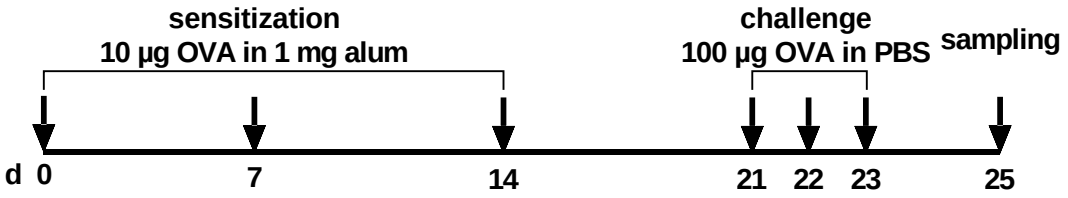

figure 12: Timeline of the experimental setup – OVA-mediated AAI.
For the induction of AAI, mice were sensitized three times *i.p.* with 10 µg OVA and 1 mg alum in weekly intervals (d 0, 7, 14). One week after the last sensitization, they were *i.n.* challenged with 100 µg OVA in 30 µl PBS once daily over three days. Forty-eight hours after the last *i.n.* challenge, the mice were sacrificed and analyzed.

To evaluate the induced AAI on a histological level lung sections were fixed in 4 % PFA, embedded in paraffin and cut into 5 µm thin slices. Afterwards, they were stained with hematoxylin and eosin and evaluated by a certified veterinary pathologist.

Histologically, in OVA-sensitized and PBS challenged mice no abnormalities were observed. In AAI, lung lesions were histologically characterized by typical bronchointerstitial pneumonia with a strong involvement of eosinophils. Mild bronchial epithelial hyperplasia and pneumocyte type II hyperplasia were observed (see figure 13, p. 60).

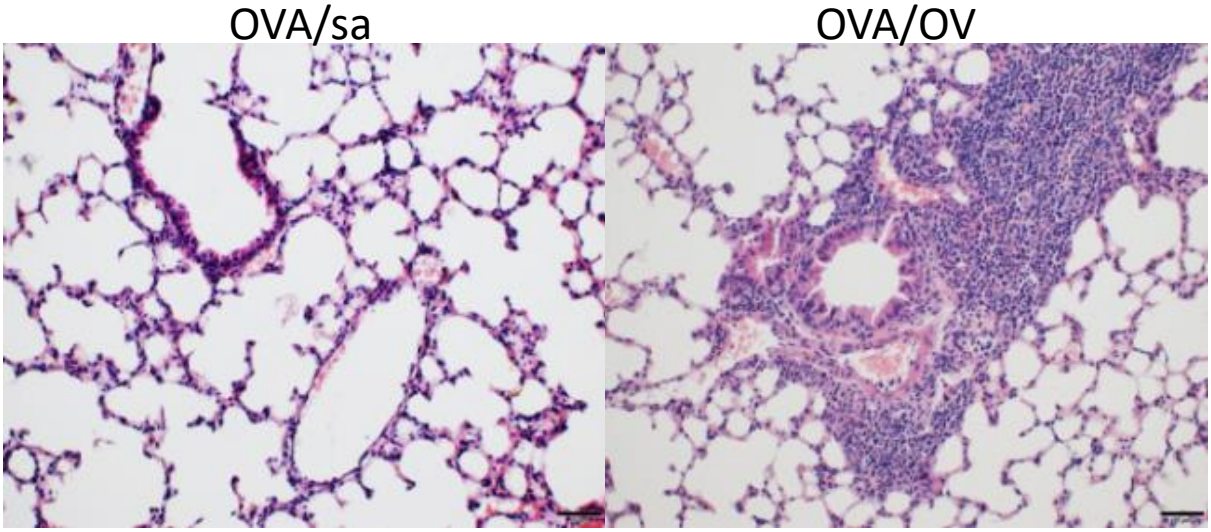

figure 13:Histological lung sections of sensitized, PBS-challenged control mice (OVA/sal) and of mice after AAI-induction (OVA/OVA).
For the induction of an AAI C57BL/6 (Janvier Labs) mice were *i.p.* sensitized with 10 µg OVA in 1 mg alum times in weekly intervals (d 0, 7, 14). One week after the last sensitization, mice were *i.n.* challenged on three consecutive days (d 21, 22, 23) with 100 µg OVA in PBS. Control mice were mock challenged with PBS. On day 25 lungs (n= 4 OVA/sal; n = 5 OVA/OVA) were prepared and tissue sections were stained with hematoxylin and eosin. Scale bar = 50 µm. The histological lung sections were evaluated by a certified veterinary pathologist.

Because literature showed that mice, purchased from different breeders differ in their specific microbiome, which could affect the outcome of experiments (Wolff *et al.*, 2020; Rasmussen *et al.*, 2021), the last step of defining a suited mouse model of AAI was to compare C57BL/6 mice purchased from Charles River and Janvier Labs using the adjusted model of AAI. As controls, mice from both vendors were combined as one group because no differences in cellular influx or cytokine production were observed.

In order to assess the induction of AAI in this adjusted OVA-mediated model, absolute numbers of total cells, DCs, eosinophils and $CD4^+$ T cells in lung and BAL were assessed using flow cytometry. Additionally, concentrations of type 2-specific cytokines in the BAL and the concentration of OVA-specific IgE in the serum were quantified.

As compared to mice of the control group, C57BL/6 mice obtained from Charles River showed a significant increase in total cells in the lung. The induction of AAI in C57BL/6 mice from Janvier Labs led to increased total cell numbers in the lung, but no significance was found (figure 14 A). Absolute numbers of DCs, eosinophils and $CD4^+$ T cells in the lung were significantly increased in C57BL/6 mice from both Charles River as well as from Janvier Labs when compared to PBS-challenged control mice (figure 14 B, C, D). There was no significant difference in absolute numbers of the prior mentioned cell types in the lungs between mice from the two suppliers (figure 14 A-D).

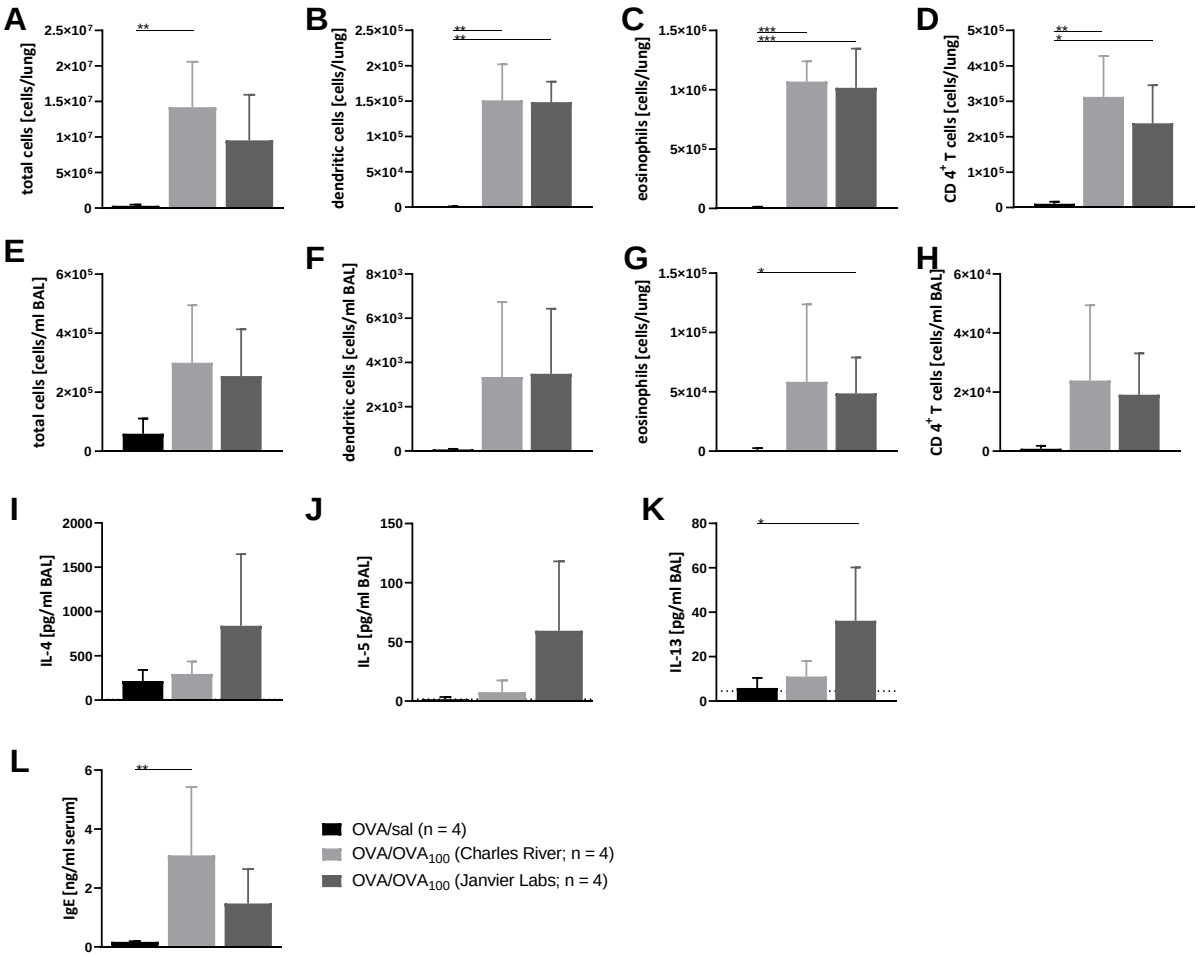

figure 14: Establishment of a murine model of OVA-mediated AAI: The peripheral sensitization with 10 µg OVA followed by an *i.n.* challenge with 100 µg OVA led to distinct cell recruitment and Th2-cytokine production in the respiratory tract as wells as antigen-specific IgE in the serum in C57BL/6 mice of two breeders.
For the induction of AAI C57BL/6J mice obtained from either Charles River (light grey bars) or Janvier Labs (dark grey bars) were *i.p.* sensitized with 10 µg OVA in 1 mg alum three times in weekly intervals (d 0, 7, 14). One week after the last sensitization, mice were *i.n.* challenged on three consecutive days (d 21, 22, 23) with 100 µg OVA in PBS. Control mice (black bars; C57BL/6 mice, obtained from Charles River and Janvier Labs) were mock challenged with PBS. On day 25, lung and BAL leukocytes were analyzed for the absolute cell count (A, E) and absolute numbers of DCs (B, F), eosinophils (C, G) and $CD4^+$ T cells (D, H). The BAL was analyzed for levels of IL-4 (I), IL-5 (J) and IL-13 (K). OVA-specific IgE-levels (L) were assessed in the serum. Treatment and control groups were compared by one-way ANOVA with Bonferroni post-hoc test or Kruskal-Wallis test with Dunn's post-hoc test depending on whether data were normally distributed or not (according to Shapiro-Wilk testing). Data are shown as mean with SD of n = 4 mice per group from a single experiment. *p < 0.05, **p < 0.01, ***p < 0.005.

Analyzed cells in the BAL also showed substantially increased numbers of total cells (figure 14 E), DCs (figure 14 F), eosinophils (figure 14 G) and $CD4^+$ T cells (figure 14 H). However, these increases were not statistically significant between the groups, except for the mean absolute number of eosinophils in AAI in mice purchased from Janvier Labs as compared to control mice.

Levels of typical type 2 cytokines (IL-4 (figure 14 I), IL-5 (figure 14 J) and IL-13 (figure 14 K)) were measured in the BAL. Mice purchased from Charles River showed only marginally increased levels of these cytokines. In contrast, mice purchased from Janvier Labs showed clearly increased concentrations of all three cytokines, with a significant increase in IL-13 as compared to the PBS-challenged control group.

Mice purchased from Charles River as well as Janvier Labs showed an increase in OVA-specific IgE-levels (figure 14 L), but only IgE-levels of mice purchased from Charles River showed a significant difference compared to control mice.

In conclusion, it was shown that a robust OVA-mediated AAI can be induced in C57BL/6 mice from Charles River as well as Janvier Labs, however with a different distribution in key parameters of AAI between mice of these two breeders. Small but distinct differences were found between mice of the two suppliers. While there were no major differences in the cell recruitment to the lung and BAL, C57BL/6 mice from Janvier Labs showed profoundly increased cytokine concentrations of type 2 cytokines in the BAL, while C57BL/6 mice from Charles River showed only a marginally increase in cytokine concentrations. In contrast, mice from Charles River showed a significantly increased OVA-specific IgE concentration in the serum, while mice from Janvier Labs showed an increase in the IgE concentration but not significant in comparison to the control group.

Because of the stable cellular inflammation in lung and BAL, the significant increase in type 2 cytokines, as well as the also detectable increase OVA-specific IgE concentration in the serum, all further experiments were performed in C57BL/6 mice from Janvier Labs.

4.2 Modulation of AAI through respiratory SEB administration

Prior to the following experiments assessing the effect of respiratory SEB administration on AAI, the optimal SEB doses for the experiments were determined. Such optimal doses would alone lead to a detectable response in the respiratory tract without adversely affecting treated mice. To this end, mice were *i.n.* treated with three doses of SEB (5 ng, 50 ng and 500 ng SEB) on three consecutive days. Forty-eight hours later, inflammatory parameters in the lung and BAL were examined, i.e. the cellular influx of eosinophils, $CD4^+$ T cells, B cells, as well as concentrations of various cytokines. The treatment with 5 ng SEB did not show detectable

inflammation with respect to these parameters in comparison to the PBS-treated control group (data not shown). The treatment with 50 ng SEB, as well as with 500 ng SEB did show detectable inflammation at different severity levels as compared to PBS-treated control mice (data not shown). For the subsequent experiments combining *i.n.* SEB administration and OVA-mediated AAI, 50 ng SEB were used as a low, yet effective dose and 500 ng SEB as a high, non-lethal dose.

The described model was now utilized to analyze how an *i.n.* SEB affects the severity and phenotype of AAI in a time- and dose-dependent manner. Fifty or 500 ng SEB were either *i.n.* administered during the allergic OVA-challenge (d 21, 22, 23) (see figure 15, p. 64) or 50 ng or 500 ng SEB were *i.n.* administered on three consecutive days prior to the first sensitization (d -3, -2, -1) (see figure 26, p. 85).

4.2.1 Intranasal SEB administered during the allergic challenge

For the examination of a possible modulation through *i.n.* SEB administration during the challenge phase of AAI induction, mice were *i.p.* sensitized with 10 µg OVA and 1 mg alum in PBS on days 0, 7 and 14 and then *i.n.* challenged with 30 µl PBS containing either 100 µg OVA alone (OVA/OVA) or 100 µg OVA in combination with 50 ng (OVA/OVA+SEB_{50}) or 500 ng SEB (OVA/OVA+SEB_{500}) (see figure 15, p. 64). Control animals were *i.p.* sensitized with OVA and alum and then *i.n.* challenged with PBS only (OVA/sal).

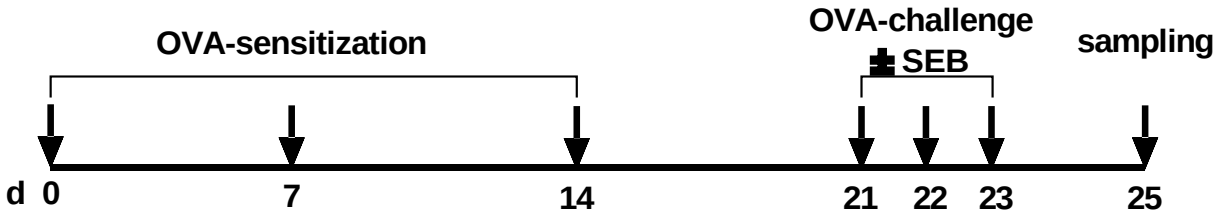

figure 15: Timeline of the experimental setup – *i.n.* SEB-treatment during the allergic challenge of OVA-mediated AAI.
For the induction of AAI, mice were sensitized three times *i.p.* with 10 µg OVA and 1 mg alum in weekly intervals (d 0, 7, 14). One week after the last sensitization they were *i.n*. challenged with 100 µg OVA in PBS. For the analysis of the modulation of AAI through SEB administered with the challenge, sensitized mice were *i.n.* challenged with OVA together with SEB (50 ng or 500 ng) on three consecutive days
Forty-eight hours after the last *i.n.* challenge the mice were sacrificed and BAL, lungs, serum, spleen, cervical and bronchial lymph nodes were harvested for further analyses.

4.2.1.1 Intranasal application of 50 ng or 500 ng SEB in combination with OVA in unsensitized mice

As a basis for further analyses with respect to the modulation of AAI, the effects of local SEB-treatment without prior sensitization were examined. For this, mice were *i.p.* mock-sensitized three times with alum only and then *i.n.* administered either 50 ng or 500 ng SEB in combination with the AAI-challenge dose of 100 µg OVA.

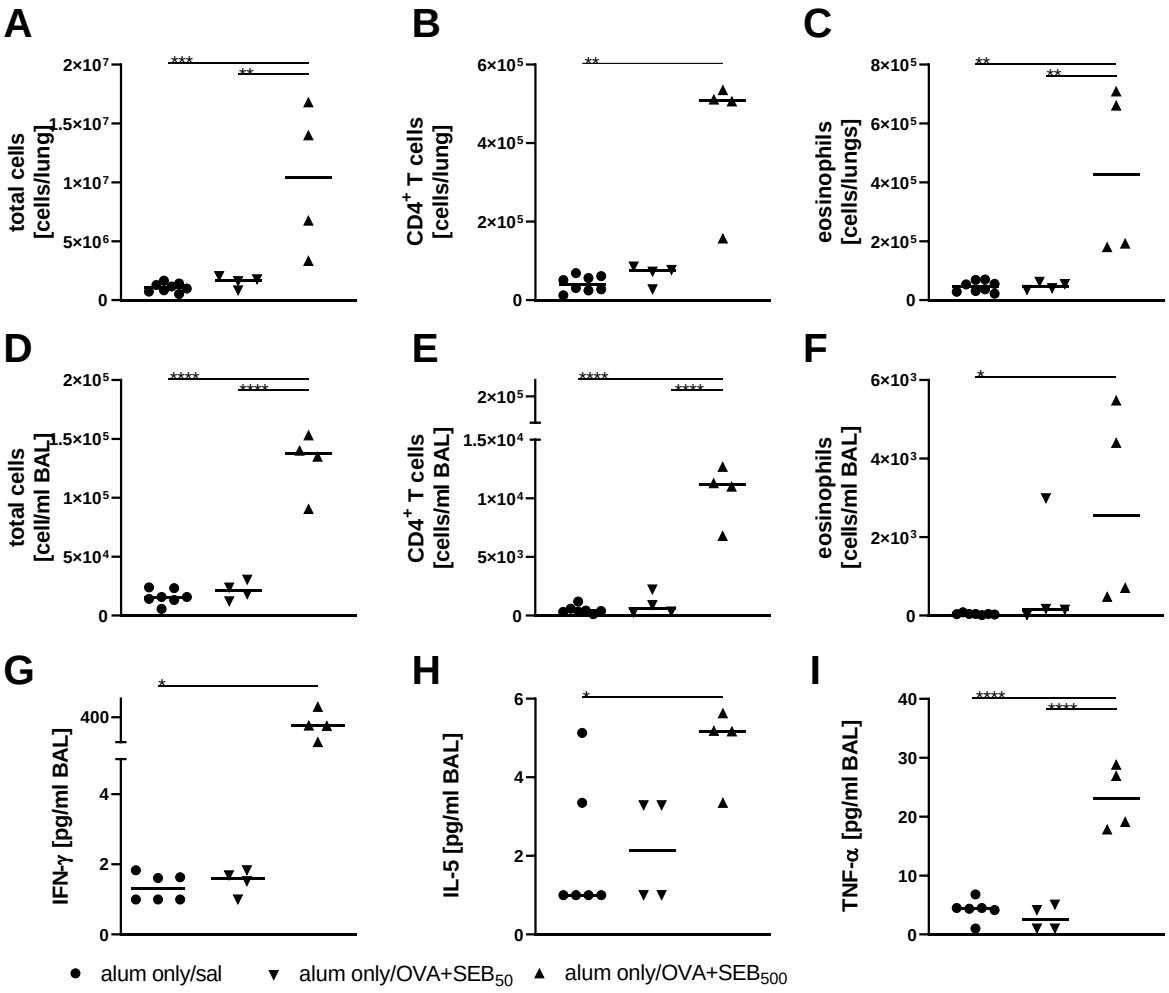

figure 16: Cell recruitment to the respiratory tract and the local cytokine response following *i.n.* SEB-administration in unsensitized mice.
Mice were mock-sensitized with alum only in weekly intervals (d 0, 7, 14). One week after the last mock-sensitization, mice were either *i.n.* treated either with PBS (control (●): alum only/sal) or with 50 ng or 500 ng of the *S. aureus* enterotoxin B (SEB) (50 ng (▼): alum only/OVA+SEB_{50}; 500 ng (▲): alum only/OVA+SEB_{500}) on three consecutive days (d 21, 22, 23). On day 25 (48h after the last treatment), leukocytes from lungs and bronchoalveolar lavage (BAL) were flow-cytometrically analyzed with respect to the total cell count ((A) lung, (D) BAL), absolute numbers of $CD4^+$ T cells ((B) lung, (E) BAL) and eosinophils ((C) lung, (F) BAL). Concentrations of IFN-γ (G), IL-5 (H) and TNF-α (I) were measured in BAL. Data are compiled from two independent experiments and show individual mice and the median. Groups ●, ▼, and ▲ were compared by one-way ANOVA with Bonferroni post-hoc test or Kruskal-Wallis test with Dunn's post-hoc test depending on whether data were normally distributed or not (according to Shapiro-Wilk testing). *p < 0.05, ** p < 0.01, *** p < 0.005, **** p < 0.0001.

For the evaluation of the SEB-mediated inflammation, different cell types and cytokines were analyzed in respiratory tract samples. Because SEB is a superantigen, the T cell response was particularly interesting. For this, absolute numbers of $CD4^+$ T cells were analyzed. Since the influence of *i.n.* SEB-application on AAI was to be investigated further, the absolute numbers of eosinophils were analyzed, as these belong to the key effector cells in AAI. As it is known that SEB is more likely to induce a type 1 immune response, the IFN-γ concentrations in the BAL were measured. IL-5 is a type 2 cytokines and TNF-α a pro-inflammatory cytokine. Concentrations of these cytokines were evaluated to fully characterize the immune response in terms of the influence of SEB in unsensitized mice.

In the lungs as well as in the BAL, there was a significant increase of total cells (figure 16 A, D), $CD4^+$ T cells (figure 16 B, E) and eosinophils (figure 16 C, F) after the treatment with 500 ng SEB compared to *i.n.* treatment with PBS. The likewise treatment with 50 ng SEB did not increase the numbers of these types of cells significantly in the lung or BAL.

I.n. treatment with 500 ng SEB led to significantly increased concentrations of IFN-γ (figure 16 G), IL-5 (figure 16 H) and TNF-α (figure 16 I) in the BAL as compared to PBS controls. The treatment with 50 ng SEB did not alter the cytokine concentrations as compared to the control group.

In conclusion these results show, that the *i.n.* treatment with 500 ng SEB in unsensitized mice led to a significant increase in total cells, absolute numbers of $CD4^+$ T cell and eosinophils in the lung and BAL, as well as significantly increased concentrations of IFN-γ, IL-5 and TNF-α compared to PBS-treated control mice. At the same time, the *i.n.* treatment with 50 ng SEB did not significantly affect these cell types and cytokine concentrations.

4.2.1.2 Effects on body weight and general condition

To assess, whether SEB administered together with the allergic challenge affected the body weight or overall disease burden in AAI, all animals were weighted and scored for their general condition on a daily basis (see 3.4, p. 36).

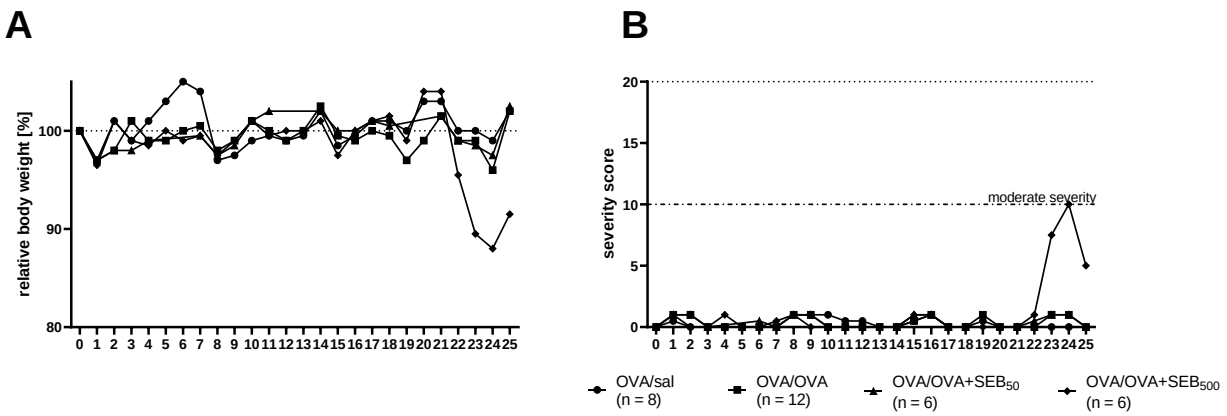

figure 17: Body weight and disease scoring during the induction of AAI and co-administration of SEB with the allergic challenge.
For the induction of AAI, mice were sensitized three times *i.p.* with 10 µg OVA and 1 mg alum in weekly intervals (d 0, 7, 14). One week after the last sensitization they were *i.n.* challenged with OVA (■ OVA/OVA) or OVA together with 50 ng or 500 ng SEB on three consecutive days (d 21, 22, 23) (50 ng SEB (▲): OVA/OVA+SEB_{50}; 500 ng SEB (♦): OVA/OVA+SEB_{500}). Control mice were sensitized but mock challenged with PBS only (● OVA/sal). All mice were weighted and scored daily according to the score sheet. For each sensitization, the relative body weight loss (A) following that sensitization was normalized to the body weight of the day prior to that sensitization. For the *i.n.* challenge, the relative body weight was calculated in relation to the body weight prior to the first challenge. The severity score (B) was determined according to the experiment-specific score sheet. Data are compiled from at least two independent experiments and shown as group median only.

Sensitized mice which were additionally treated with 50 ng SEB during the allergic challenge did not show any differences in relative body weight (figure 17 A) or severity score (figure **17** B) as compared to PBS-challenged control mice or mice with AAI alone. The additional treatment with 500 ng SEB during the allergic challenge led to a more pronounced weight loss (figure 17 A) in the respective experimental animals. The maximal weight loss was approximately 12 % and the maximum severity score (figure 17 B) was 10 one day after the last *i.n.* challenge + 500 ng SEB. From one day after the last *i.n.* challenge, mice began to re-gain weight.
To sum up, the additional SEB treatment during the allergic challenge did not overtly affect body weight or overall scoring. After repeated treatment with 500 ng SEB in combination with the allergic challenge, a moderate and short-lasting weight loss could be observed.

4.2.1.3 Histological effects

For the first evaluation of the effects of an *i.n.* administration of 50 ng or 500 ng SEB on AAI, lungs from mice with AAI alone and AAI with additional SEB administration together with the allergic challenge were fixed in 4 % PFA, embedded in paraffin and cut into 5 µm slices.

Subsequently, they were stained with hematoxylin and eosin and AAI and effects of SEB administration on AAI were evaluated by a certified veterinary pathologist.

In AAI (OVA/OVA), lung lesions were histologically characterized by typical bronchointerstitial pneumonia with a strong involvement of eosinophils. Mild bronchial epithelial hyperplasia and pneumocyte type II hyperplasia were observed. After the *i.n.* administration of either 50 ng (OVA/OVA+SEB_{50}) or 500 ng SEB (OVA/OVA+SEB_{500}) together with the allergic challenge, these changes were similar, however with milder perivascular lymphocytic infiltrates but moderate interstitial lymphocytic infiltrates and moderate type II pneumocyte hyperplasia. Accumulation of mucus was observed only sporadically but goblet cell hyperplasia in the medium sized and predominantly large bronchi was present in AAI and was not affected by SEB-treatment during the challenge (see figure 18, p. 68, scoring data not shown).

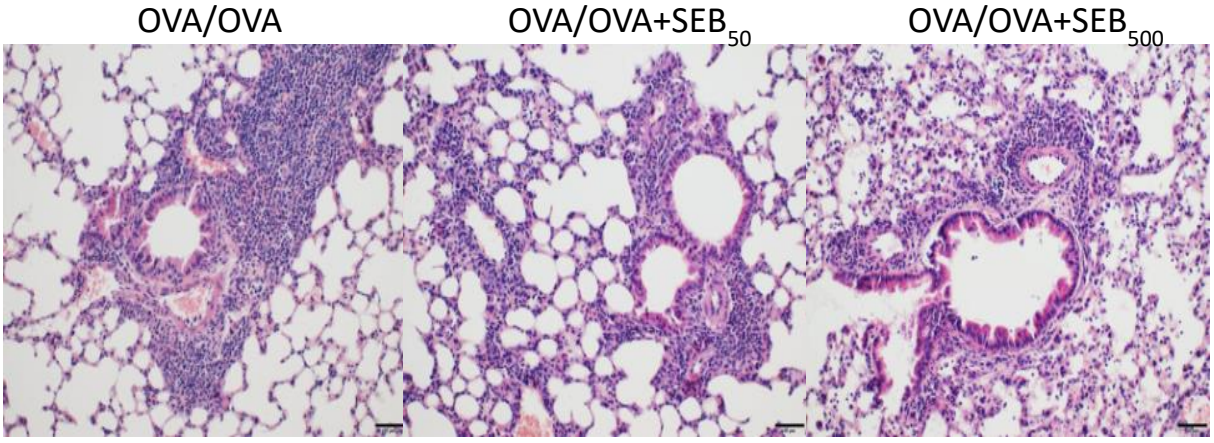

figure 18: Histological lung sections of mice after AAI induction (OVA/OVA) and after *i.n.* administration of 50 ng SEB (OVA/OVA+SEB_{50}) or 500 ng SEB (OVA/OVA+SEB_{500}) together with the allergic challenge.
For the induction of AAI, C57BL/6 mice were *i.p.* sensitized with 10 µg OVA in 1 mg alum three times in weekly intervals (d 0, 7, 14). One week after the last sensitization, mice were *i.n.* challenged on three consecutive days (d 21, 22, 23) with 100 µg OVA in PBS or OVA together with 50 ng (OVA/OVA+SEB_{50}) or 500 ng SEB (OVA/OVA+SEB_{500}). Control mice were mock challenged with PBS. On day 25 lungs (n = 5) were stained with hematoxylin and eosin. Scale bar = 50 µm. The histological lung sections were evaluated by a certified veterinary pathologist.

4.2.1.4 Effects on innate effector cells of AAI in the respiratory tract and spleen

During allergic inflammation, cells of the innate immune system are one of the first in line to encounter the allergen (Kubo, 2017). To comprehensively analyze the cellular innate immune response following AAI induction and the effect of intranasal SEB administered with the allergic challenge, absolute numbers of macrophages, DCs, eosinophils, mast cells, M2-

polarized monocytes/macrophages as well as basophils were examined in the respiratory tract as well as the spleen.

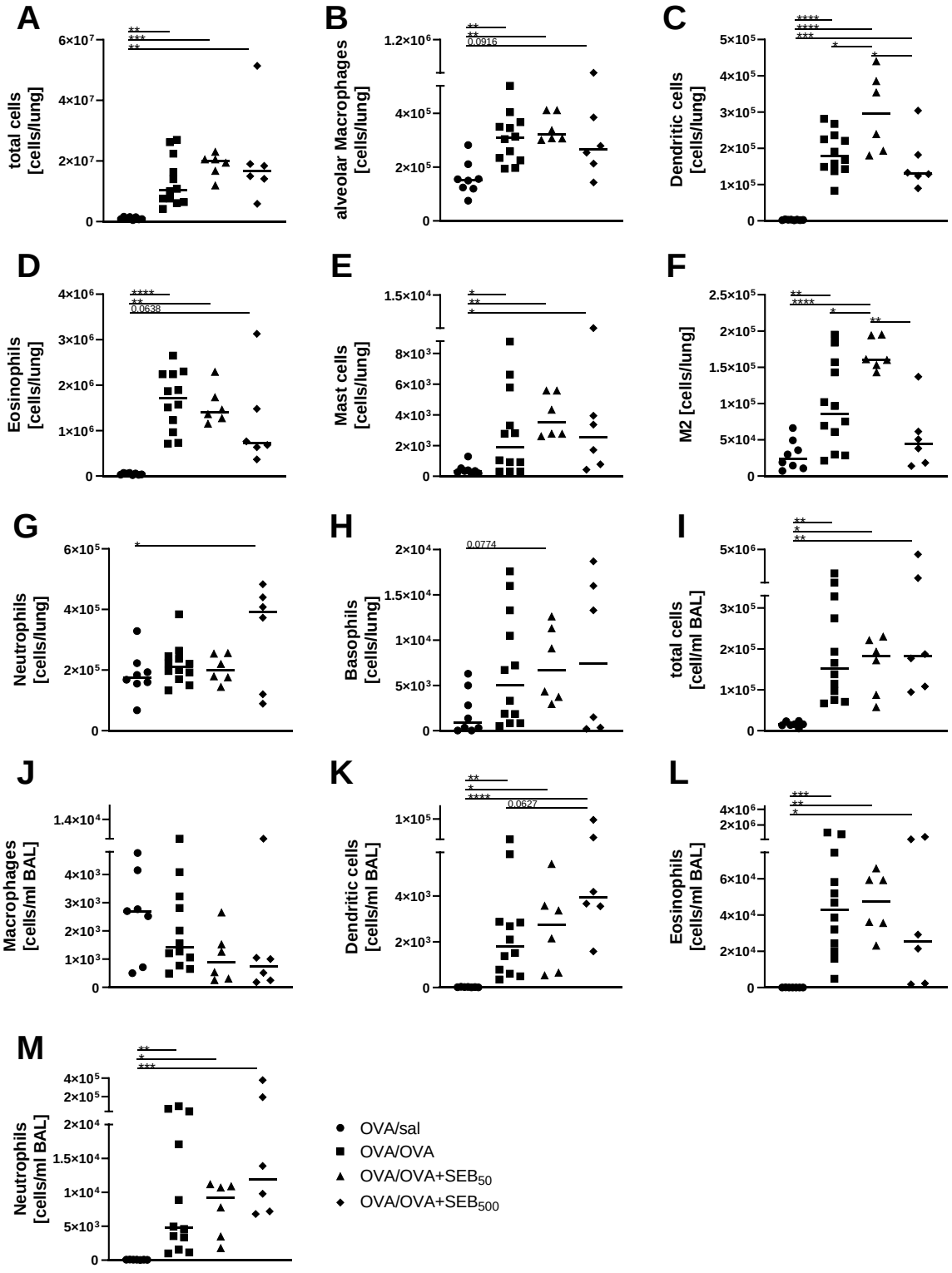

figure 19: Innate effector cells of AAI in the respiratory tract in AAI alone and following administration of SEB with the allergic challenge.

For the induction of AAI, mice were sensitized three times *i.p.* with 10 µg OVA and 1 mg alum in weekly intervals (d 0, 7, 14). One week after the last sensitization they were *i.n.* challenged with 100 µg OVA (■ OVA/OVA) or 100 µg OVA together with 50 ng or 500 ng SEB on three consecutive days (d 21, 22, 23) (50 ng SEB (▲): OVA/OVA+SEB$_{50}$; 500 ng SEB (♦): OVA/OVA+SEB$_{500}$). Control mice were sensitized but mock challenged with PBS only (● OVA/sal). On day 25, leukocytes from lung and BAL were analyzed for total cell counts (A, I) and total

numbers of (alveolar) macrophages (B, J), DCs (C, K), eosinophils (D, L), mast cells (E), M2-polarized monocytes/macrophages (F), neutrophils (G, M) and basophils (L). Data compiled from at least two independent experiments are shown for individual mice with the median. *p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.0001.

In the lungs, the induction of AAI alone led to a significant increase in total cells (figure 19 A), alveolar macrophages (figure 19 B), DCs (figure 19 C), eosinophils (figure 19 D), mast cells (figure 19 E) and M2-polarized monocytes/macrophages (figure 19 F).

In the BAL, the induction of AAI alone led to significant increased total numbers of total cells (figure 19 I), DCs (figure 19, K), eosinophils (figure 19, L) and neutrophils (figure 19 M).

In the lung and BAL the *i.n.* administration of 50 ng or 500 ng SEB during the allergic challenge did not alter the number of total cells (figure 19 A, I), macrophages (figure 19 B, J), eosinophils (figure 19 D, L) or neutrophils (figure 19 G, M). In the lung, total numbers of mast cells (figure 19 E) and basophils (figure 19 H) were not affected by the treatment with 50 ng or 500 ng SEB during the allergic challenge. Nevertheless, the administration of 50 ng SEB led to a significant increase in total numbers of DCs (figure 19 C) as well as M2-polarized monocytes/macrophages (figure 19 F) in the lungs as compared to AAI alone. Treatment with 500 ng SEB led to rather reduced numbers of these cells as compared to AAI alone and to significantly reduced their numbers as compared to the treatment with 50 ng SEB. Also, the administration of 500 ng SEB during the allergic challenge led to a significant increase in neutrophil numbers as compared to control animals (figure 19 G), while the induction of AAI alone did not significantly affect this cell type.

In the BAL, the *i.n.* administration of 50 ng or 500 ng during the allergic challenge did not significantly affect absolute numbers of the analyzed innate immune cells. Nevertheless, absolute numbers of DCs were rather increased after the administration of 500 ng SEB as compared to AAI alone (figure 19 K).

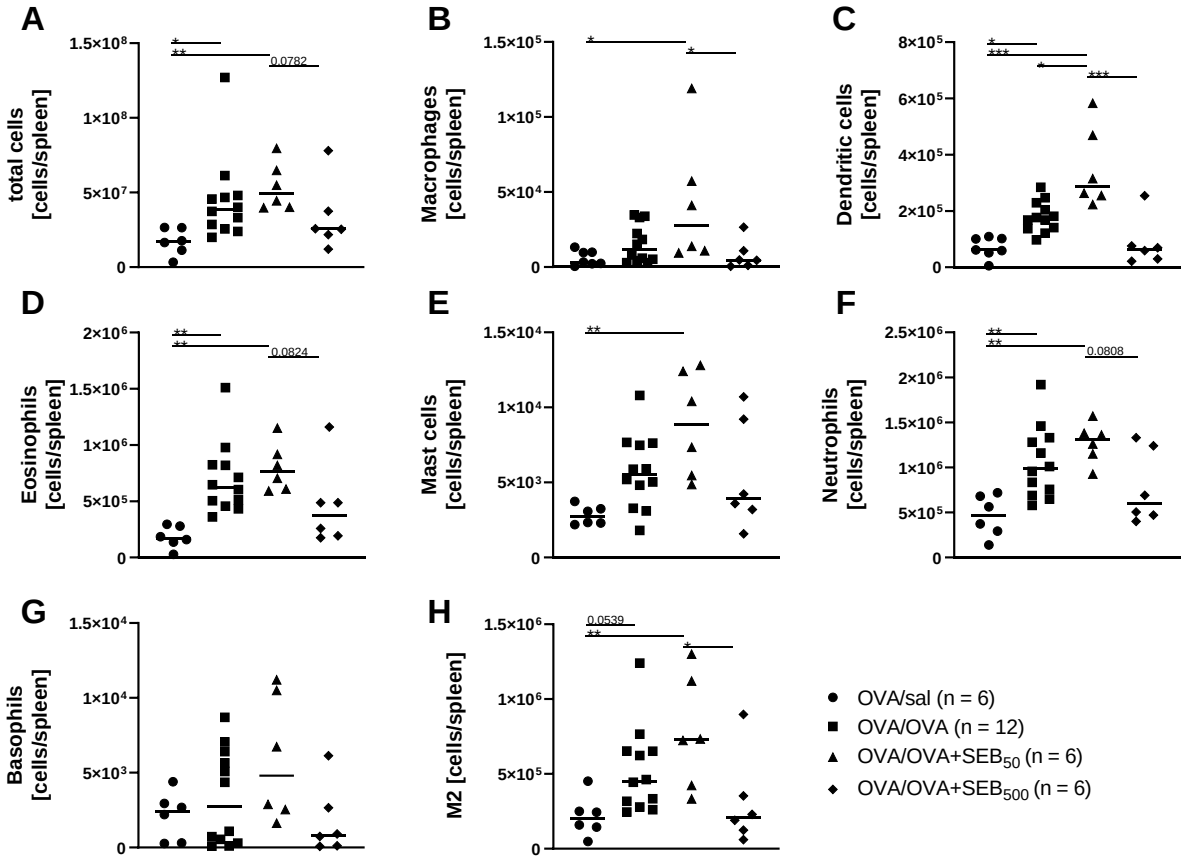

figure 20: Innate effector cells of AAI in the spleen in AAI alone and following administration of SEB with the allergic challenge.

For the induction of AAI, mice were sensitized three times *i.p.* with 10 µg OVA and 1 mg alum in weekly intervals (d 0, 7, 14). One week after the last sensitization, they were *i.n.* challenged with OVA (■ OVA/OVA) or OVA together with 50 ng or 500 ng SEB on three consecutive days (d 21, 22, 23) (50 ng SEB (▲): OVA/OVA+SEB_{50}; 500 ng SEB (♦): OVA/OVA+SEB_{500}). Control mice were sensitized but mock challenged with PBS only (● OVA/sal). On day 25, cells from the spleen were analyzed for total cells (A), absolute numbers of macrophages (B), DCs (C), eosinophils (D), mast cells (E), neutrophils (F), basophils (G) and M2-polarized monocytes/macrophages (H). Data compiled from at least two independent experiments are shown for individual mice with the median. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, ****$p < 0.0001$.

To evaluate the possible effects of the *i.n.* SEB administration during the allergic challenge on relevant peripheral innate immune cell populations, splenocytes were isolated and examined. The induction of AAI alone led to a significant increase in spleen total cell numbers (figure 20 A), absolute numbers of DCs (figure 20 C), eosinophils (figure 20 D), as well as neutrophils (figure 20 F) as compared to controls. Compared to AAI alone, administration of 50 ng SEB during the allergic challenge significantly increased absolute numbers of DCs in the spleen (figure 20 C). As compared to PBS-treated control mice, the number of totals cells (figure 20 A) as well as the absolute numbers of macrophages (figure 20 B), DCs (figure 20 C), eosinophils (figure 20 D), mast cells (figure 20 E), neutrophils (figure 20 F) and M2-polarized

monocytes/macrophages (figure 20 H) in the spleen were significantly increased in AAI combined with the administration of 50 ng SEB during the allergic challenge. The *i.n.* administration of 500 ng SEB during the allergic challenge led to rather reduced numbers of total cells (figure 20 A), macrophages (figure 20 B), DCs (figure 20 C), neutrophils (figure 20 F) and M2-polarized monocytes/macrophages (figure 20 H) as compared to AAI alone and to significantly reduced numbers compared to AAI in combination with 50 ng SEB. Furthermore, the examination of absolute numbers of eosinophils (figure 20 D), mast cells (figure 20 E) and basophils (figure 20 G) showed tendencies, but no significant effect, that *i.n.* administration of 500 ng SEB during the allergic challenge led to a decrease in these cell types as compared to AAI alone.

To summarize, the *i.n.* administration of 50 ng or 500 ng SEB during the challenge phase of allergic asthma showed versatile potential to modulate the innate cellular immune response in AAI. AAI combined with the administration of 50 ng SEB led to increased absolute numbers of DCs (lung, spleen) and M2-polarized monocytes/macrophages (lung) as compared to AAI alone. In contrast, the *i.n.* administration of 500 ng SEB led to rather reduced absolute cell numbers of DCs (lung, spleen), eosinophils (lung, BAL, spleen), M2-polarized monocytes/macrophages (lung, spleen) and mast cells (lung, spleen) as compared to AAI alone and significantly decreased numbers of DCs and M2-polarized monocytes/macrophages in the lung and spleen as compared to mice with *i.n.* treatment with 50 ng SEB together with the OVA-challenge.

4.2.1.5 Effect on T and B cells in the respiratory tract, draining lymph nodes and spleen

With the aim of further analysis of the cell composition in the respiratory tract, local draining lymph nodes and spleen in AAI and in order evaluate the effects of *i.n.* SEB administered during the allergic challenge, lymphocytes were isolated from lungs, BAL, cervical and bronchial lymph nodes as well as the spleen and analyzed using flow cytometry.

T and B lymphocytes belong to the crucial immune cells of the adaptive immune system during allergic sensitization as well as allergic inflammation. Additionally, SEB belongs to the family of superantigens which specifically interact with T cells and thereby modulate the adaptive

immune response. For these reasons, the detailed analysis of these cells was of great interest in the context of modulation of AAI through *i.n.* SEB administration.

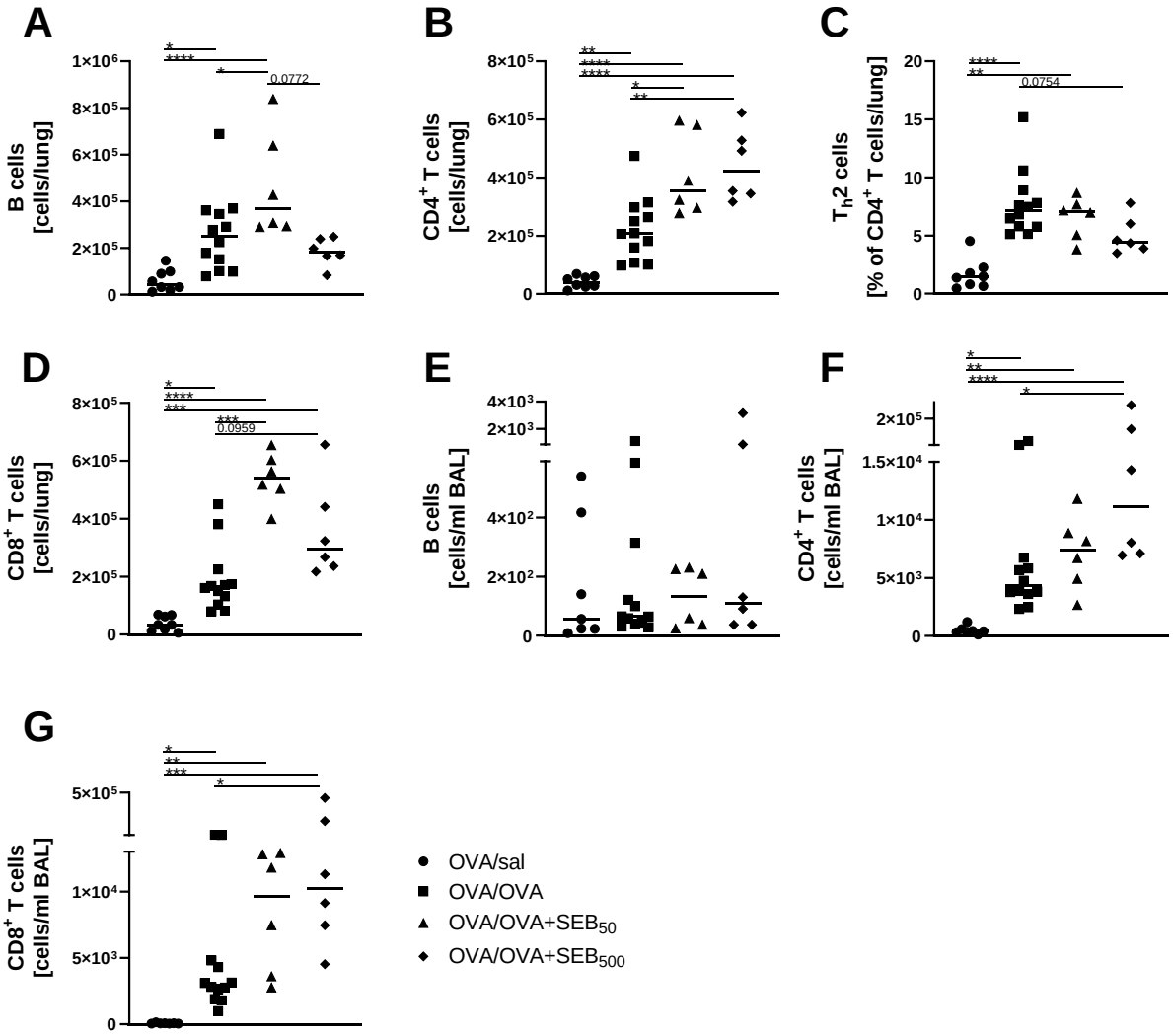

figure 21: Lymphocytes in the respiratory tract in AAI alone and following administration of SEB with the allergic challenge.

Mice were sensitized three times *i.p.* with 10 µg OVA and 1 mg alum in weekly intervals (d 0, 7, 14). One week after the last sensitization they were *i.n.* challenged with OVA (■ OVA/OVA) or OVA together with 50 ng or 500 ng SEB on three consecutive days (d 21, 22, 23) (50 ng SEB (▲): OVA/OVA+SEB_{50}; 500 ng SEB (♦): OVA/OVA+SEB_{500}). Control mice were sensitized but mock challenged with PBS only (● OVA/sal). On day 25, lymphocytes from lung and BAL were analyzed for total numbers of B cells (A, E), $CD4^+$ T cells (B, F), the frequency of Th2 cells within $CD4^+$ T cells (C) and $CD8^+$ T cells (D, G). Data compiled from at least two independent experiments are shown for individual mice with the median. *p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.0001.

In the lungs, the induction of AAI alone led to a significant increase in total numbers of B cells (figure 21 A), $CD4^+$ T cells (figure 21 B), the frequency of Th2 cells in $CD4^+$ T cells (figure 21 C), as well as $CD8^+$ T cells (figure 21 D). In the BAL, total numbers of $CD4^+$ T cells (figure 21 F) and

$CD8^+$ T cells (figure 21 G) were significantly increased by AAI induction as compared to control mice.

Compared to AAI alone, the administration of 50 ng SEB together with the allergic challenge significantly increased the absolute numbers of B cells, $CD4^+$ T cells and $CD8^+$ T cells but not the frequency of Th2 cells (figure 21 A, B, C, D) in the lungs. Absolute cell numbers of B cell, $CD4^+$ T cells and $CD8^+$ T cells analyzed in the BAL were not affected by the administration of 50 ng SEB together with the allergic challenge.

Compared to AAI alone, the *i.n.* administration of 500 ng SEB together with the allergic challenge led to significantly increased absolute numbers of $CD4^+$ T cells in the lungs (figure 21 B) and BAL (figure 21 F), as well as $CD8^+$ T cells in the BAL (figure 21 G). Additionally, after *i.n.* administration of 500 ng SEB together with the allergic challenge, the frequency of Th2 cells was no longer significantly increased as compared to the PBS-treated control group despite the significant increase of $CD4^+$ T cells in mice with additional administration of 500 ng SEB as compared to mice with AAI alone (figure 21 C).

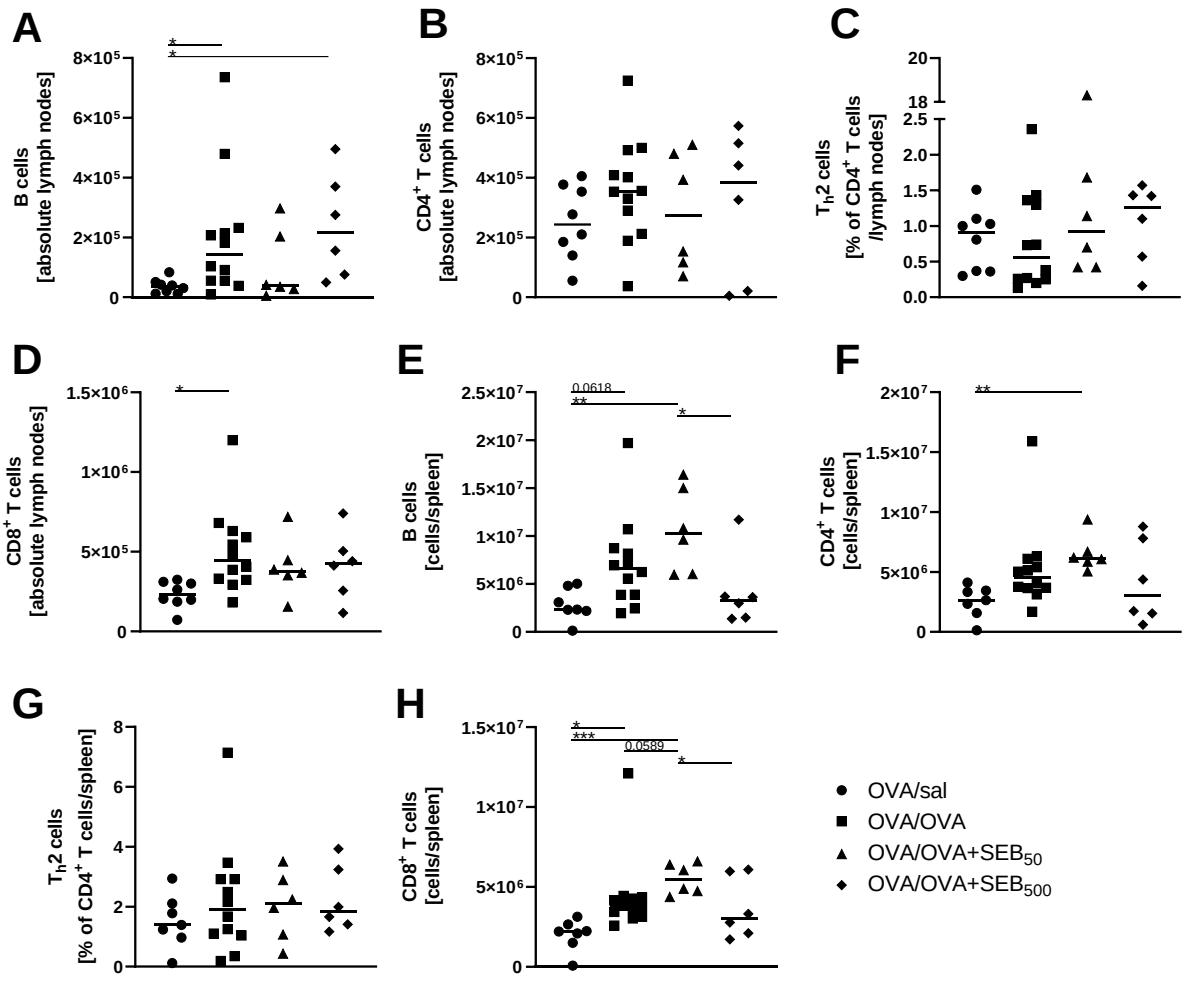

figure 22: Lymphocytes in the draining lymph nodes and spleen in AAI alone and following administration of SEB with the allergic challenge.
Mice were sensitized three times *i.p.* with 10 µg OVA and 1 mg alum in weekly intervals (d 0, 7, 14). One week after the last sensitization, they were *i.n.* challenged with OVA (■ OVA/OVA) or OVA together with 50 ng or 500 ng SEB on three consecutive days (d 21, 22, 23) (50 ng SEB (▲): OVA/OVA+SEB_{50}; 500 ng SEB (♦): OVA/OVA+SEB_{500}). Control mice were sensitized but mock challenged with PBS only (● OVA/sal). On day 25, lymphocytes from pooled cervical and bronchial lymph nodes and spleen were analyzed for total numbers of B cells (A, E), $CD4^+$ T cells (B, F), the frequency of Th2 cells within $CD4^+$ T cells (C, G) and $CD8^+$ T cells (D, H). Data compiled from at least two independent experiments are shown for individual mice with the median. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, ****$p < 0.0001$.

During an allergic inflammation in the respiratory tract, lymphocytes migrate to the draining lymph nodes, where they accumulate and are activated with the help of APCs (Carson *et al.*, 2008; Doganci *et al.*, 2008). In order to completely evaluate the cellular response after the experimental induction of AAI and *i.n.* SEB administration during the allergic challenge, cervical and bronchial lymph nodes draining the respiratory tract were collected, pooled and lymphocytes were isolated. AAI alone led to a significant increase in absolute B cell numbers (figure 22 A). And while absolute numbers of $CD4^+$ T cells (figure 22 B) and the frequency of

Th2 cells (figure 22 C) were not affected by AAI induction alone, absolute numbers of $CD8^+$ T cells (figure 22 D) were also significantly increased in these lymph nodes. I.n. administration of both tested doses of SEB had no effects on absolute numbers of the above-mentioned cell types in cervical and bronchial lymph nodes as compared to AAI alone or the control group (figure 22 A-D).

In the spleen, AAI alone did not affect absolute cell numbers of B cells, $CD4^+$ T cells or the frequency of Th2 cells (figure 22 E-G). AAI alone led to significantly increased absolute numbers of $CD8^+$ T cells (figure 22 H) in the spleen as compared to the control. Compared to AAI alone, neither administration of 50 ng nor 500 ng SEB had an effect on lymphocyte numbers in the spleen. In comparison to the PBS-treated control group, the administration of 50 ng SEB during the allergic challenge led to significantly increased absolute numbers of B cells (figure 22 E), as well as absolute numbers of $CD4^+$and $CD8^+$ T cells (figure 22 F, H). For B cells and $CD4^+$ T cells this was not the case in AAI alone. Treatment with 500 ng SEB led to rather decreased numbers of these cell types as compared to AAI alone and significantly decreased numbers as compared to administration 50 ng SEB in absolute numbers of B cells and $CD8^+$ T cells (figure 22 E, H). Of note, Th2 cell numbers in lymph nodes and spleen were not significantly affected in AAI alone or in combination with SEB administered together with the allergen challenge.

To conclude, the i.n. administration of 50 ng or 500 ng SEB during the allergic challenge significantly affected absolute numbers of T and B lymphocytes in the respiratory tract. Compared to AAI alone, the administration of SEB led to increased numbers of B cells (50 ng SEB), $CD4^+$ (50 ng and 500 ng SEB) and $CD8^+$ (50 ng SEB) T cells in the lung, as wells as increased $CD4^+$ and $CD8^+$ T cell numbers (500 ng SEB) in the BAL. In the spleen, the i.n. administration of 500 ng SEB led to significantly decreased B cell and $CD8^+$ T cell numbers as compared to administration of 50 ng SEB. Lymphocyte numbers in the draining lymph nodes were not affected by *i.n.* administration of 50 ng or 500 ng SEB together with the allergic challenge.

During a typical specific immune response, B cells are activated and mature into effector cells able to produce antigen-specific antibodies. Naïve T helper cells are activated when peptide antigens presented *via* MHCII receptors are recognized and co-stimulation occurs. Once activated, they rapidly divide and secrete cytokines to further regulate and enhance the immune response (Romagnani, 2000).

During allergic inflammation T and B lymphocytes are activated in the same manner. In order to comprehensively asses the influence of *i.n.* SEB administration together with the allergic challenge on AAI, the activation status of T and B lymphocytes was examined using CD69 as an early activation marker expressed on the cell surface. Due to an insufficient number of cells in the BAL, the activation status of T and B cells was examined in lung, spleen and draining lymph nodes only.

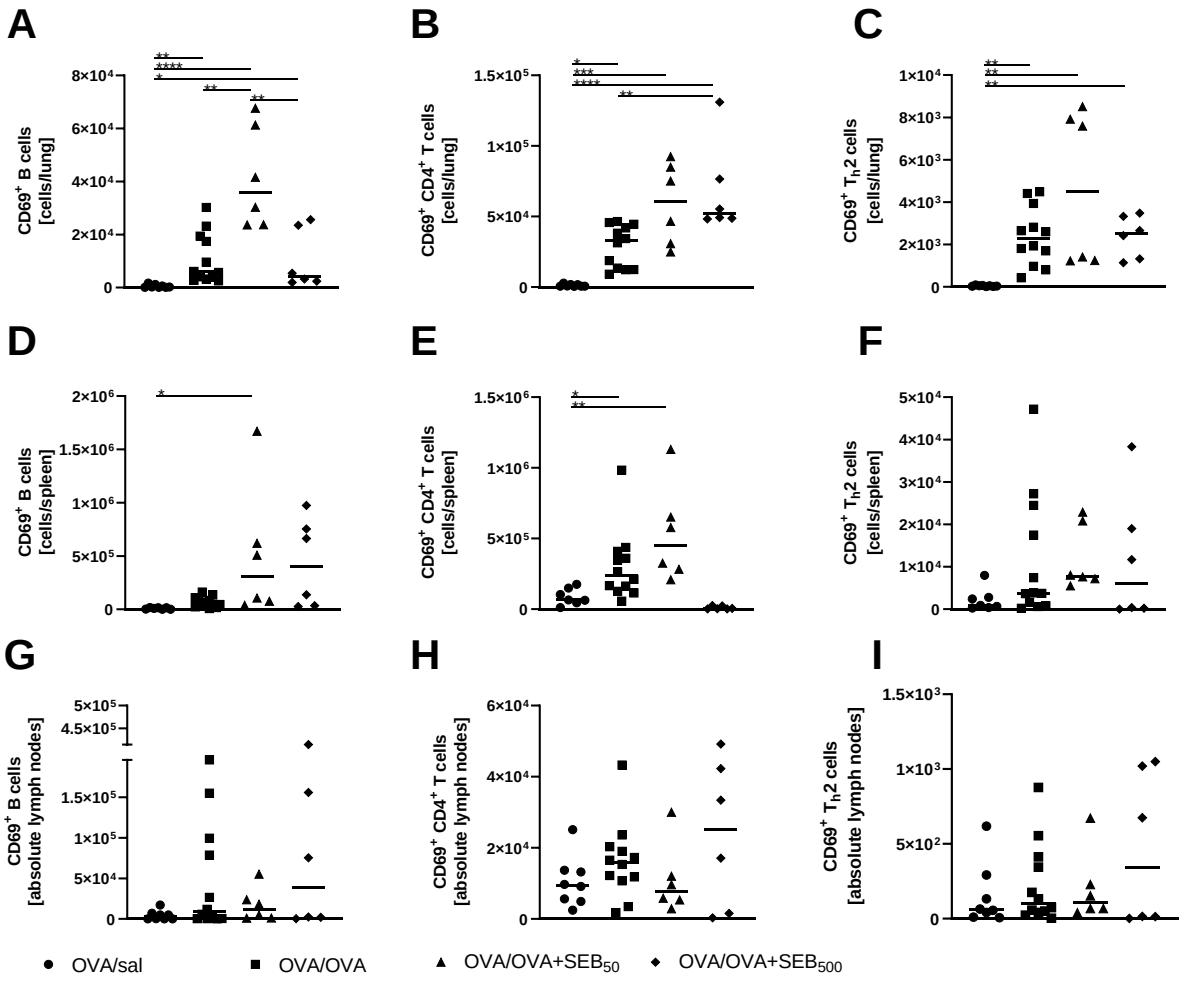

figure 23: Lymphocyte activation in the lung, draining lymph nodes and spleen in AAI alone and following administration of SEB with the allergic challenge.
Mice were sensitized three times *i.p.* with 10 µg OVA and 1 mg alum in weekly intervals (d 0, 7, 14). One week after the last sensitization, they were *i.n.* challenged with OVA (■ OVA/OVA) or OVA together with 50 ng or 500 ng SEB on three consecutive days (d 21, 22, 23) (50 ng SEB (▲): OVA/OVA+SEB_{50}; 500 ng SEB (♦): OVA/OVA+SEB_{500}). Control mice were sensitized but mock challenged with PBS only (● OVA/sal). On day 25, lymphocytes from lung, cervical and bronchial lymph nodes and spleen were analyzed for total numbers of $CD69^+$ B cells (A, D, G), $CD69^+$ $CD4^+$ T cells (B, E, H) and $CD69^+$ Th2 cells (C, F, I). Data compiled from at least two independent experiments are shown for individual mice with the median. *p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.0001.

In the lungs, AAI alone led to significantly increased numbers of activated, CD69-expressing B cells (figure 23 A), $CD4^+$ T cells (figure 23 B) as well as Th2 cells (figure 23 C). Peripheral lymphocytes isolated from the spleen showed a significant increase of activated $CD4^+$ T cells (figure 23 E). Mean absolute numbers of activated B cells or Th2 cells in the spleen were marginally increased but not significantly affected in AAI (figure 23 D, F). Lymphocytes isolated from the draining lymph nodes showed marginally increased numbers of CD69-expressing B cells, $CD4^+$ T cells and Th2 cells, but did not show any significant changes as compared to control mice and AAI alone (figure 23 G, H, I).

The administration of 50 ng SEB with the allergic challenge significantly increased the number of activated B cells (figure 23 A) in the lungs when compared to AAI alone. Also mean total numbers of $CD69^+$ $CD4^+$ T cells and $CD69^+$ Th2 cells in the lungs were increased, however not significantly affected by the *i.n.* administration of 50 ng SEB with the allergic challenge (figure 23 B, C). Activated B cell numbers in the spleen were not affected by AAI induction, however the administration of 50 ng SEB during the allergic challenge led to significantly increased absolute numbers of $CD69^+$ B cells in the spleen as compared to PBS-treated control animals (figure 23 D). $CD69^+$ $CD4^+$ T cells in the spleen were marginally increased in mice with *i.n.* treatment with 50 ng SEB together with the allergic challenge as compared to mice with AAI alone (figure 23 E). Mean numbers of activated Th2 cells in the spleen were not affected by administration of 50 ng SEB (figure 23 F). In the draining lymph nodes, CD69-expression of B cells, $CD4^+$ T cells, as well as Th2 cells was not significantly affected by administration of 50 ng SEB with the allergic challenge (figure 23 G-I).

Mice, *i.n.* administered 500 ng SEB together with the allergic challenge showed significantly increased numbers of activated $CD4^+$ T cells (figure 23 B) in the lungs as compared to AAI alone. At the same time, this treatment did not affect the number of activated B cells or Th2 cells in the lungs (figure 23 B, C). As compared to AAI alone, cells isolated from the spleen (figure 23 D-F) and the local draining lymph nodes (figure 23 G-I) were not affected by the *i.n.* administration of 500 ng SEB during the allergic challenge.

In conclusion, the *i.n.* administration of 50 ng or 500 ng SEB together with the allergic challenge affected the absolute numbers of B and T cells, as well as their activation status, using CD69 as an early marker for activation, in the respiratory tract in a significant manner. Absolute cell numbers and activation status of cells isolated from local draining lymph nodes

and the spleen were not significantly affected by the additional SEB treatment together with the allergic challenge.

4.2.1.6 Effects on respiratory cytokines and the specific IgE-response in AAI

Cytokines are essential for the progress of an immune response. During an allergic immune response, cells of the innate immune system, as well as cells of the adaptive immune system are involved. At all times, cytokines are of paramount importance. They ensure the activation and then proliferation of activated immune cells and promote the further course of the immune response, including its resolution (Banyer *et al.*, 2000).

In addition to pro-inflammatory cytokines like IL-6 and TNF-α, typical cytokines during a type 2-immune response are IL-4, IL-5 and IL-13. Among other functions, they are responsible for the activation of Th2 cell and eosinophils, as well as the class switch from naïve B cells to IgE secreting plasma cells. Cytokine concentrations in the BAL were measured using a panel of relevant cytokines for examining the inflammatory phenotype in AAI and the effect resulting from an *i.n.* SEB administration with the allergic challenge.

The induction of AAI alone led to significantly increased concentrations of IL-4, IL-5 and TNF-α (figure 24 A, B, C), while concentrations of IL 13, IFN-γ, IL-6 and IL-17A were not significantly affected (figure 24 D, E, F, G).

When comparing BAL cytokine levels following the administration of 50 ng SEB together with the allergic challenge to the induction of AAI alone, no effects on any of the cytokines analyzed were detected. However, there were significant changes in the concentrations of IL-4, IL-5, TNF-α, IFN-γ and IL-6 when compared to the control group (figure 24 A, B, C, E, F). Of note, in case of IFN-γ and IL-6 no significant differences between PBS-treated control animals and mice with AAI alone were shown (figure 24 E, F).

Mice *i.n.* administered 500 ng SEB during the allergic challenge showed a significant increase in BAL concentrations of TNF-α, IFN-γ and IL-6 (figure 24 C, E, F) as compared to the AAI only group. Additionally, after the *i.n* administration of 500 ng SEB together with the allergic challenge the concentrations of IL-4 and IL-5 are no longer significantly increased as compared to control mice (figure 24 A, B).

During allergic inflammation, naïve B cells differentiate into IgE-producing plasma cells. The secreted IgE can bind to the surfaces of e.g. mast cells. Due to cross-linking of the surface IgE

molecules the degranulation of the cells is triggered and different inflammatory mediators are released.

Compared to the PBS-treated control group, the concentration of OVA-specific IgE was significantly increased in AAI. The *i.n.* administration of 50 ng or 500 ng SEB together with the allergic challenge had no effect on the OVA-specific IgE-response detected in the serum (figure 24 H).

In summary, the *i.n.* administration of 50 ng SEB during the allergic challenge did not alter the BAL cytokine concentrations when compared to AAI alone but did lead to a significant increase in IFN- γ and IL-6 as compared to controls, that was not apparent in AAI alone. However, after administration of 500 ng SEB with the allergic challenge, concentrations of the pro-inflammatory and Th1 cytokines TNF-α, IL-6 and IFN-γ, respectively were significantly increased as compared to AAI alone. The specific serum IgE-response was not altered due to *i.n.* administration of 50 ng or 500 ng SEB when compared with the AAI only group.

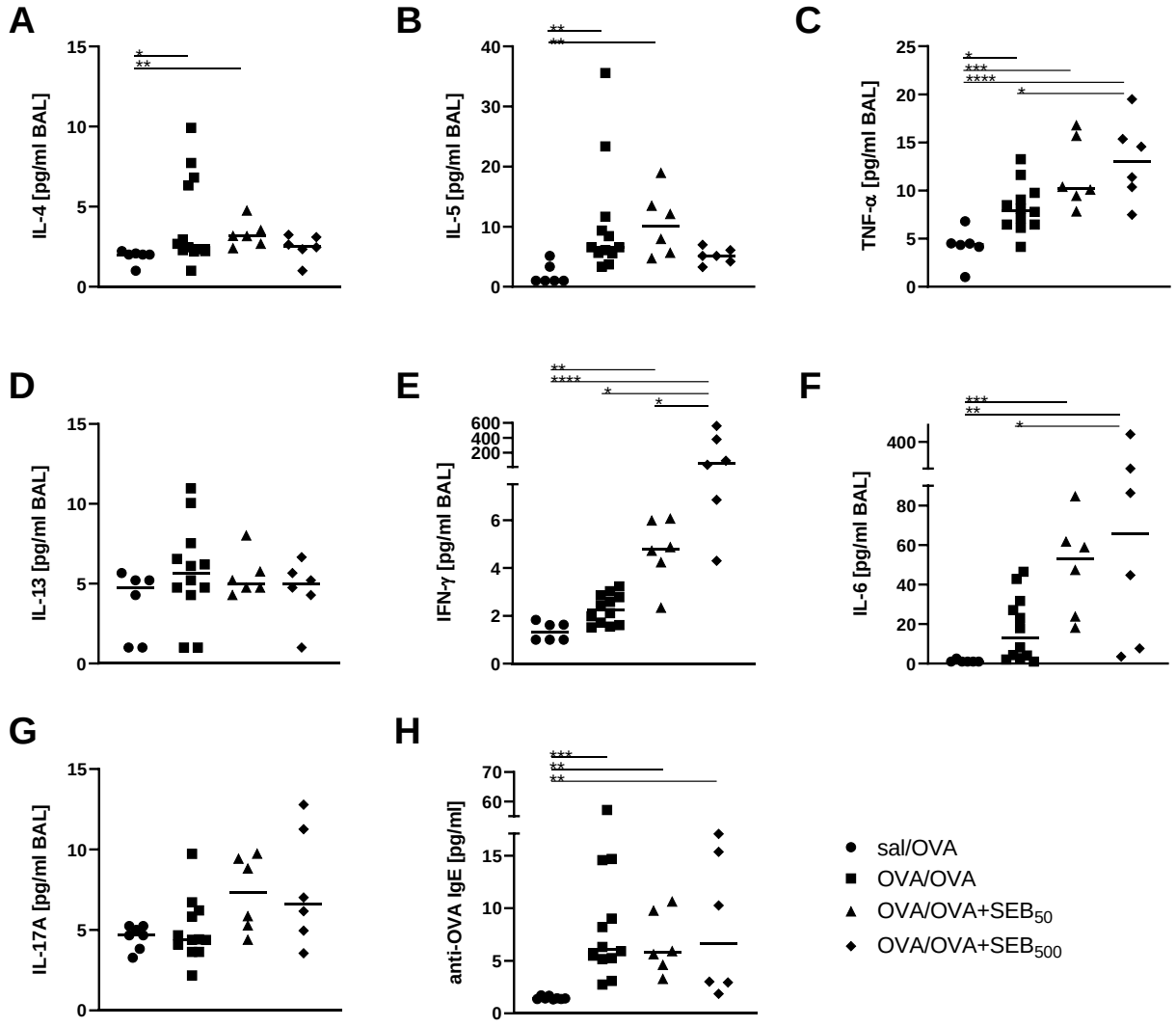

figure 24: Cytokine levels in BAL and OVA-specific IgE in the serum in AAI alone and following administration of SEB with the allergic challenge.
For the induction of AAI, mice were sensitized three times *i.p.* with 10 µg OVA and 1 mg alum in weekly intervals (d 0, 7, 14). One week after the last sensitization they were *i.n.* challenged with OVA (■ OVA/OVA) or OVA together with 50 ng or 500 ng SEB on three consecutive days (d 21, 22, 23) (50 ng SEB (▲): OVA/OVA+SEB_{50}; 500 ng SEB (♦): OVA/OVA+SEB_{500}). Control mice were mock-challenged with PBS only (● OVA/sal). On day 25, bronchoalveolar lavage (BAL) was analyzed for the concentrations of IL-4 (A), IL-5 (B), IL-13 (C), IFN-γ (D), IL-6 (E), TNF-α (F) and IL-17A (G). OVA-specific IgE antibodies were assessed in the serum (H). Data compiled from at least two independent experiments are shown for individual mice with the median. *p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.0001.

4.2.1.7 Effects on airway hyperresponsiveness in AAI

The assessment of airway hyperresponsiveness to unspecific triggers is one of the most common methods of diagnosing bronchial asthma and AAI (Cockcroft, 2010). After assessing lung functional parameters in the resting state, increasing doses of the parasympathomimetic bronchoconstrictor methacholine are nebulized and administered *via* inhalation.

Methacholine has a bronchoconstrictive effect in all subjects, but which is more pronounced in asthmatic patients and animal models of AAI.

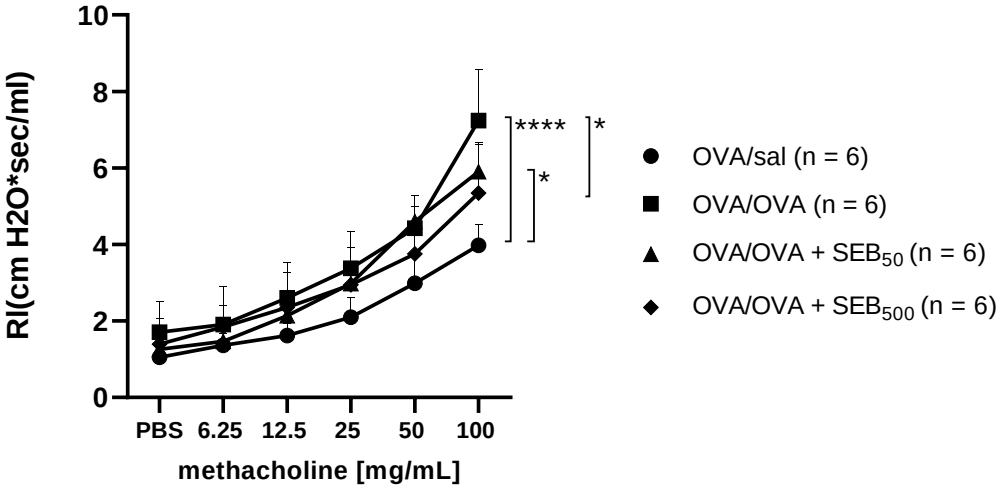

figure 25: Airway hyperreactivity in response to methacholine in AAI alone and following administration of SEB with the allergic challenge.
Mice were sensitized three times *i.p.* with 10 µg OVA and 1 mg alum in weekly intervals (d 0, 7, 14). One week after the last sensitization, they were *i.n.* challenged with OVA (■ OVA/OVA) or OVA together with 50 ng or 500 ng SEB on three consecutive days (d 21, 22, 23) (50 ng SEB (▲): OVA/OVA+SEB_{50}; 500 ng SEB (♦): OVA/OVA+SEB_{500}). Control mice were sensitized but mock challenged with PBS only (● OVA/sal). On day 25, airway hyperreactivity in response to methacholine was assessed. Data (from two individual experiments) are shown as mean + SD. P-values (*$p < 0.05$, ****$p < 0.0001$) refer to inspiratory lung resistance (RI) after inhalation of 100 mg/ml methacholine.

In AAI alone, mice showed significantly increased airway responsiveness to methacholine, measured by the inspiratory lung resistance, as compared to PBS treated control mice.

Mice *i.n.* administered 50 ng SEB together with the allergic challenge also showed significantly increased airway responsiveness to methacholine as compared to PBS treated control mice and the *i.n.* administration of 50 ng SEB did not show any significant effects as compared with mice with AAI alone.

The *i.n.* administration of 500 ng SEB during the allergic challenge significantly attenuated airway responsiveness as compared to AAI alone. The responsiveness to methacholine was attenuated to such an extent that there was no longer any significant difference as compared to the PBS treated control group.

In conclusion, the *i.n.* administration of 50 ng SEB together with the asthmatic challenge did not alter airway responsiveness after methacholine inhalation compared with AAI alone.

However, 500 ng SEB administered with the asthmatic challenge led to a significant amelioration of airway hyperresponsiveness as compared to the AAI only group.

To condense the results of the additional SEB-treatment during the challenge phase of AAI induction, the significant effects are summarized in the following table:

table 25: Summary of the significant effects of SEB *i.n.* administered during the allergic challenge on AAI

SEB dose	Organ	AAI parameter	Effect (compared to AAI alone)
50 ng	lung	dendritic cells	increased
		M2-polarized monocytes/macrophages	increased
		$CD4^+$ T cells	increased
		$CD8^+$ T cells	increased
		$CD69^+$ B cells	increased
	spleen	dendritic cells	increased
		$CD8^+$ T cells	increased
500 ng	lung	$CD4^+$ T cells	increased
		$CD69^+$ T cells	increased
	lung function	airway hyperreactivity	decreased
	BAL	dendritic cells	increased
		$CD4^+$ T cells	increased
		$CD8^+$ T cells	increased
		IFN-γ	increased
		IL-6	increased
		TNF-α	increased

4.2.2 Intranasal SEB administered prior to allergic sensitization

The analysis of possible modulation of AAI through *i.n.* SEB treatment together with the allergic challenge showed clear immunomodulatory effects of SEB. However, *S. aureus*

colonization and allergic challenges do not necessarily occur together, for this reason the possible modulatory potential of *i.n.* SEB administration prior to the allergic sensitization was examined.

To assess whether the *i.n.* SEB administration prior to sensitization modulates AAI, SEB or PBS were *i.n.* administered three times on consecutive days, then the mice were *i.p.* sensitized with OVA in combination with alum or alum only and *i.n.* challenged OVA or PBS. This setup resulted in the following experimental groups: AAI alone (sal/OVA/OVA), administration of 50 ng SEB followed by AAI induction (SEB_{50}/OVA/OVA), administration of 500 ng SEB followed by AAI induction (SEB_{500}/OVA/OVA). Control groups were *i.n.* pre-treated with PBS, *i.p.* sensitized with OVA and alum and then *i.n.* challenged with PBS (sal/OVA/sal) (see figure 26, p. 85).

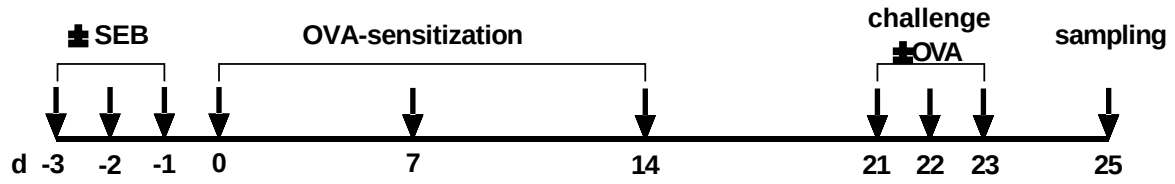

figure 26: Timeline of the experimental setup – *i.n.* SEB-treatment prior to allergic sensitization in combination with OVA mediated AAI.
For the induction of AAI, mice were sensitized three times *i.p.* with 10 µg OVA and 1 mg alum in weekly intervals (d 0, 7, 14). One week after the last sensitization they were *i.n.* challenged with 100 µg OVA in PBS. To investigate effects of i.n. SEB administration on allergic sensitization, mice were treated i.n. with SEB (50 ng or 500 ng) or PBS on three consecutive days, then *i.p*. sensitized with OVA and alum and *i.n*. challenged with OVA. Forty-eight h after the last *i.n.* challenge, the mice were sacrificed and BAL, lungs, serum, spleen, cervical and bronchial lymph nodes were harvested for further analyses.

4.2.2.1 Long-term effects of intranasal administration of 50 ng or 500 ng SEB on an intranasal OVA-challenge in mock sensitized mice

As a basis for these analyses, the long-term effects of *i.n.* SEB administration on the *i.n.* OVA-challenge alone were investigated in unsensitized mice.

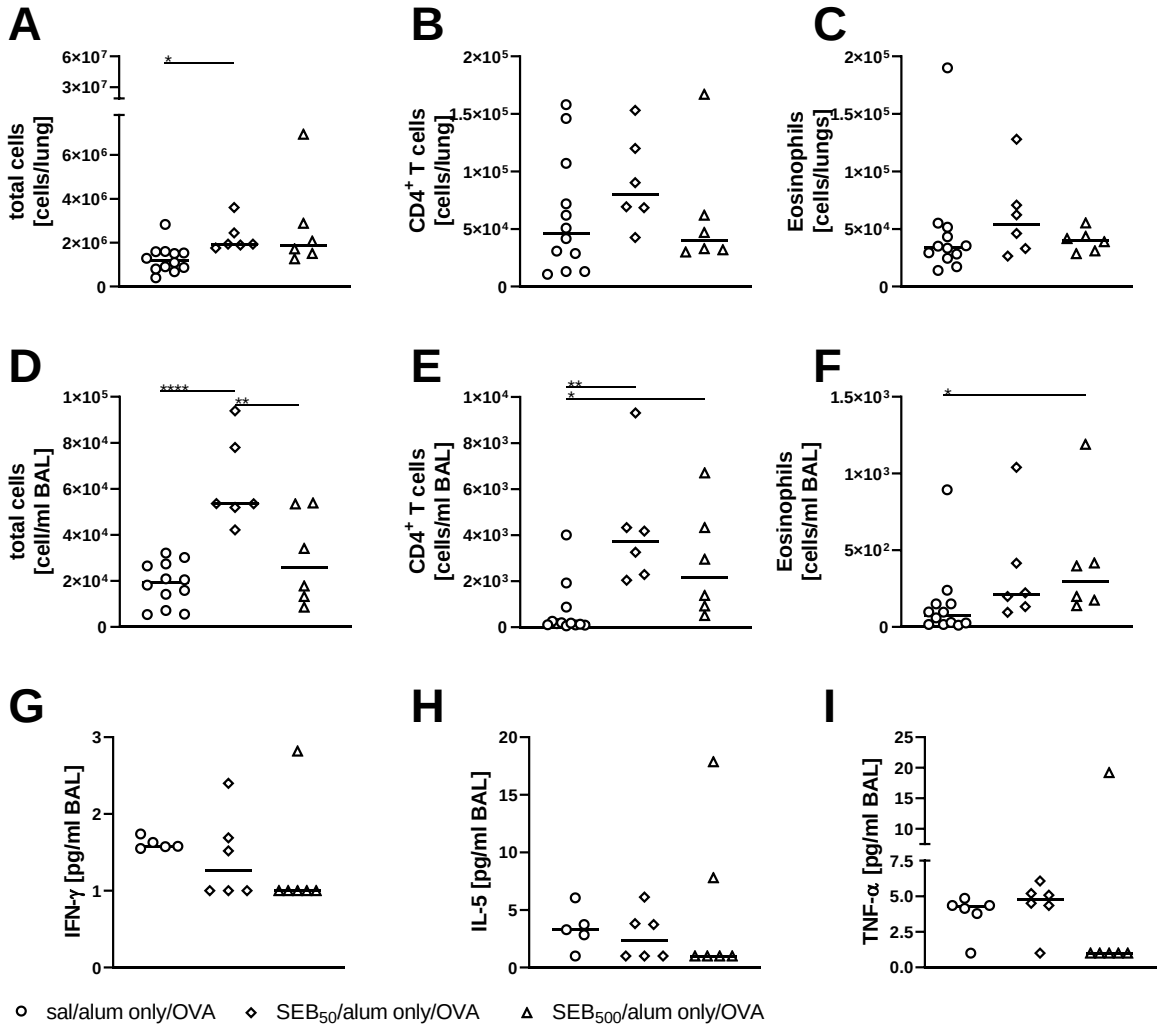

figure 27: Long-term immune cell recruitment to the respiratory tract and the local respiratory cytokine response following *i.n.* SEB-administration in unsensitized mice.
To examine long-term effects of *i.n.* administration of the different doses of SEB alone, mice were *i.n*. administered either 50 ng (◇; SEB_{50}/alum only/OVA) or 500 ng (△; SEB_{500}/alum only/OVA) SEB on three consecutive days (d -3, -2, -1), *i.p.* injected with 1 mg alum only in weekly intervals (d 0, 7, 14) and challenged *i.n.* three times with 100 µg OVA (d 21, 22, 23) one week later. The control group (○; sal/alum only/OVA) was *i.n.* treated with PBS, *i.p*. injected with 1 mg alum only in weekly intervals (d 0, 7, 14) and also challenged *i.n.* three times with OVA (d 21, 22, 23) one week later. On day 25, leukocytes from lungs and bronchoalveolar lavage (BAL) were analyzed using flow-cytometry with respect to the total cell count ((A) lung, (D) BAL), absolute numbers of $CD4^+$ T cells ((B) lung, (E) BAL) and eosinophils ((C) lung, (F) BAL). Concentrations of IFN-γ (G), IL-5 (H) and TNF-α (I) were measured in BAL. Data are compiled from at least two independent experiments and are shown for individual mice with the group median. Groups were compared by one-way ANOVA with Bonferroni post-hoc test or Kruskal-Wallis test with Dunn's post-hoc test depending on whether data were normally distributed or not (according to Shapiro-Wilk testing). *$p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

The *i.n.* administration of 50 ng SEB prior to mock sensitization led to significantly increased total cell numbers in the lungs (d 25) as compared to PBS-treated, mock-sensitized control mice (figure 27 A). Absolute numbers of $CD4^+$ T cells and eosinophils in the lung were not

significantly affected by the administration of 50 ng SEB before mock-sensitization, although a marginally increase was observed (figure 27 B, C).

In the BAL, 50 ng SEB administered prior to mock-sensitization led to a significant increase of total cells, as well as $CD4^+$ T cells (figure 27 D, E) on day 25. However, absolute numbers of eosinophils were not affected (figure 27 F).

The *i.n.* administration of 500 ng SEB before mock-sensitization led to no significant effects in cell populations analyzed in the lung (figure 27 A-C) as compared to control mice. Though, in the BAL, by day 25 a significant increase in absolute numbers of $CD4^+$ T cells and eosinophils was observed after the *i.n.* administration of 500 ng SEB before mock-sensitization (figure 27 E, F). Interestingly, *i.n.* administration of 500 ng SEB led to significantly decreased total cell numbers in the BAL as compared to mice administered 50 ng SEB (figure 27 D).

Upon analysis on day 25, the concentrations of IFN-γ, IL-5 and TNF-α were not significantly affected by the *i.n.* administration of 50 ng or 500 ng SEB prior to mock-sensitization. However, the administration of 500 ng SEB led to marginally decreased concentrations of IFN-γ, IL-5 and TNF-α (figure 27 G-I).

In conclusion, the *i.n.* administration of 50 ng and 500 ng SEB followed by mock-sensitization and OVA-challenge had long-term effects of cell recruitment to the respiratory tract. Especially in the BAL, absolute numbers of $CD4^+$ T cells (50 ng and 500 ng SEB) as wells as eosinophils (500 ng SEB) were significantly increased after SEB administration.

4.2.2.2 Effects on body weight and general condition in AAI

To ensure the well-being of the experimental animals throughout the whole length of the experiment, they were weighted and scored according to the experiment-specific score sheet once every 24 h. The relative body weight was calculated in relation to the body weight prior to the first *i.n.* treatment (d -3), the body weight of the day of each sensitization (d 0, 7, 14) and the body weight of the day of the first *i.n.* allergic challenge (d 21).

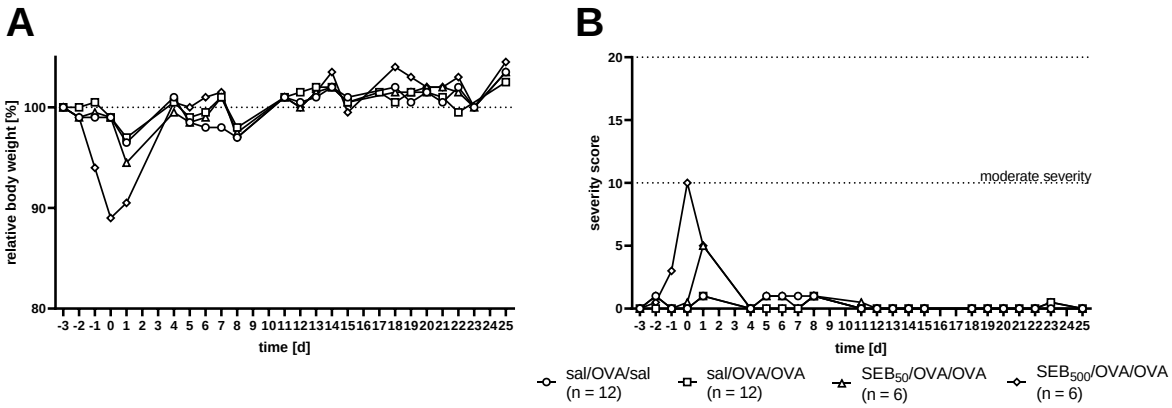

figure 28: Body weight and disease scoring in the course of the induction of AAI following prior *i.n.* administration of SEB.
Mice were treated *i.n.* with 50 ng or 500 ng SEB or PBS (control; sal) on three consecutive days (d -3, -2, -1), then *i.p.* sensitized with 10 µg OVA (d 0, 7, 14) and 1 mg alum and *i.n.* challenged with OVA (50 ng SEB (Δ): SEB_{50}/OVA/OVA; 500 ng SEB(◊): SEB_{500}/OVA/OVA; AAI (□): sal/OVA/OVA) on three consecutive days (d 21, 22, 23). Control mice were mock challenged *i.n.* with PBS only (○ sal/OVA/sal). All mice were weighted (A) and scored (B) daily according to the score sheet. For *i.n.* administration of SEB or PBS prior to sensitization, the relative weight loss was always calculated in relation to the body weight of the day of the first administration. For each sensitization, the relative body weight loss following that sensitization was normalized to the body weight of the day prior to that sensitization. For the *i.n.* challenge, the relative body weight was calculated in relation to the body weight prior to the first challenge. The cumulative severity score (B) was determined according to the score sheet. Data are shown as group median of n ≥ 6 mice form a minimum of two independent experiments.

The induction of AAI alone did not affect the relative body weight or the severity score when compared to the PBS-treated control group. Comparing the relative body weight and severity score of mice following *i.n.* administration of 50 ng SEB prior to allergic sensitization to mice with AAI alone, a minor weight loss of about 6 % (figure 28 A) and a minor increase of the severity score (figure 28 B) to 5 on day 1 after the first sensitization were observed. The *i.n.* administration of 500 ng SEB before allergic sensitization led to a moderate weight loss on day 0 of about 11 % (figure 28 A) and an increased severity score of 10 (figure 28 B). On day 4, mice administered 500 ng SEB prior to allergic sensitization reached their starting weight again. The *i.n.* administration of 50 ng or 500 ng SEB before allergic sensitization did not affect relative body weight or severity score during the following peripheral sensitization or the *i.n.* OVA-challenge at later time points during the experiments.

To summarize, *i.n.* administered SEB (50 ng or 500 ng) did not sustainably alter the body weight or the general condition of treated mice. Only *i.n.* administration of 500 ng SEB led to a short-term weight loss and a medium severity score of 10 for a short period of time (one day).

4.2.2.3 Histological effects in AAI

To evaluate the effects of an *i.n.* SEB administration prior to the allergic sensitization on subsequently induced AAI, lungs were harvested and fixed in 4 % PFA. After they were embedded in paraffin and cut into 5 µm thick slices, they were stained with hematoxylin and eosin. The cellular influx and structural changes were evaluated by a certified veterinary pathologist.

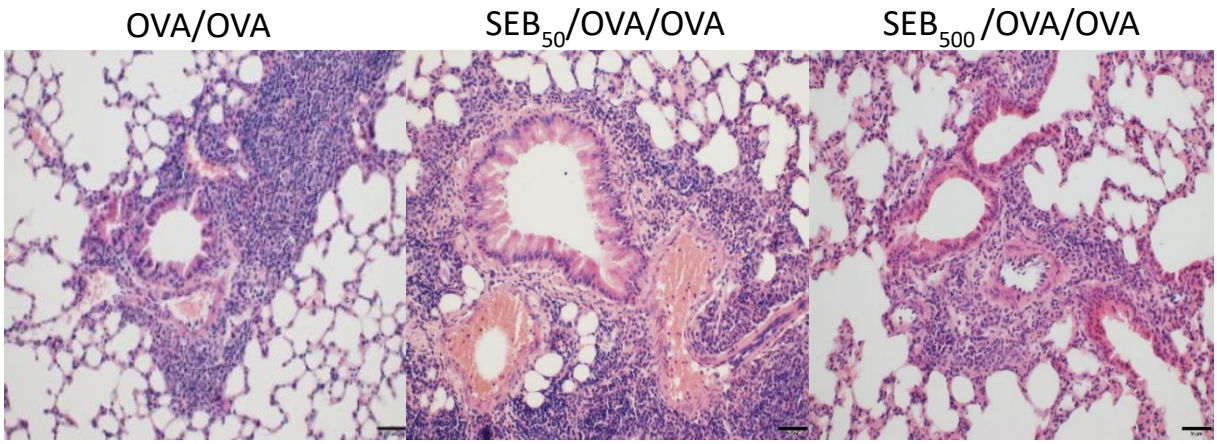

figure 29: Histological lung sections after AAI induction alone (OVA/OVA) and after *i.n.* administration of 50 ng SEB (SEB_{50}/OVA/OVA) or 500 ng SEB (SEB_{500}/OVA/OVA) prior to the allergic sensitization.
SEB was *i.n.* administered on three consecutive days (d -3, -2, -1) prior to the allergic sensitization. For the induction of AAI, C57BL/6 mice were *i.p.* sensitized with 10 µg OVA in 1 mg alum three times in weekly intervals (d 0, 7, 14). One week after the last sensitization, mice were *i.n.* challenged on three consecutive days (d 21, 22, 23) with 100 µg OVA in PBS. Control mice were mock challenged with PBS. On day 25, lungs (n = 5) were stained with hematoxylin and eosin. Scale bar = 50 µm. The histological lung sections were evaluated by a certified veterinary pathologist.

Histologically, lung lesions characterized by a typical bronchointerstitial pneumonia with a strong involvement of eosinophils were found in mice with AAI (OVA/OVA). Additionally, a mild bronchial epithelial hyperplasia and pneumocyte type II hyperplasia were observed.
The administration of 50 ng or 500 ng SEB prior to the allergic sensitization led to overall similar histological changes. However, mice administered 50 ng SEB before allergic sensitization (SEB_{50}/OVA/OVA) showed only moderately activated alveolar macrophages as compared to clearly activated alveolar macrophages in AAI alone and after the administration of 500 ng SEB prior to allergic sensitization. Additionally, mice administered 500 ng SEB before allergic sensitization (SEB_{500}/OVA/OVA) showed a less pronounced interstitial infiltration of eosinophils as compared to AAI alone and after the administration of 50 ng SEB prior to the allergic sensitization (see figure 29, p. 89, scoring data not shown).

4.2.2.4 Effects on innate effector cells of AAI in the respiratory tract and spleen

To date, it is not fully understood, what factors drive allergen-specific Th2-sensitization that is underlying subsequent allergic responses.

To examine the potential modulation of the phenotype of AAI through *i.n.* administration of 50 ng or 500 ng SEB before the allergic sensitization, mice were *i.n.* treated with SEB on three consecutive days (d -3, -2, -1) before the peripheral sensitization.

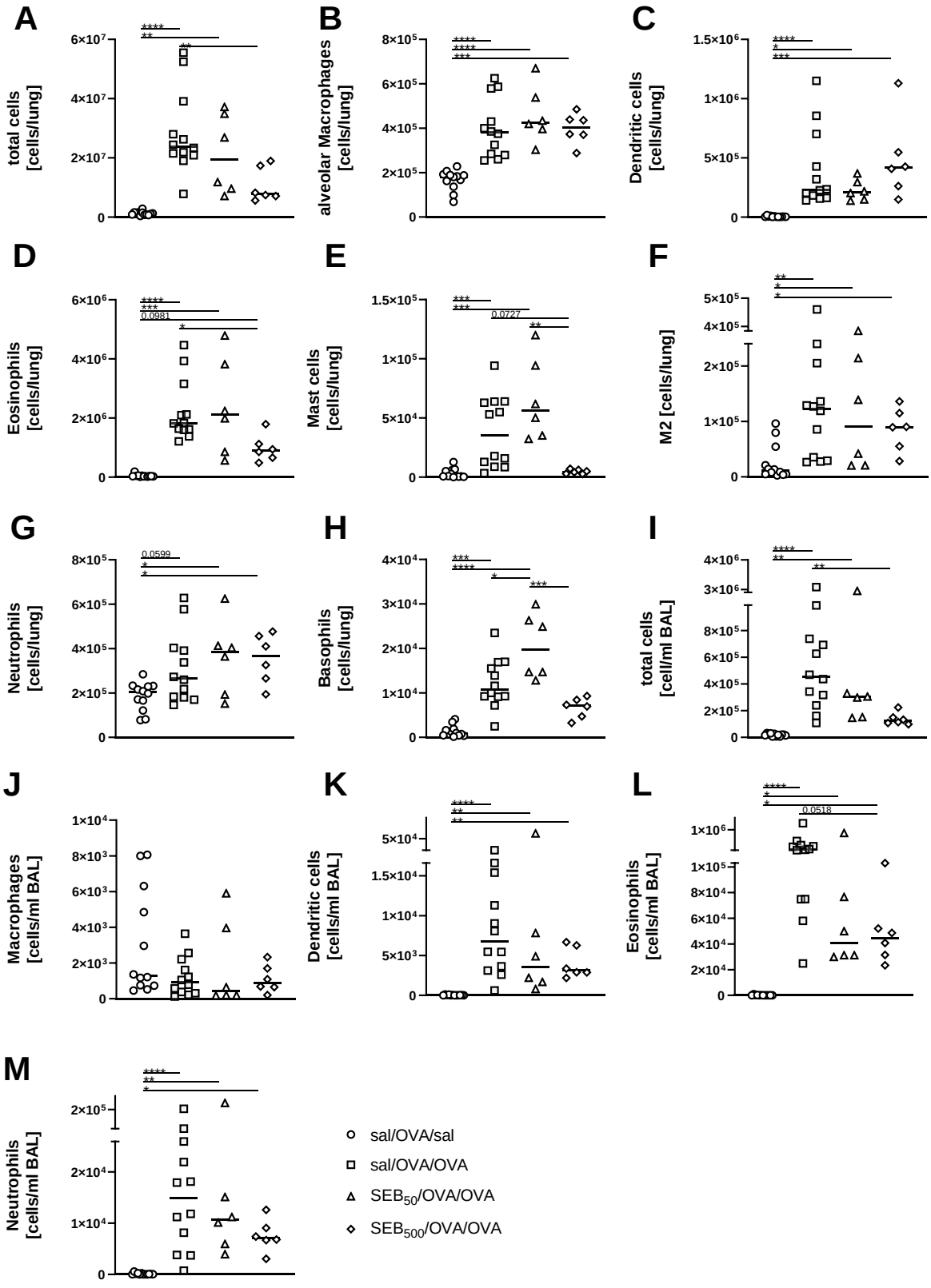

figure 30: Innate immune cells in the respiratory tract in AAI alone and following administration of SEB prior to allergic sensitization.

Mice were treated *i.n.* with 50 ng or 500 ng SEB or PBS (control; sal) on three consecutive days, then *i.p.* sensitized with 10 µg OVA and 1 mg alum and *i.n.* challenged with OVA (50 ng SEB (Δ): SEB_{50}/OVA/OVA; 500 ng SEB (◊): SEB_{500}/OVA/OVA; AAI (□): sal/OVA/OVA). Control mice were mock challenged *i.n.* with PBS only (○ sal/OVA/sal). On day 25, leukocytes from lung and BAL were analyzed for total cell count (A, I) and total numbers of (alveolar)

macrophages (B, J), DCs (C, K), eosinophils (D, L), mast cells (E), M2-polarized monocytes/macrophages (F), neutrophils (G, M) and basophils (H). Data compiled from at least two independent experiments are shown for individual mice with the median. *p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.0001.

In the lungs, AAI alone led to a significant increase in total cell number, absolute numbers of alveolar macrophages, DCs, eosinophils, mast cells, M2-polarized monocytes/macrophages, as well as basophils (figure 30 A, B, C, D, E, F, H). Absolute numbers of neutrophils were increased, but not significantly affected (figure 30 G).

Compared to AAI alone, the i.n. administration of 50 ng SEB prior to allergic sensitization did not affect the numbers of the above-mentioned cell populations, with the exception of absolute numbers of basophils, which were significantly increased (figure 30 H).

Mice i.n. administered 500 ng SEB before allergic sensitization showed significantly decreased total cell numbers (figure 30 A), along with significantly decreased numbers of eosinophils (figure 30 D) as compared to AAI alone. Absolute numbers of basophils and mast cells were not significantly affected as compared to AAI alone. However, they were significantly decreased when compared to i.n. administration of 50 ng SEB prior to allergic sensitization (figure 30 E, H).

In the BAL, AAI alone also led to a significant increase in total cell numbers, DCs, eosinophils as well as neutrophils (figure 30 I, K, L, M).

The i.n. administration of 50 ng SEB prior to allergic sensitization did not affect the innate immune cell composition in the BAL. When mice were i.n. administered 500 ng SEB before allergic sensitization, as in the lungs a significant decrease in total cell numbers (figure 30 I) was observed as compared to AAI alone. The i.n. administration of 500 ng SEB led to marginally decreased absolute cell numbers of DCs, eosinophils and neutrophils (figure 30 K, L, M), however it was not significant.

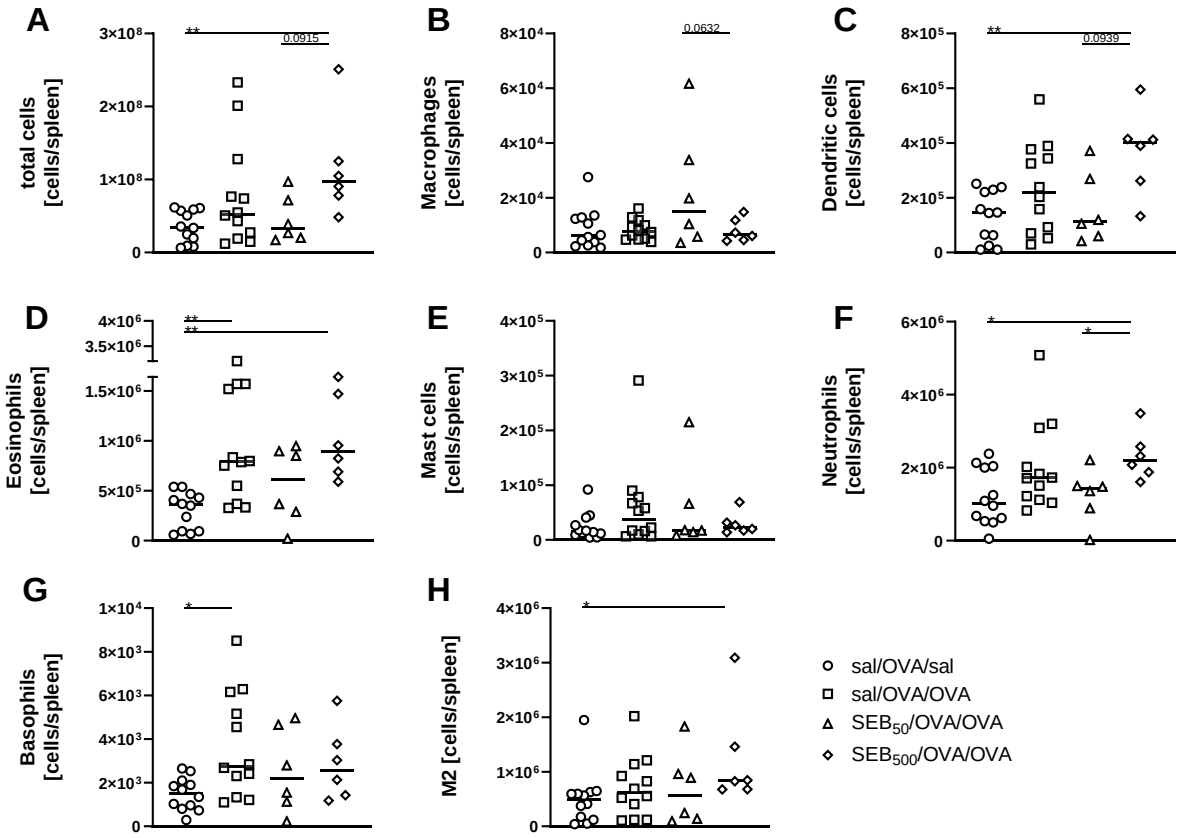

figure 31: Innate immune cells in the spleen in AAI alone and following administration of SEB prior to the allergic sensitization.

Mice were treated *i.n.* with 50 ng or 500 ng SEB or PBS (control; sal) on three consecutive days, then *i.p.* sensitized with 10 µg OVA and 1 mg alum and *i.n.* challenged with OVA (50 ng SEB (Δ): SEB_{50}/OVA/OVA; 500 ng SEB (◊): SEB_{500}/OVA/OVA; AAI (□): sal/OVA/OVA). Control mice were mock challenged *i.n.* with PBS only (○ sal/OVA/sal). On day 25, leukocytes from the spleen were analyzed for total cell numbers (A), absolute numbers of macrophages (B), DCs (C), eosinophils (D), mast cells (E), neutrophils (F), basophils (G) and M2-polarized monocytes/macrophages (H). Data compiled from at least two independent experiments are shown for individual mice with the median. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, ****$p < 0.0001$.

In the periphery, which is displayed here through the analysis of splenocytes, AAI alone led to a significant increase of eosinophils and basophils (figure 31 D, G) as compared to PBS-challenged control mice.

The *i.n.* administration of either 50 ng or 500 ng SEB prior to allergic sensitization did not significantly affect the analyzed cell populations in the spleen as compared to AAI alone. However, as compared to PBS-pre-treated and PBS mock–challenged control mice, the *i.n.* administration of 500 ng SEB prior to allergic sensitization led to a significant increase in total cell numbers, DCs, eosinophils, neutrophils, as well as M2-polarized monocytes/macrophages in the spleen (figure 31 A, C, D, F, H). Of note, except for eosinophils, these cells were not significantly elevated in the AAI alone group.

To conclude, the composition of innate immune cells in the respiratory tract upon induction of AAI was significantly affected into a pro-inflammatory direction by the *i.n.* administration of 50 ng SEB before sensitization. On the other hand, *i.n.* administration of 500 ng SEB prior to sensitization showed tendencies to attenuate AAI.

The *i.n.* administration of 50 ng or 500 ng SEB prior to the allergic sensitization did not significantly affect the composition of innate immune cells in the spleen as compared to AAI alone. However, administration of 500 ng SEB before sensitization exclusively led to the accumulation of significantly increased numbers of DCs, neutrophils and M2-polarized monocytes/macrophages in the spleen as compared to controls.

4.2.2.5 Effects on T and B cells in the respiratory tract, draining lymph nodes and spleen

AAI induced after the *i.n.* administration of 50 ng or 500 ng SEB prior to allergic sensitization was further characterized by a more detailed analysis of lymphocyte recruitment to the respiratory tract, the draining cervical and bronchial lymph nodes as well as lymphocyte numbers in the periphery.

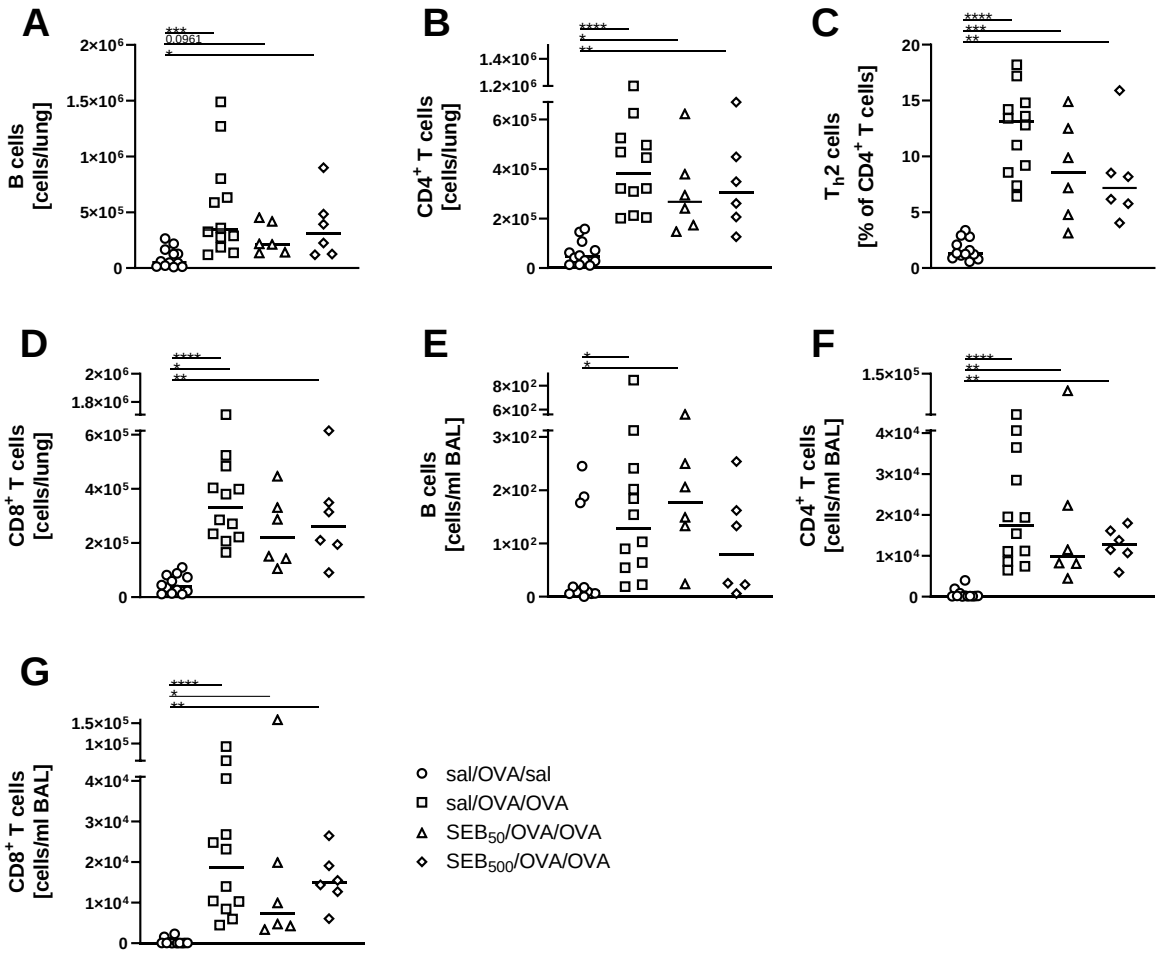

figure 32: Lymphocytes in the respiratory tract in AAI alone and following administration of SEB prior to the allergic sensitization.

Mice were treated *i.n.* with 50 ng or 500 ng SEB or PBS (control; sal) on three consecutive days, then *i.p*. sensitized with 10 µg OVA and 1 mg alum and *i.n*. challenged with 100 µg OVA (50 ng SEB (Δ): SEB_{50}/OVA/OVA; 500 ng SEB (◊): SEB_{500}/OVA/OVA; AAI (□): sal/OVA/OVA). Control mice were mock challenged *i.n*. with PBS only (○ sal/OVA/sal). On day 25, leukocytes from lung and BAL were analyzed for total numbers of B cells (A, E), $CD4^+$ T cells (B, F), the frequency of Th2 cells within $CD4^+$ T cells (C) and $CD8^+$ T cells (D, G). Data compiled from at least two independent experiments are shown for individual mice with the median. *p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.0001.

In the lung and BAL, AAI alone led to a significant increase in absolute numbers of B cells (figure 32 A, E), $CD4^+$ T cells (figure 32 B, F) and the frequency of Th2 cells (figure 32 C; only analyzed in the lung) as well as absolute numbers of $CD8^+$ T cells (figure 32 D, G). The absolute numbers of the above-mentioned cell population were not affected by the *i.n.* treatment with 50 ng or 500 ng SEB before allergic sensitization as compared to AAI alone (figure 32). However, in the BAL, 500 ng SEB administered prior to allergic sensitization resulted in absolute B cell numbers no longer significant as compared to the control group (figure 32 E).

In these experiments, the analysis of splenocytes showed no effects of AAI induction on absolute numbers of B cells, $CD4^+$ T cells and the frequency of Th2 cells as well as absolute numbers of $CD8^+$ T cells (figure 33 A-D).

The *i.n.* administration of 50 ng or 500 ng SEB prior to allergic sensitization showed no significant effects on the absolute numbers these lymphocyte subsets as compared to AAI alone. However, *i.n.* administration of 500 ng SEB before allergic sensitization led to significantly increased absolute cell numbers of B cells, $CD4^+$ and $CD8^+$ T cells, along with a significant decrease in the frequency of Th2 cells in the spleen as compared to mice that had been *i.n.* administered 50 ng SEB prior to allergic sensitization (figure 33 A-D).

In lymphocytes from pooled cervical and bronchial lymph nodes, AAI alone led to significantly increased absolute cell numbers of B cells, $CD4^+$ and $CD8^+$ T cells (figure 33 E, F, H). As compared to AAI alone, the *i.n.* administration of 50 ng SEB prior to allergic sensitization led to a significant decrease in $CD4^+$ T cells (figure 33 F). In addition, absolute numbers of B cells, $CD4^+$ and $CD8^+$ T cells were no longer significantly increased as compared to control mice (figure 33 E, F, H). *i.n.* administration of 500 ng SEB before allergic sensitization led to a significant decrease of the frequency of Th2 cells (figure 33 G) in the draining lymph nodes as compared to AAI alone. Additionally, absolute numbers of $CD4^+$ and $CD8^+$ T cells were no longer significantly increased as compared to the control group (figure 33 F, H).

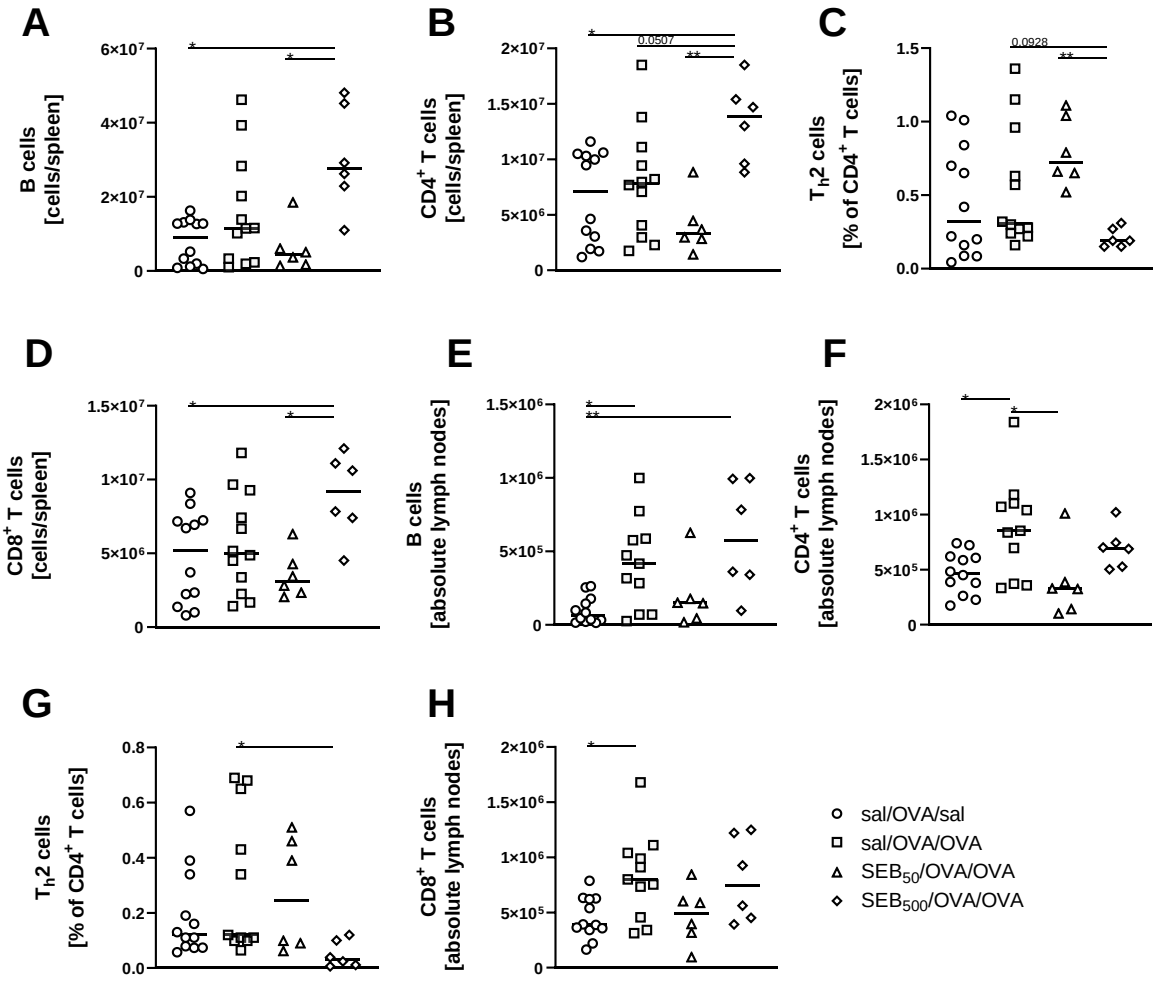

figure 33: Lymphocytes in the spleen and draining lymph nodes in AAI alone and following administration of SEB prior to the allergic sensitization.

Mice were treated *i.n.* with 50 ng or 500 ng SEB or PBS (control; sal) on three consecutive days, then *i.p.* sensitized with 10 µg OVA and 1 mg alum and *i.n*. challenged with OVA (50 ng SEB (Δ): SEB_{50}/OVA/OVA; 500 ng SEB (◊): SEB_{500}/OVA/OVA; AAI (□): sal/OVA/OVA). Control mice were mock challenged *i.n.* with PBS only (○ sal/OVA/sal). On day 25, leukocytes from spleen and pooled cervical and bronchial lymph nodes were analyzed for total numbers of B cells (A, E), $CD4^+$ T cells (B, F), the frequency of Th2 cells within $CD4^+$ T cells (C) and $CD8^+$ T cells (D, H). Data compiled from at least two independent experiments are shown for individual mice with the median. *p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.0001.

With respect to lymphocyte activation, in AAI alone the absolute numbers of activated, CD69-expressing B cells, $CD4^+$ T cells and Th2 cells in the lung were significantly increased as compared to PBS-challenged control mice (figure 34 A, B, C). The *i.n.* administration of 50 ng or 500 ng SEB prior to allergic sensitization led to no significant effects on the absolute numbers of the above-mentioned cell populations.

In the spleen, AAI alone led to no significant changes in absolute numbers of $CD69^+$ B cells, $CD69^+$ $CD4^+$ T cells or $CD69^+$ Th2 cells (figure 34 D, E, F). After the *i.n.* administration of 50 ng SEB prior to allergic sensitization marginally but not significantly increased numbers of $CD69^+$

Th2 cells were observed as compared to AAI alone (figure 34 F). The administration of 500 ng SEB before the allergic sensitization led to increased numbers of activated B cells, as well as activated $CD4^+$ T cells as compared to the control group. Compared to AAI alone, there were no significant effects of *i.n.* administration of 500 ng SEB prior to the allergic sensitization (figure 34 D, E, F). Nevertheless, a significant increase in $CD69^+$ $CD4^+$ T cells was observed as compared to mice administered 50 ng SEB prior to allergic sensitization (figure 34 E).

The analysis of CD69 expression in lymphocytes isolated from the draining lymph nodes showed no significant effects of AAI alone as compared to mice from the control group. However, compared to the control group, 50 ng SEB administered before allergic sensitization led to a significant increase in in absolute numbers of $CD69^+$ B cells. Compared to AAI alone, the increase was also observed, but it was not statistically significant (figure 34 G). The *i.n.* administration of 500 ng SEB prior to allergic sensitization did not affect the absolute numbers of activated B cell, $CD4^+$ T cells or Th2 cells as compared to AAI alone. Compared to mice administered 50 ng SEB before allergic challenge, absolute numbers of $CD69^+$ B cells were significantly decreased (figure 34 G H I).

Taken together, mice pre-treated with 50 ng or 500 ng SEB before sensitization did not show any changes in the numbers of activated lymphocytes in the lung, cells of cervical and bronchial lymph nodes or in splenocytes as compared to mice with AAI alone.

In conclusion, the *i.n.* administration of 50 ng or 500 ng SEB prior to allergic sensitization had no substantial effects of B and T cell numbers and activation in the respiratory tract, draining lymph nodes and the spleen as compared to AAI alone. *i.n.* administration of 500 ng SEB before allergic sensitization had a decreasing effect on absolute numbers of B cells in the BAL, as well as increasing effects on B cells, $CD4^+$ and $CD8^+$ T cells in the spleen, although they were observed also as compared to AAI alone, they were only significant as compared to the control group.

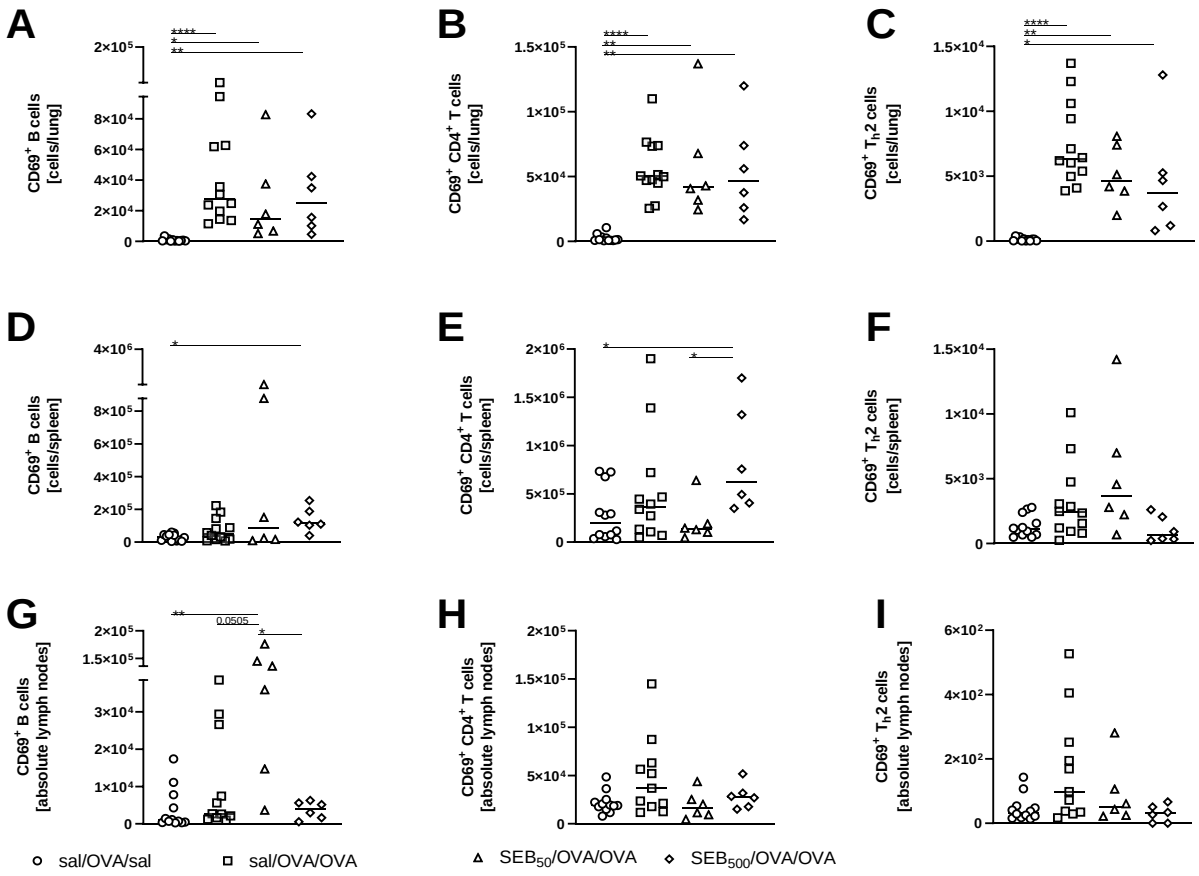

figure 34: Lymphocyte activation in the lung, draining lymph nodes and spleen in AAI alone and following administration of SEB prior to the allergic sensitization.
Mice were treated *i.n.* with 50 ng or 500 ng SEB or PBS (control; sal) on three consecutive days, then *i.p*. sensitized with 10 µg OVA and 1 mg alum and *i.n*. challenged with 100 µg OVA (50 ng SEB (Δ): SEB_{50}/OVA/OVA; 500 ng SEB(◊): SEB_{500}/OVA/OVA; AAI (□): sal/OVA/OVA). Control mice were mock challenged *i.n*. with PBS only (○ sal/OVA/sal). On day 25, leukocytes from lung, cervical and bronchial lymph nodes and spleen were analyzed for total numbers of $CD69^+$ B cells (A, D, G), $CD69^+$ $CD4^+$ T cells (B, E, H) and $CD69^+$ Th2 cells (C, F, I). Data compiled from at least two independent experiments are shown for individual mice with the median. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$.

4.2.2.6 Effects on respiratory cytokines and the specific IgE-response

In experiments where mice were *i.n.* treated with SEB prior to the allergic sensitization, there was a significant increase in the concentrations of IL-4, IL-5, IL-6 and TNF-α in the supernatant of the BAL in AAI alone as compared to control mice (figure 35 A, B, E, F). The concentrations of IL-13, IFN-γ and IL-17A were not affected in AAI (figure 35 C, D, H).

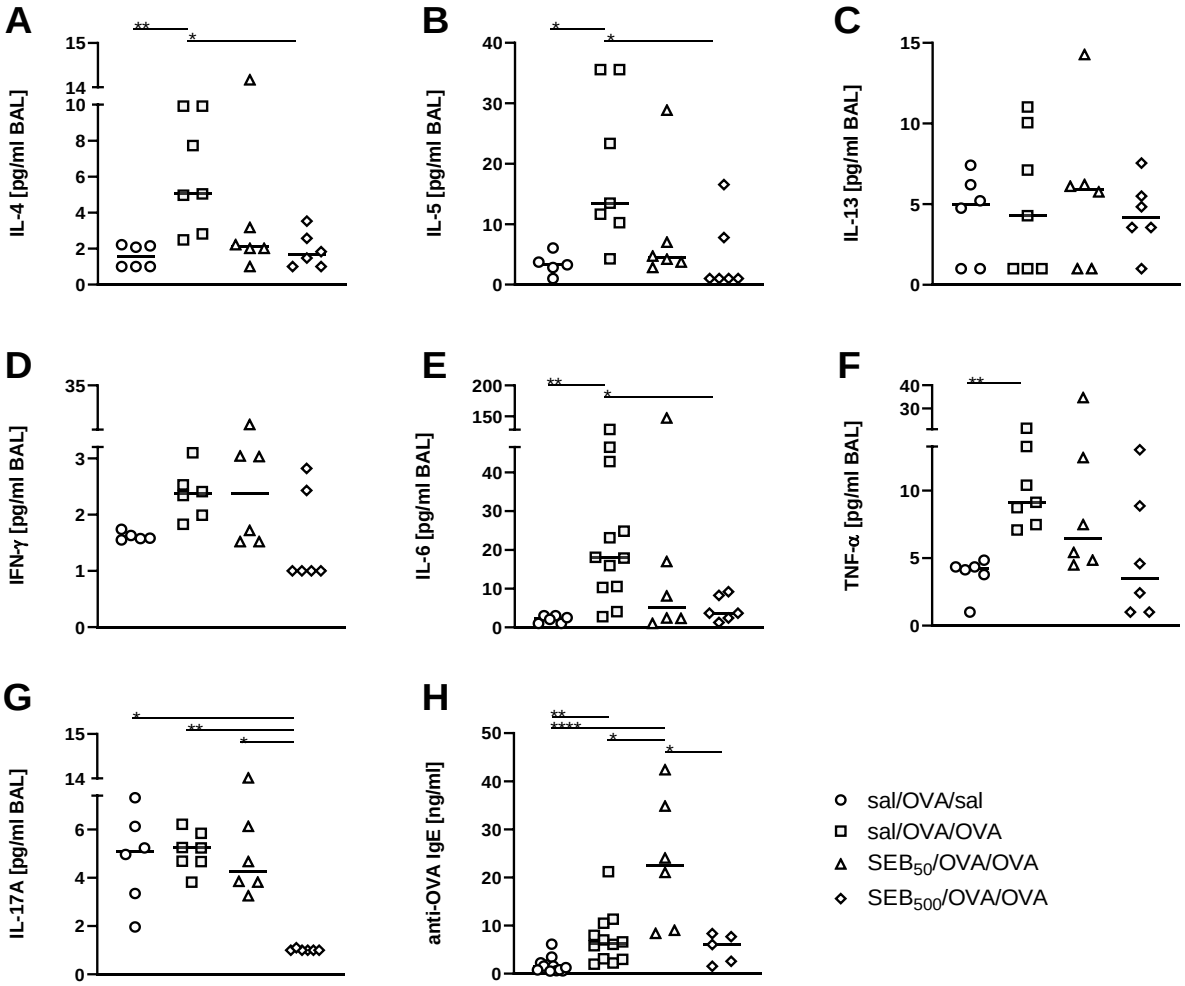

figure 35: Cytokine levels in the BAL and OVA-specific IgE in the serum in AAI alone and following administration of SEB prior to the allergic sensitization.

Mice were treated *i.n.* with 50 ng or 500 ng SEB or PBS (control; sal) on three consecutive days, then *i.p*. sensitized with 10 µg OVA and 1 mg alum and *i.n*. challenged with 100 µg OVA (50 ng SEB (Δ): SEB_{50}/OVA/OVA; 500 ng SEB (◊): SEB_{500}/OVA/OVA; AAI (□): sal/OVA/OVA). Control mice were mock challenged *i.n*. with PBS only (○ sal/OVA/sal). On day 25, bronchoalveolar lavage (BAL) was analyzed for the concentrations of IL-4 (A), IL-5 (B), IL-13 (C), IFN-γ (D), IL-6 (E), TNF-α (F) and IL-17A (G). OVA-specific IgE antibodies were assessed in the serum (H). Data compiled from at least two independent experiments are shown for individual mice with the median. *p < 0.05, **p < 0.01, ****p < 0.0001.

While the *i.n.* administration of 50 ng SEB prior to the allergic sensitization did not show any effects on the concentrations of the above-mentioned cytokines as compared to AAI alone, the *i.n.* administration of 500 ng SEB before allergic sensitization led to a significant decrease in IL-4, IL-5, IL-6 and IL-17A concentrations as compared to AAI alone (figure 35 A, B, E, G). Additionally, when compared to mice administered 50 ng SEB prior to allergic sensitization, the administration of 500 ng SEB led to a significantly decreased concentration of IL-17A. Interestingly, the *i.n.* administration of 50 ng or 500 ng SEB before allergic sensitization

resulted in no longer significantly increased TNF-α concentrations as compared to the control group (figure 35 F).

After the induction of AAI alone, there was a significant increase of OVA-specific IgE antibodies in the serum, compared to PBS-treated control mice (figure 35 H). Although the *i.n.* administration of 50 ng SEB prior to allergic sensitization did not affect the cytokine concentrations in the respiratory tract, it led to a significant increase in OVA-specific IgE antibodies as compared to mice with AAI alone, in the serum (figure 35 H). The administration of 500 ng before allergic sensitization did not show any effects on the IgE-production compared to mice with AAI alone however the OVA-specific IgE concentration was significantly reduced as compared to mice *i.n.* administered 50 ng SEB prior to allergic sensitization (figure 35 H).

Taken together, the *i.n.* administration of 50 ng SEB prior to allergic sensitization did not affect the concentrations of respiratory cytokines (IL-4, IL-5, IL-13, IFN-γ, IL-6, TNF-α and IL-17A) measured in the BAL, however it significantly increased the OVA-specific IgE response measured in the serum. *I.n.* administration of 500 ng SEB before allergic sensitization led to significantly decreased concentrations of typical type 2 cytokines (IL-4, IL-5) as well as the pro-inflammatory cytokines IL-6 and IL-17A in the BAL as compared to AAI alone.

4.2.2.7 Effects on airway hyperresponsiveness in AAI

Compared to the PBS-treated control group, AAI alone led to significantly increased airway hyperreactivity also in these experiments. The pre-treatment with 50 ng or 500 ng SEB prior to allergic sensitization did not significantly affect the airway hyperreactivity compared to mice with AAI alone.

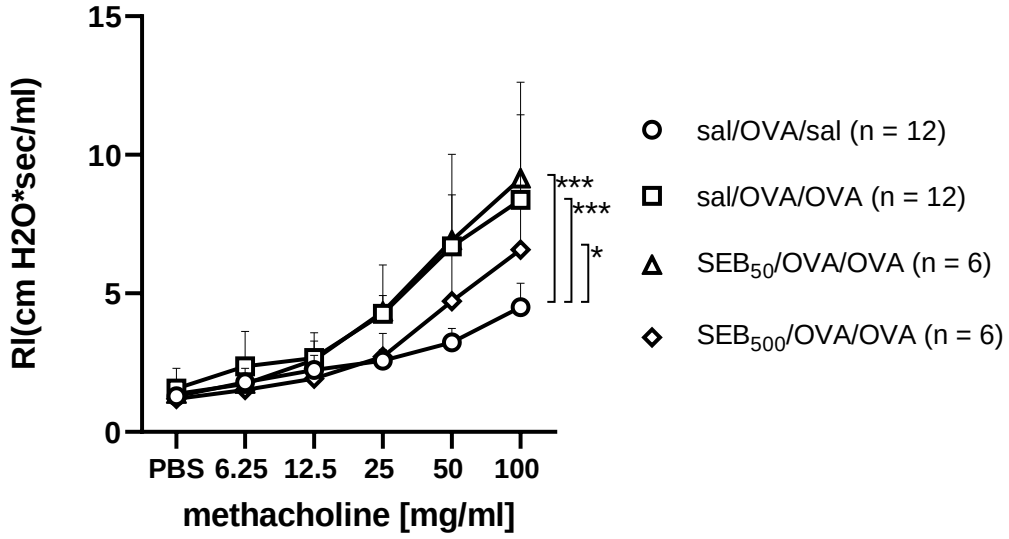

figure 36: Airway hyperreactivity in response to methacholine in AAI alone and following administration of SEB prior to the allergic sensitization.
Mice were treated *i.n.* with 50 ng or 500 ng SEB or PBS (control; sal) on three consecutive days, then *i.p*. sensitized with 10 µg OVA and 1 mg alum and *i.n*. challenged with 100 µg OVA (50 ng SEB (Δ): SEB_{50}/OVA/OVA; 500 ng SEB (◊): SEB_{500}/OVA/OVA; AAI (□): sal/OVA/OVA). Control mice were mock challenged *i.n*. with PBS only (○ sal/OVA/sal). On day 25, airway hyperreactivity in response to methacholine was assessed. Data (from two individual experiments) are shown as mean + SD. P-values (*p < 0.05, ***p < 0.005) refer to inspiratory lung resistance (RI) after inhalation of 100 mg/ml methacholine.

The significant effects after the *i.n.* administration of 50 ng or 500 ng SEB prior to the allergic sensitization described above as compared to mice with AAI alone are summarized in the following table:

table 26: Summary of the significant effects of the *i.n.* pre-treatment with 50 ng or 500 ng SEB before sensitization on key parameters of AAI.

SEB dose	Organ	AAI parameter	Effect (compared to AAI alone)
50 ng	lung	basophils	increased
	lymph nodes	$CD4^+$ T cells	decreased
	serum	OVA-specific IgE	increased
500 ng	lung	total cells	decreased
		eosinophils	decreased
	BAL	total cells	decreased
		eosinophils	decreased
		IL-4	decreased
		IL-5	decreased
		IL-6	decreased
	lymph nodes	Th2 cells (% of $CD4^+$ T cells)	decreased

4.3 Effects of intranasal SEB-treatment on the allergen-specific IgE sensitization

I.n. treatment with 50 ng SEB before allergic sensitization led to significantly increased OVA-specific IgE levels in the serum upon allergen challenge. To assess, whether the *i.n.* SEB-treatment affected the Ig-production during the allergic sensitization period itself, mice were *i.n.* administered 50 ng or 500 ng SEB or PBS on days -3, -2 and -1 as in the previous experiments and as described in 3.3.2, p. 36. From one day after the last *i.n.* SEB-treatment, mice were *i.p.* sensitized three times with 10 µg OVA in combination with 1 mg alum in weekly intervals (see 3.2.2, p. 34) (see figure 37, p. 104). This setup resulted in the following experimental groups: Sensitized mice without SEB pre-treatment (sal/OVA), sensitized mice with 50 ng SEB pre-treatment (SEB_{50}/OVA) and sensitized mice with 500 ng SEB pre-treatment (SEB_{500}/OVA). Control mice were pre-treated with PBS and sensitized with alum only (sal/alum only) (see figure 37, p. 104). Six days after the last treatment (day 20), mice were sacrificed and analyzed for the OVA-specific IgG- and IgE-response.

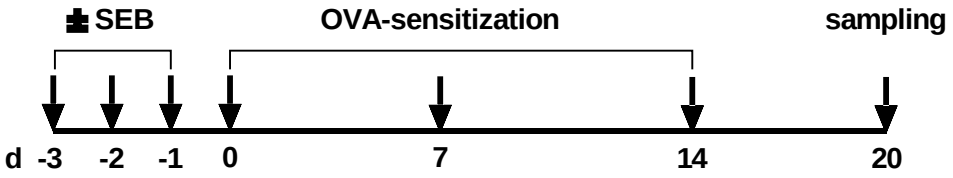

figure 37: Timeline of the experimental setup – effects of *i.n.* administration of SEB on the allergic sensitization. Mice were *i.n.* administered SEB (50 ng or 500 ng) on three consecutive days (d -3, -2, -1) and then *i.p.* sensitized in weekly intervals (d 0, 7, 14). On day 20, mice were sacrificed and further analyzed for OVA-specific IgG- and IgE-responses.

4.3.1 Effects of intranasal SEB administered prior to the allergic sensitization on the OVA-specific IgG and IgE-response without allergen challenge

During the first encounter of the immune system with an exogenous antigen, antigen-specific IgM antibodies are secreted from B cells upon activation. When the specific immune response progresses, a class switch to antigen-specific IgG takes place. In the course of anti-parasitic Th2 responses as well as an allergic immune reaction there is also a class switch to IgE-producing plasma cells (Poulsen & Hummelshoj, 2007). Now, allergen-specific IgE antibodies are secreted, which are important for the further course of the immune response, i.e. the allergic reaction. For example, they can bind to specific IgE-receptors on the surface of mast cells, where the cross-linking of the IgE molecules upon allergen recognition leads to the release of other allergic mediators like histamine (Poulsen & Hummelshoj, 2007).

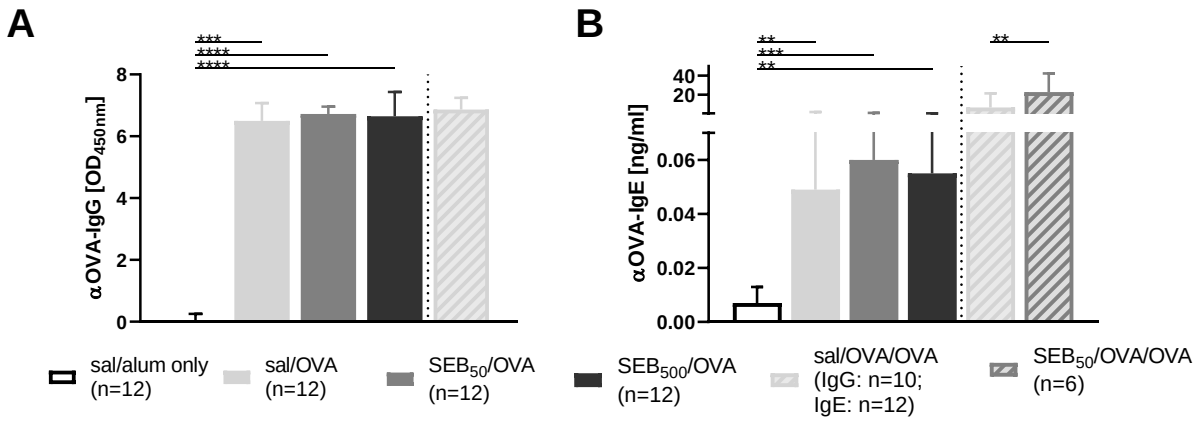

figure 38: OVA-specific serum IgG and IgE concentrations after *i.n.* SEB administration followed by *i.p.* OVA-sensitization.
Mice were *i.n.* administered 50 ng or 500 ng SEB or PBS on three consecutive days, then *i.p*. sensitized with OVA and alum. Control mice were sensitized with alum only. On day 20, OVA-specific IgG (A) and IgE (B) antibodies were detected in the serum. For comparison, previous data from mice analyezed following full AAI-induction (sal/OVA/OVA) and mice administered 50 ng SEB prior to AAI-induction (SEB_{50}/OVA/OVA) are shown without consideration in the statistical comparison. Data compiled from at least two independent experiments are shown as mean +SD. **p < 0.01, ***p < 0.005, ****p < 0.0001.

Three sensitizations against OVA alone led to a significant increase of OVA-specific IgG antibodies detectable in the serum (figure 38 A). Mice *i.n.* administered 50 ng or 500 ng SEB prior to this peripheral allergic sensitization did not show any differences in the concentration of OVA-specific IgG as compared to mice sensitized alone without SEB pre-treatment.

Following the sensitization, a minor but significant increase of OVA-specific IgE levels could be observed in mice sensitized alone without SEB pre-treatment. The same was true for mice sensitized after the *i.n.* administration of 50 ng or 500 ng SEB (figure 38 B). There was no significant difference between mice sensitized alone or after the *i.n.* administration of 50 ng or 500 ng SEB. As opposed to the IgG response, the concentration of OVA-specific IgE in the serum of sensitized, but not challenged mice was approximately 500-fold lower as compared to mice which were sensitized and *i.n.* challenged with OVA (figure 38 B).

In summary, the *i.n.* administration of 50 ng or 500 ng SEB did not alter the OVA-specific IgG or IgE production during the allergic sensitization alone as compared to sensitized mice without prior administration of SEB.

According to the results of these experiments, the previously observed significant increase of OVA-specific IgE concentrations in the serum after the *i.n.* administration of 50 ng SEB prior

to the allergic sensitization as compared to mice with AAI alone most likely manifests rather upon completion of the allergic challenge.

4.4 Long-term effects of intranasal SEB-treatment on the respiratory tract immune milieu

The clear effects of SEB administered before sensitization on the phenotype of subsequently induced AAI suggested possible long-term effects of SEB administration on the respiratory tract immune milieu. To investigate whether and how *i.n.* administration of SEB conferred long term effects on cellular and humoral immune factors in the respiratory tract and the kinetics of these effects, SEB (50 ng or 500 ng) was *i.n.* administered on three consecutive days as in the previous experiments (day -3, -2 and -1). Control groups were *i.n.* treated with PBS (sal) only (see figure 39, p. 106). At different time points (day 0, 7 or 14), mice were sacrificed and BAL, lung, spleen, cervical and bronchial lymph nodes were harvested for analysis.

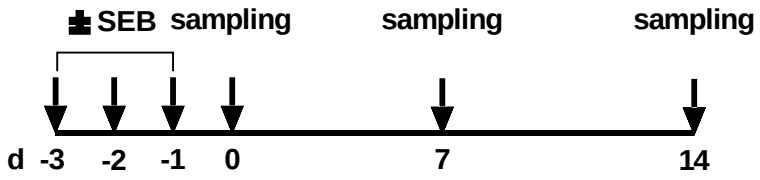

figure 39: Timeline of the experimental setup – long term effects of *i.n.* SEB-treatment on the respiratory tract immune milieu.
For the analysis of effects of *i.n.* SEB-treatment on the respiratory tract, mice were *i.n.* treated with SEB (50 ng or 500 ng) on three consecutive days (d -3, -2, -1). At different time points (d 0, d 7, d 14) mice were sacrificed and BAL, lung, spleen, cervical and bronchial lymph nodes were harvested for analysis.

4.4.1 Long-term effects of intranasal SEB administration on body weight and general condition

In the experiments addressing possible long-term effects of SEB alone, all animals were closely monitored, weighted and scored according to the score sheet daily.

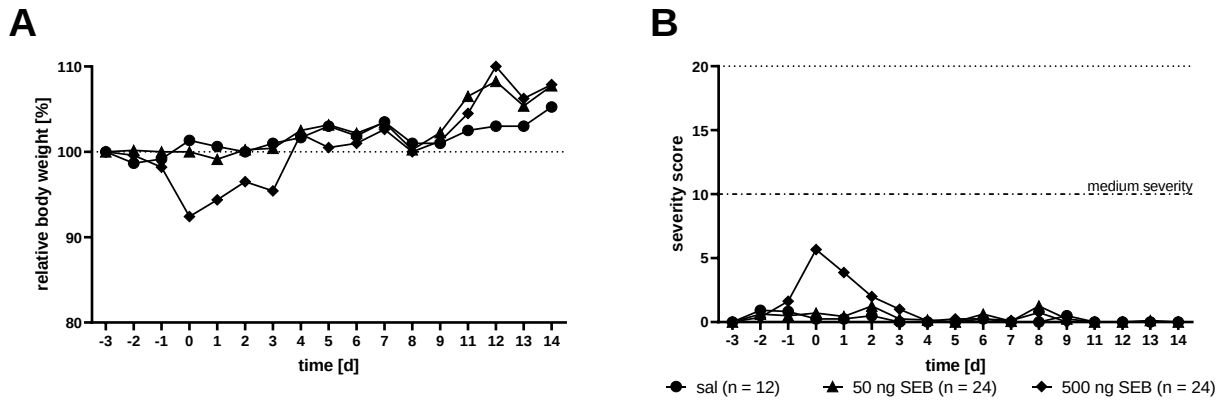

figure 40: Body weight and disease scoring after *i.n.* administration of SEB alone in naïve mice.
Mice were treated *i.n.* with 50 ng (▲) or 500 ng (♦) SEB or PBS (sal; control) on three consecutive days (d -3, -2, -1). All mice were weighted and scored daily according to the score sheet. The relative weight loss (A) was always calculated from the body weight on day -3. The cumulative severity score (B) was determined according to the score sheet. Data are shown as group median.

The repeated *i.n.* administration of 50 ng SEB did not affect the relative body weight or increase the severity score when compared to PBS-treated control mice (figure 40 A, B).

The *i.n.* administration of 500 ng SEB on three consecutive days resulted in a moderately decreased relative body weight on day 0 of about 92 %. However, these mice reached their starting weight by day 4 (figure 40 A). The same trend was observed in the severity score (figure 40 B).

In summary, only the *i.n.* administration of 500 ng SEB led to a light severity score due to a medium body weight loss following *i.n.* SEB administration.

4.4.2 Long-term effects of intranasal SEB administration on innate effector cells of AAI in the respiratory tract

As described, SEB belongs to the superantigens family of toxins, which are potent immune activators leading to unspecific lymphocyte activation and are usually associated with pro-inflammatory, mainly type 1 responses that are known to suppress Th2- and allergic responses (Balaban, 2000). However, recently it was shown that the production of SEB can be associated with several atopic diseases, including allergic asthma. The question, if *i.n.* administration of SEB has any long-term immune imprinting effects in the cellular composition of the respiratory tract immune milieu and the kinetics of such changes was not addressed until now.

In the lung, on day 0 (i.e. one day after the third *i.n.* SEB administration), the *i.n.* administration of 50 ng SEB did not significantly affect total cell numbers or the absolute numbers of alveolar macrophages, eosinophils, neutrophils, basophils, mast cells or M2-polarized monocytes/macrophages as compared to the PBS-treated control group (figure 41 A, B, D-H). However, absolute numbers of DCs were significantly increased after the *i.n.* administration of 50 ng SEB as compared to the control group (figure 41 C). Seven days after the last *i.n.* administration of 50 ng SEB total cells as well as the absolute numbers of DCs, eosinophils as well as M2-polarized monocytes/macrophages were significantly increased as compared to the control group (figure 41 A, C, H). Fourteen days after the last *i.n.* administration of 50 ng SEB there were no significant differences in total cells and absolute numbers of alveolar macrophages, DCs, eosinophils, neutrophils, basophils, mast cells and M2-polarized monocytes/macrophages as compared to the PBS-treated control group (figure 41 A-H).

The repeated *i.n.* administration of 500 ng SEB led to significantly increased total cell numbers, as well as absolute numbers of DCs, eosinophils and neutrophils one day after the last administration (day 0), as compared to the control group (figure 41 A, C-E). Absolute numbers of alveolar macrophages, basophils, mast cells and M2-polarized monocytes/macrophages were not affected one day after the last *i.n.* administration of 500 ng SEB in the lung (figure 41 B, F-H) as compared to PBS-treated control mice. A significant increase of absolute numbers of basophils, as well as M2-polarized monocytes/macrophages was observed seven days after the last administration of 500 ng SEB as compared to the control group (figure 41 F, H). There were no significant effects of the *i.n.* administration of 500 ng SEB on the absolute cell numbers of the above-mentioned cell populations in the lung 14 days after the last treatment (figure 41 A-H).

In the BAL, the *i.n.* administration of 50 ng SEB did not significantly affect total cell numbers, as well as absolute numbers of macrophages, DCs, eosinophils or neutrophils as compared to PBS-treated control mice (figure 41 I-M). Seven days after the last SEB administration (50 ng SEB), absolute numbers of eosinophils were significantly increased as compared to the control group (figure 41 L). After 14 days, total cell numbers as well as absolute numbers of DCs were significantly increased due to the *i.n.* administration of 50 ng SEB as compared to control mice (figure 41 I, K).

The repeated *i.n.* administration of 500 ng SEB led to a significant increase in total cells, along with absolute numbers of DCs, eosinophils and neutrophils as compared to PBS-treated

control mice on day 0 (figure 41 I, K, L M). On day seven and fourteen, after 500 ng SEB were *i.n.* administered the last time, absolute numbers of eosinophils were significantly increased in the BAL as compared to the control group (figure 41 L).

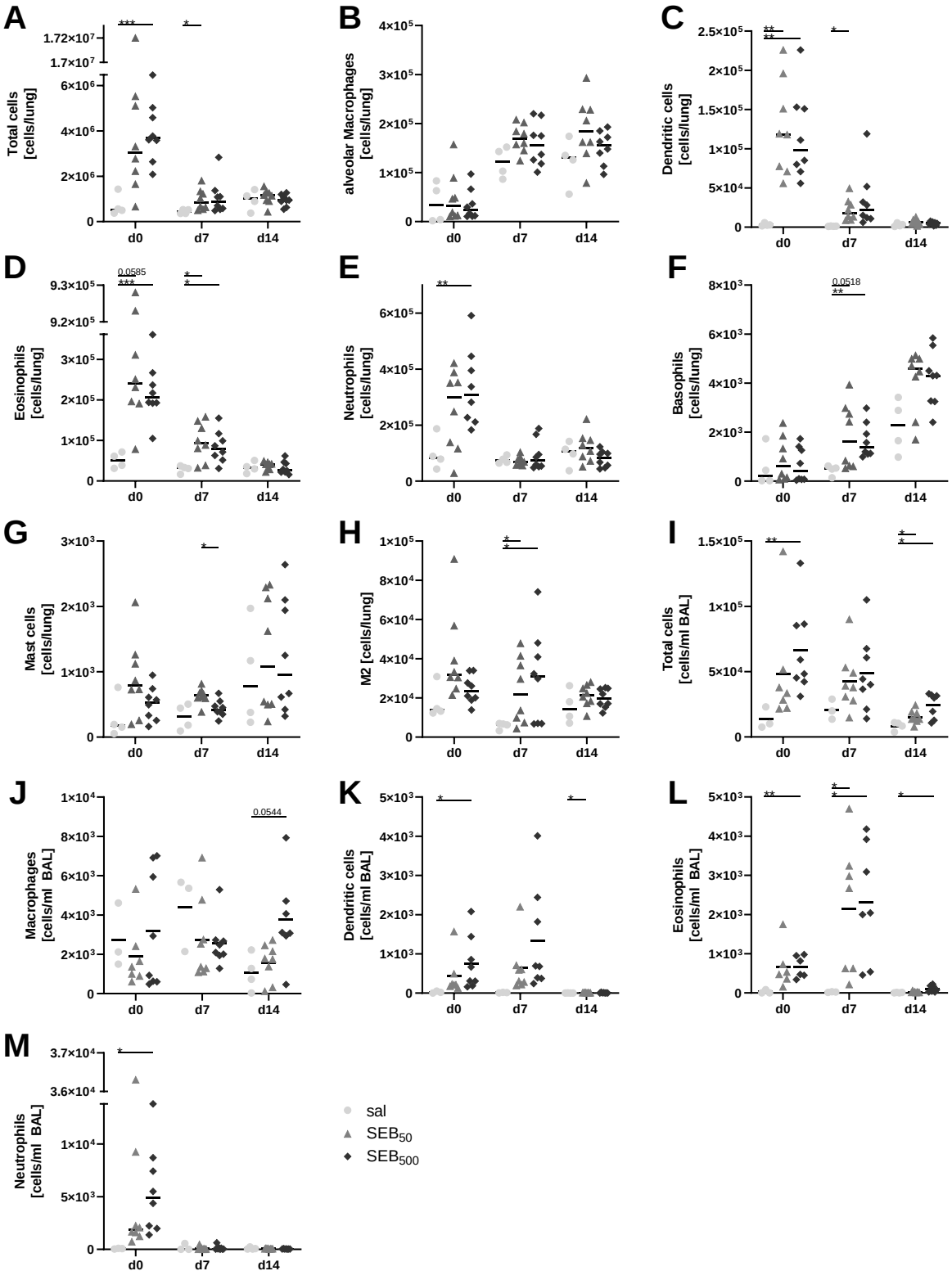

figure 41: Innate immune cells in the respiratory tract 14 days after *i.n.* SEB administration.

Mice were treated *i.n.* with 50 ng (▲) or 500 ng (♦) SEB or PBS (sal; control) on three consecutive days (d -3, -2, -1). On days 0, 7 and 14 leukocytes from lung and BAL were analyzed for total cell count (A, I) and absolute numbers of (alveolar) macrophages (B, J), DCs (C, K), eosinophils (D, L) and neutrophils (E, M). Cells, isolated from the lung were further analyzed for absolute cell numbers of basophils (F), mast cells (G) and M2-polarized monocytes/macrophages. Data compiled from at least two independent experiments are shown for individual mice with the median. Data for all experimental groups were tested for normality using the Shapiro-Wilk

normality test. In the case of Gaussian distribution for all groups in a comparison, two-way ANOVA and Tukey's multiple comparison test were performed. In case of non-Gaussian distribution in at least one of the groups in a comparison, mixed-effects analysis with Tukey's multiple comparison test was performed. *p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.0001.

In conclusion, the *i.n.* administration of SEB affected the innate effector cell composition of the respiratory tract in a significant and long-term manner up to 14 days. One day after the last SEB administration significantly increased numbers of various innate effector cells of AAI were observed as compared to the control group. One, respectively two weeks after the last *i.n.* administration of SEB distinct cell populations (e.g. basophils, eosinophils) remained significantly increased as compared to PBS-treated control mice.

4.4.3 Long-term effects of *i.n.* SEB administration on B and T cells in the respiratory tract and draining lymph nodes

In order to draw a comprehensive picture of the long-term changes in the local immune environment after *i.n.* SEB administration, the absolute numbers and activation status of lymphocytes in the respiratory tract and the draining lymph nodes were evaluated.

One day after the last *i.n.* administration of 50 ng SEB in the lung, absolute numbers of B cells were not affected on day 0 (figure 42 A). However, a significant increase of absolute numbers of $CD4^+$ T cells, the frequency of Th2 cells as well as $CD8^+$ T cells was observed after *i.n.* administration of 50 ng SEB as compared to the control group (figure 42 B-D). Seven days after the last *i.n.* administration of 50 ng SEB, absolute numbers of $CD4^+$ T cells were still significantly increased (figure 42 B). Additionally, $CD8^+$ T cells were increased, however not in a significant manner (figure 42 D). Fourteen days after the last SEB (50 ng) administration, no significant effects were observed in the lung (figure 42 A-D).

Following *i.n.* administration of 500 ng SEB, absolute numbers of B cells, $CD4^+$ T cells, the frequency of Th2 cells and $CD8^+$ T cells were significantly increased in the lung as compared to PBS-treated control mice on day 0 (figure 42 A-D). Seven, as well as fourteen days after the last *i.n.* administration of 500 ng SEB, no significant effects on lymphocyte numbers were observed as compared to the control group (figure 42 A-D).

In the BAL, the *i.n.* administration of 50 ng SEB led to increased absolute numbers of B cells, $CD4^+$ and $CD8^+$ T cells on day 0, however these changes were not significant as compared to control animals (figure 42 E-G). This trend continued seven days after the last *i.n.* administration of 50 ng SEB, with a significant increase of $CD4^+$ T cells (figure 42 F). Fourteen

days after the last *i.n.* administration of 50 ng SEB, the treatment did not affect the absolute numbers of the above-mentioned cell populations in a significant manner (figure 42 E-G).

The *i.n.* administration of 500 ng SEB did not affect absolute B cell numbers in the BAL on day 0, 7 or 14 as compared to the control group (figure 42 E). One day after the last *i.n.* administration of 500 ng SEB, absolute numbers of $CD4^+$ and $CD8^+$ T cells were significantly increased as compared to control mice (figure 42 F, G). Absolute numbers of $CD4^+$ T cells were still significantly increased seven days after the last administration of 500 ng SEB. On day 14, absolute numbers of $CD4^+$, as well as $CD8^+$ T cells were not significantly altered as compared to PBS-treated control mice (figure 42 F, G).

In pooled lymphocytes from cervical and bronchial lymph nodes, on day 0 the *i.n.* administration of 50 ng SEB led to significantly increased absolute numbers of B cells, as well as increased absolute numbers of $CD4^+$ T cells, however this increase was not statistically significant as compared to control mice (figure 42 H, I). The frequency of Th2 cells and absolute numbers of $CD8^+$ T cells were not affected on day 0 (figure 42 J, K). On day 7 and 14, no significant effects of *i.n.* administration of 50 ng SEB were observed (figure 42 H-K).

The repeated *i.n.* administration of 500 ng SEB did not affect absolute numbers of B cells, $CD4^+$ T cells or the frequency of Th2 cells in isolated lymphocytes from the draining lymph nodes on days 0, 7 and 14 as compared to the control group (figure 42 H-J). On day 0, absolute numbers of $CD8^+$ T cells were significantly increased after *i.n.* administration of 500 ng SEB as compared to PBS-treated control animals. Seven or fourteen days after the last treatment, no significant differences between mice *i.n.* administered 500 ng SEB and control mice could be observed (figure 42 K).

To conclude, the *i.n.* administration of 50 ng or 500 ng SEB affected absolute numbers of B cells, $CD4^+$ and $CD8^+$ T cells as well as the frequency of Th2 cells in the respiratory tract in a long-term manner (up to 7 days), thereby possibly acting on later induced AAI. In the draining lymph nodes, a short-term effect of *i.n.* SEB administration, especially in absolute numbers of B cells was observed.

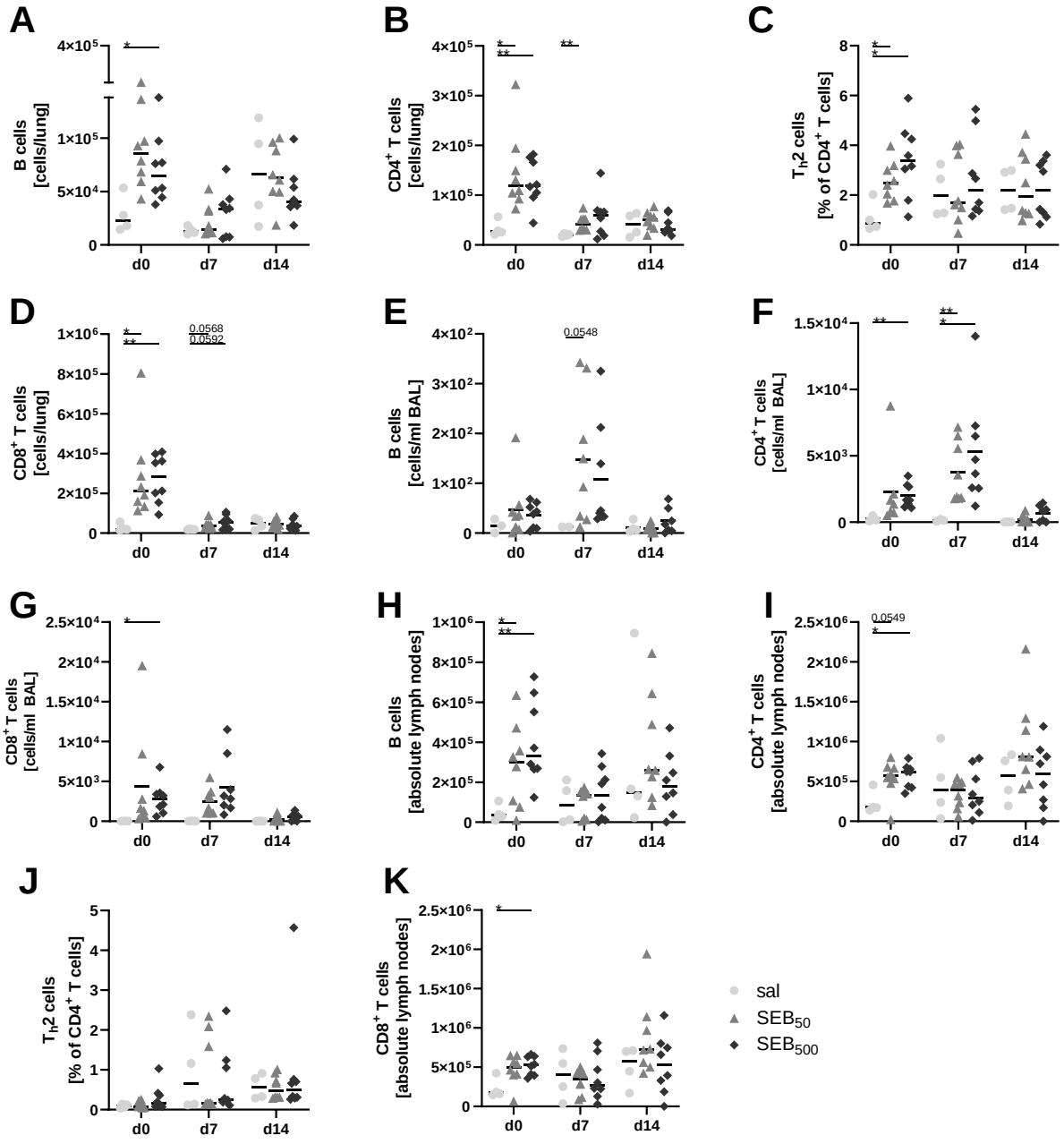

figure 42: Lymphocytes in the respiratory tract and draining lymph nodes up to 14 days after *i.n.* SEB administration.

Mice were treated *i.n.* with 50 ng (▲) or 500 ng (♦) SEB or PBS (sal; control) on three consecutive days (d -3, -2, -1). On days 0, 7 and 14, lymphocytes from lung, BAL and pooled cervical and bronchial lymph nodes were analyzed for absolute numbers of B cells (A, E, H), $CD4^+$ T cells (B, F, I), the frequency of Th2 cells in $CD4^+$ T cells (C, J) and $CD8^+$ T cells (D, G, K). Data compiled from at least two independent experiments are shown for individual mice with the group median. Data for all experimental groups were tested for normality using the Shapiro-Wilk normality test. In the case of Gaussian distribution for all groups in a comparison, two-way ANOVA and Tukey's multiple comparison test were performed. In case of non-Gaussian distribution in at least one of the groups in a comparison, mixed-effects analysis with Tukey's multiple comparison test was performed. *$p < 0.05$, **$p < 0.01$.

Next to absolute lymphocyte counts, the activation status was evaluated in lymphocyte populations of the lung and the draining lymph nodes. As described above, CD69 was used as a cell surface marker of early activation.

In the lung, the *i.n.* administration of 50 ng SEB led to marginally increased absolute numbers of $CD69^+$ B cells (not significant) and significantly increased numbers of activated $CD4^+$ T cells and Th2 cells as compared to the control group on day 0 (figure 43 A-C). Seven days after the last *i.n.* administration of 50 ng SEB, absolute numbers of activated B cells, $CD4^+$ T cells and Th2 cells were significantly increased as compared to control mice (figure 43 A-C). On day fourteen, no significant effects of the *i.n.* administration of 50 ng SEB were observed in the $CD69^+$ B cells, $CD69^+$ $CD4^+$ T cells and $CD69^+$ Th2 cells (figure 43 A-C).

The repeated *i.n.* administration of 500 ng SEB resulted in significantly increased absolute cell numbers of activated B cells, $CD4^+$ T cells and Th2 cells as compared to PBS-treated control mice on day 0 (figure 43 A-C). On day seven after the last SEB administration, absolute numbers of $CD69^+$ B cells and $CD69^+$ $CD4^+$ T cells were still significantly increased (figure 43 A, B). On day 14, the activation status of none of the above-mentioned cell populations was affected by the *i.n.* administration of 500 ng SEB in the lung as compared to the control group (figure 43 A-C).

Isolated lymphocytes of the draining lymph nodes showed an increase in absolute numbers of activated $CD4^+$ T cells, compared to the control group, one day after the last *i.n.* administration of 50 ng SEB (figure 43 E). For the absolute numbers of $CD69^+$ B cells and $CD69^+$ Th2 cells, a marginal increase was observed, however this was not significant. Seven and fourteen days after the last *i.n.* administration of 50 ng SEB, no significant effects in isolated lymphocytes from the draining lymph nodes were observed with respect to CD69-expression as compared to the control group (figure 43 D-F).

The repeated *i.n.* administration of 500 ng SEB led to significantly increased absolute numbers of activated B cells and $CD4^+$ T cells in the draining lymph nodes on day 0 when compared to the control group (figure 43 D, E). After 7 and 14 days, no significant effects on the activation status were observed in cells of the draining lymph nodes after the *i.n.* administration of 500 ng SEB (figure 43D-F).

To summarize, the *i.n.* administration of 50 ng or 500 ng SEB significantly affected the activation status of lymphocytes in the lung up to seven days after the last administration. In

the draining lymph nodes, CD69$^+$ B cells, as well as CD69$^+$ CD4$^+$ T cells were significantly increased one day after the last *i.n.* administration.

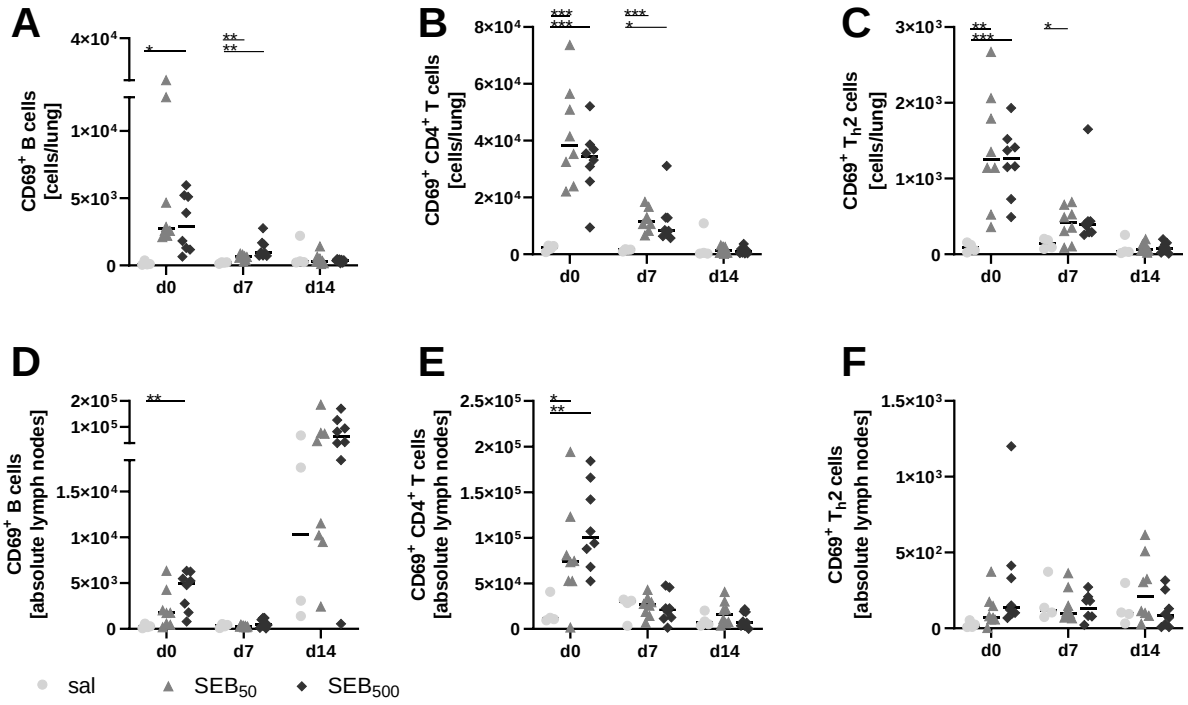

figure 43: Lymphocyte activation in the lung and draining lymph nodes up to 14 days after *i.n.* SEB administration.

Mice were treated *i.n.* with 50 ng (▲) or 500 ng (♦) SEB or PBS (sal; control) on three consecutive days (d -3, -2, -1). On days 0, 7 and 14 lymphocytes from lung and pooled cervical and bronchial lymph nodes were analyzed for absolute numbers of CD69$^+$ B cells (A,), CD69$^+$ CD4$^+$ T cells (B, E) and CD69$^+$ Th2 cells (C, F). Data compiled from at least two independent experiments are shown for individual mice with the group median. Data for all experimental groups were tested for normality using the Shapiro-Wilk normality test. In the case of Gaussian distribution for all groups in a comparison, two-way ANOVA and Tukey's multiple comparison test were performed. In case of non-Gaussian distribution in at least one of the groups in a comparison, mixed-effects analysis with Tukey's multiple comparison test was performed. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$.

4.4.4 Long-term effects of intranasal SEB administration on respiratory tract cytokines

Following *i.n.* SEB administration, levels of the respiratory cytokines IL-4, IL-5, IL-13, IFN-γ, IL-6, TNF-α and IL-17A were measured in the supernatant of the BAL using a multiplex-based immunoassay to assess effects on the local cytokine milieu.

After the *i.n.* administration of 50 ng or 500 ng SEB, one day after the last administration (d 0) no significant changes in the concentrations of the above-mentioned cytokines were observed as compared to the control group (figure 44 A-G).

However, one week after the last *i.n.* administration of 50 ng SEB as well as after the administration of 500 ng SEB significant increases in the levels of IL-4, IFN-γ, IL-6, TNF-α and IL-17A (figure 44 A, D, E, F, G) were detected as compared to PBS-treated control mice. The concentration of IL-5 was also marginally increased after the *i.n.* administration of 50 ng SEB. However, this change was not significant (figure 44 B).

Fourteen days after the last *i.n.* administration of 50 ng or 500 ng SEB, no significant effects in the cytokine concentrations were observed, with the exception of IL-13. This Th2 cytokine was significantly increased fourteen days after *i.n.* administration of 500 ng SEB as compared to PBS-treated control mice (figure 44 C).

Taken together, the *i.n.* administration of 50 ng or 500 ng SEB led to significant changes in the respiratory cytokine profile affecting Th1, Th2 as well as pro-inflammatory cytokines, particularly one week after the last *i.n.* administration of SEB.

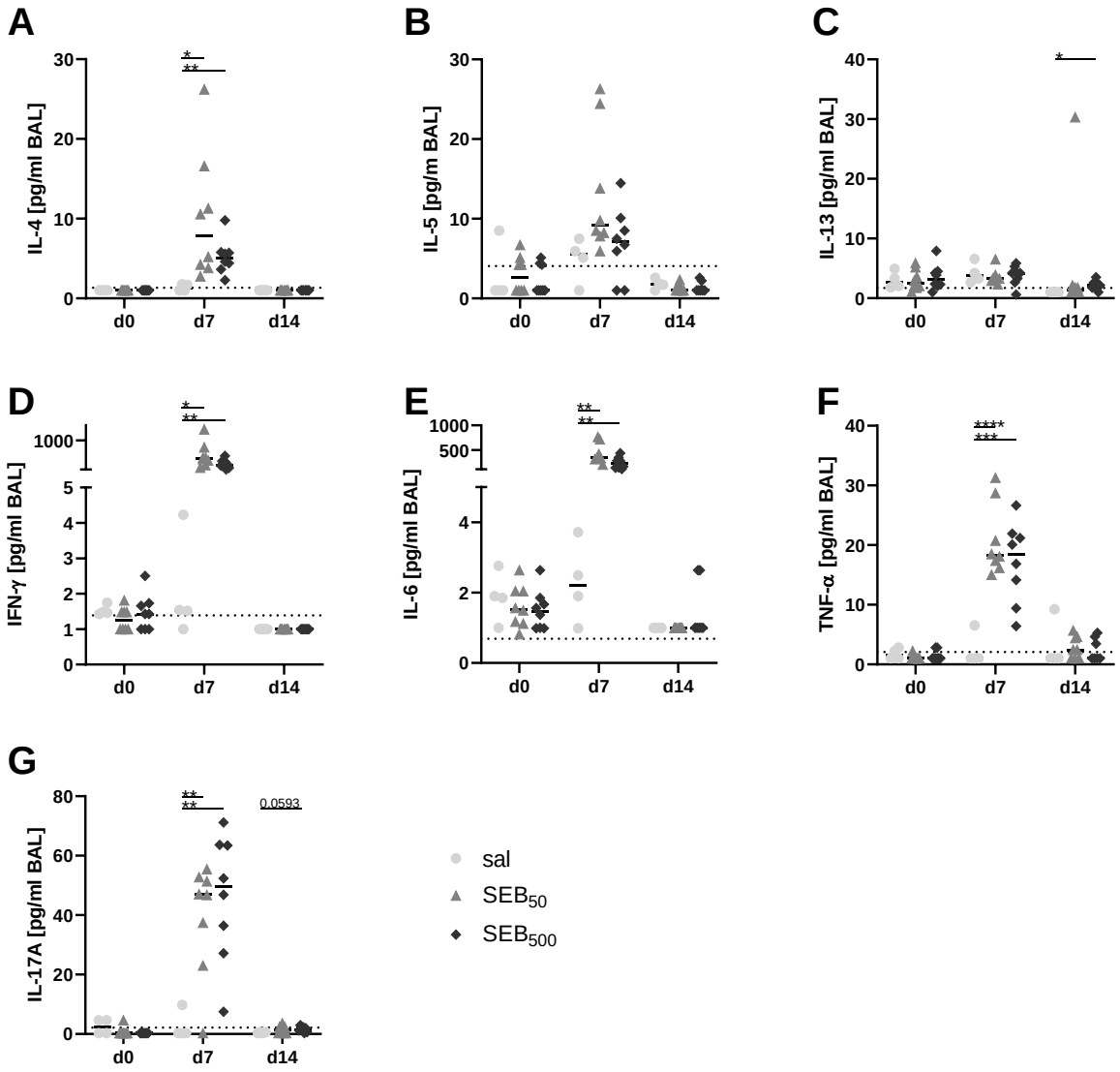

figure 44: Th1, Th2 and pro-inflammatory cytokine levels in the BAL up to 14 days after *i.n.* SEB administration. Mice were treated *i.n.* with 50 ng (▲) or 500 ng (♦) SEB or PBS (sal; control) on three consecutive days (d -3, -2, -1). On days 0, 7 and 14, bronchoalveolar lavage (BAL) was analyzed for the concentrations of IL-4 (A), IL-5 (B), IL-13 (C), IFN-γ (D), IL-6 (E), TNF-α (F) and IL-17A (G). Data compiled from at least two independent experiments are shown for individual mice with the median. Data for all experimental groups were tested for normality using the Shapiro-Wilk normality test. In the case of Gaussian distribution for all groups in a comparison, two-way ANOVA and Tukey's multiple comparison test were performed. In case of non-Gaussian distribution in at least one of the groups in a comparison, mixed-effects analysis with Tukey's multiple comparison test was performed. *p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.0001.

The described significant long-term effects of *i.n.* administration of 50 ng and 500 ng SEB are summarized in the following tables:

table 27: Summary of the significant effects of the *i.n.* administration of 50 ng SEB as compared to PBS-treated mice.

Organ	sampling day	Parameter	Effect (compared to PBS treatment)
lung	d 0	dendritic cells	increased
		eosinophils	increased
		$CD4^+$ T cells	increased
		Th2 cells (% of $CD4^+$ T cells)	increased
		$CD8^+$ T cells	increased
		$CD69^+$ $CD4^+$ T cells	increased
		$CD69^+$ Th2 cells	increased
	d 7	total cells	increased
		dendritic cells	increased
		eosinophils	increased
		M2 cells	increased
		$CD4^+$ T cells	increased
		$CD69^+$ B cells	increased
		$CD69^+$ $CD4^+$ T cells	increased
		$CD69^+$ Th2 cells	increased
BAL	d 7	eosinophils	increased
		B cells	increased
		$CD4^+$ T cells	increased
		IL-4	increased
		IFN-γ	increased
		IL-6	increased
		TNF-α	increased
		IL-17A	increased
	d 14	dendritic cells	increased
lymph nodes	d 0	B cells	increased
		$CD4^+$ T cells	increased
		$CD69^+$ $CD4^+$ T cells	increased

table 28: Summary of the significant effects of the *i.n.* administration of 500 ng SEB in comparison with PBS-treated mice
(table to be continued on the next page)

Organ	sampling day	Parameter	Effect (compared to PBS treatment)
lung	d 0	total cells	increased
		dendritic cells	increased
		neutrophils	increased
		eosinophils	increased
		B cells	increased
		$CD4^+$ T cells	increased
		Th2 cells (% of $CD4^+$ T cells)	increased
		$CD8^+$ T cells	increased
		$CD69^+$ B cells	increased
		$CD69^+$ $CD4^+$ T cells	increased
		$CD69^+$ Th2 cells	increased
	d 7	eosinophils	increased
		basophils	increased
		M2 cells	increased
		$CD69^+$ B cells	increased
		$CD69^+$ $CD4^+$ T cells	increased
BAL	d 0	total cells	increased
		dendritic cells	increased
		neutrophils	increased
		eosinophils	increased
		$CD4^+$ T cells	increased
		$CD8^+$ T cells	increased
	d 7	eosinophils	increased
		$CD4^+$ T cells	increased
		IL-4	increased
		IFN-γ	increased
		IL-6	increased

		TNF-α	increased
		IL-17A	increased
	d 14	total cells	increased
		eosinophils	increased
		IL-13	increased
lymph nodes	d 0	B cells	increased
		$CD4^+$ T cells	increased
		$CD8^+$ T cells	increased
		$CD69^+$ B cells	increased
		$CD69^+$ $CD4^+$ T cells	increased

Overall, these results demonstrate a significant potential for SEB to modulate the respiratory immune network in a sustained manner, possibly imprinting the immune milieu towards altered reactions upon the subsequent induction of AAI.

4.5 Utilization of the established OVA-mediated mouse model of AAI for the investigation of effects of resolved influenza A infection

The OVA-mediated mouse model of AAI established in this thesis for the analysis of SEB-mediated modulation of airway inflammation was further used to investigate the effects of a resolved sublethal influenza A (IAV) infection on lung immune homeostasis and subsequently induced AAI. This work, employing the OVA-mediated model for AAI established here, was performed by the medical student Qingyu Wu for her thesis (Dr. med.).

In that study, mice *i.n.* infected with a sublethal dose of 0.31 $TCID_{50}$ IAV A/PR/8/34 (H1N1). Two weeks after the IAV infection, mice had recovered from infection and AAI was induced as described. Shortly, mice were sensitized three times through *i.p.* injection of 10 µg OVA and 1 mg alum and were *i.n.* challenged with 100 µg OVA in PBS on three consecutive days from one week after the last sensitization.

Control mice, IAV infection alone, AAI alone and AAI after IAV infection were compared with respect to histology, IgE levels, respiratory cells and cytokines as well as airway hyperreactivity. Resolved IAV infection led to distinct changes in the lung immune network 39 days post IAV infection. These included the accumulation of $CD4^+$ as well as $CD8^+$ lymphocytes as well as MHCII expressing macrophages in the respiratory tract. Also, subsequently induced AAI was affected by resolved IAV infection with respect to several key parameters including histology. In contrast to AAI alone, histologically a moderate as opposed to mild accumulation of alveolar eosinophils and a severe as opposed to mild accumulation of activated macrophages (giant cells) were observed. On a cellular level analyzed using flow cytometry, it was observed that resolved IAV infection significantly reduced the recruitment of $CD4^+$ and $CD8^+$ T cells to the respiratory tract, reduced the activation of Th2 cells in the lung and significantly increased lung B cell numbers in AAI. Furthermore, resolved IAV infection followed by the induction of AAI led to the significant amelioration of pro-inflammatory cytokines like IL-6, IL-17A, IFN-γ and TNF-α, as well as the type 2 cytokines IL-13 as compared to AAI alone.

Taken together the study described prolonged effects of IAV infection in respiratory immunity resulting in the significant modulation of subsequently induced AAI (Wu, Jorde *et al.* 2020).

5 Discussion

Bronchial asthma is a highly heterogeneous chronic inflammatory disease of the airways which affects over 3 million patients worldwide. About 60 % of adult asthmatics suffer from allergic asthma, which is dominated by a Th2-driven immunoreaction against aeroallergens (Global Initiative for Asthma, GINA; 2020).

The pathophysiology of allergic asthma involves complex inflammatory responses orchestrated by the innate and adaptive immune system in which both cellular and humoral components are of great importance. Amongst others, epithelial cells, eosinophils, mast cells as well as Th2 cells and B cells, alongside with soluble factors like e.g. IL-4 and IL-5 are involved in the immune reaction in allergic asthma (Hirose *et al.*, 2017, Papi *et al.*, 2018).

Since the heterogenous inflammation in allergic asthma makes the treatment of asthmatic patients difficult, current goals in allergic asthma research are to elucidate the complex underlying mechanisms and highly heterogenous disease triggering factors. Research into those factors could lead to the development of prophylactic treatments to prevent the development of allergic asthma in high-risk patients.

Over the last decade, new factors have been recognized that are involved in the development of allergic asthma. The nasal colonization with *S. aureus* is one of these factors. For a long time, it has been known that patients with atopic diseases, e.g. AD, are more frequently colonized with *S. aureus*. Today this is known also for patients with allergic asthma (Davis *et al*., 2015; Kim *et al*., 2019). Furthermore, different proteins produced by *S. aureus* came into focus with respect to the induction of Th2-biased immune responses, including SEB. SEB belongs to the superantigen family of toxins produced by *S. aureus* and is usually associated with a pro inflammatory type 1 immune response. However, recently various clinical and experimental studies could show that the production of SEB can be associated with several atopic diseases, including allergic asthma (Perez-Novo *et al*., 2004; Bachert *et al*., 2007).

In this thesis, the possible immune modulatory effects of an *i.n.* SEB-treatment on the development and phenotype of allergic asthma was examined at different time-points of AAI-induction using an OVA-mediated mouse model of AAI.

Establishing a mouse model for AAI suited to study SEB-mediated effects:

To identify and understand the underlying pathomechanisms of a multifactorial disease like allergic asthma and in order to develop appropriate treatments, up to date, the use of animal models is indispensable. Especially for studies addressing single pre-disposing and modulating factors such as SEB in this thesis, the controlled setting of animal experiments allowing e.g. exact timing and dosing of treatments and elimination of confounding factor is of great advantage. For the induction of AAI, numerous animal models can be found in the current literature and are being routinely employed (Nials *et al.*, 2008; Motta *et al.*, 2004; Towards *et al.*, 2004; Gascoigne *et al.*, 2003; Ayanoglu *et al.*, 2010; van Scott *et al.*, 2004).

For the experimental induction of AAI, in this thesis a murine OVA-induced model of AAI was used. The use of mice as model animals is common because they are relatively easy and inexpensive in breeding, they have a short generation time and there are many genetic variants and genetically engineered strains available. Also, research reagents for this species are largely at hand (Karol, 1994; Aun *et al.*, 2017). With respect of overall structure, the immune system in mice and humans is quite similar. However, several differences in innate and adaptive immunity have been observed. For example, the balance of lymphocytes and neutrophils differs between humans (50-70 % neutrophils, 30-50 % lymphocytes) and mice (10-25 % neutrophils, 75-90 % lymphocytes), moreover the immune system of mice and humans often shows different susceptibility loci for infections, e.g. for infection with the cytomegalovirus. Adaptive immunity differs in humans and mice, for example humans express two type of IgA, while mice only express one (Mestas & Hughes, 2004; Haley, 2003; Webb *et al.*, 2002; Martin & Lew, 1998). Genomes of humans and mice are highly conserved and, although the human genome is larger than the genome of the mouse, 80 % of mouse coding sequences have direct orthologs in the human genome (Mouse Genome Sequencing Consortium *et al.*, 2002; Rosenberg & Druey, 2018). Although, the human immune system changes under pathogen-directed external influences, which may differ between closely related species given habitats and distinct environments (Kapetanovic R. *et al.,* 2014). Also, mice do not develop AAI spontaneously, which can be a major disadvantage when mouse-related research results are to be related to the human pathophysiology of allergic asthma (Rosenberg & Druey, 2018). Despite these differences, mice as model animals have contributed in the exposition and examination of important pathomechanisms of various

diseases like systemic lupus erythematosus, malaria parasite infection, psoriasis and asthma (Wagner *et al*., 2010; Minkah *et al*., 2018; Ohlemiller, 2006; Morel, 2010; Sagar *et al*., 2015)

OVA is the major protein component of chicken egg-white and belongs to the serpin family even though it lacks any protease inhibitory activity (Huntington & Stein, 2001). Due to many advantages, OVA is widely used as a model antigen in immunological studies. It can easily be produced in high quantity, is non-toxic and a wide range of research reagents are available. Therefore, although it is not a natural allergen, OVA is one of the most commonly used model-antigens for the induction of AAI and such models are well characterized (Mine & Yang, 2006; Lloyed, 2008). The most common protocols of AAI-induction using OVA are structured as follows: The allergic sensitization and allergic challenge are clearly separated experimental procedures and phases in the induction of experimental AAI. According to the compartments targeted, e.g. *i.p.* sensitization and *i.n.* challenge, separate phases (or possible influences/treatments in these) can be examined independently from each other (Kumar *et al.*, 2008; Shin *et al.* 2009; Conrad *et al.*, 2009).

In this thesis, different treatment regimens were tested to identify the optimal treatment regimen for experimental and control groups to investigate the aims of this study, i.e. modulation of AAI by *i.n.* SEB. Allergic inflammation of the airways was demonstrated in all of the tested treatment regimens, characterized by accumulation of DCs, eosinophils and $CD4^+$ T cells as well as increased concentrations of IL-4 and IL-5 in the respiratory tract and the detection of OVA-specific IgE in the serum. However, no significant differences between treatment and control groups were observed. Possible reasons are the substantial heterogeneity within the individual groups, the relatively small number of animals analyzed for these preliminary experiments and the large number of experimental groups compared in the statistical analyses. Nevertheless, induction of different hallmark parameters was clearly evident, and based on these results an adjusted treatment regimen was chosen for the subsequent experiments.

In the lung and BAL, especially mice challenged three times, regardless of the OVA dose used for the challenge, showed a tendency towards higher absolute eosinophil and $CD4^+$ T cell counts in the respiratory tract. No association between the number of sensitizations and the severity of the allergic inflammation could be determined in the respiratory system. Regarding respiratory cytokine concentrations, the allergic challenge with 250 µg OVA by trend led to higher concentrations of IL-4 and IL-5. Concentrations of IL-13 were not affected. Gueders *et*

al. showed that C57/Bl6 mice showed significantly lower amounts of IL-13 measured in BAL as compared to BALB/c mice in an OVA-mediated mouse model of AAI, suggesting genetic factors, amongst others, to be involved in this phenomenon (Eum *et al.*, 2005; Gueders *et al.*, 2009). The OVA-specific IgE-response measured in the serum was also most pronounced after a challenge with 250 µg OVA.

In published OVA-mediated models for AAI, the most common route for sensitization is the *i.p.* injection of OVA in combination with an adjuvant. The dose of antigen used for the sensitization varies between 1 µg to 8000 µg OVA (Nakajima *et al.*, 1992; Wilder *et al.*, 1999; Chen *et al.*, 2018). However, doses between 10 µg and 50 µg OVA are most commonly employed (Takeda *et al*., 2013; Nakashima *et al.*, 2014; dos Santos *et al.*, 2018). The use of an adjuvant, which is intended to boost and shift the immune reaction towards a Th2-response during sensitization, is one potential source for differing results between OVA-induced models of AAI. Alum is the most commonly used adjuvant promoting the production of Th2 cytokines during the immune response (Epstein, 2004). Typically, OVA is absorbed in 150 µg to 40 mg of alum for *i.p.* injection (Sakai *et al.*, 1999; Lunding *et al.*, 2015). In most cases, mice are *i.p.* sensitized two times in intervals of seven to fourteen days to induce a strong Th2-biased immune response (van Rijt *et al.*, 2005; Zhou *et al.*, 2014; Nakashima *et al*., 2014). For route, dosing and frequency of the airway challenge a wide range of options have been published. Mice can be challenged by *i.n.* application, *i.t.* installation as well as by inhalation of aerosolized OVA (Park *et al.*, 2018; Yosri *et al.*, 2018; Hong *et al.*, 2019). A major advantage of the *i.n.* application is, that the amount of antigen administered to the airways can be precisely determined, while during inhalation it is difficult to estimate exactly how much antigen is being inhaled. Due to the tendency towards a stronger accumulation of eosinophils and $CD4^+$ T cells in the respiratory tract following three sensitizations and the similar results between two and three challenges, it was decided to *i.p.* sensitize mice three times with 10 µg OVA and 1 mg alum and challenge them three times *i.n.* with 100 µg OVA in PBS for subsequent experiments. Because a challenge with 10 µg OVA might induce an insufficient AAI, while a challenge with 250 µg OVA could lead to a strong AAI, the challenge dose of 100 µg was chosen as a medium dosage which allowed detection of both possible aggravating as well as attenuating effects of the additional SEB-treatment.

Because literature showed that the genetic background, as well as the specific microbiome in mice of different breeders may have possible effects on the induction of different disease

models, C57BL/6 mice purchased from Charles River and Janvier Labs were compared using the adjusted model of AAI. Hilbert *et al*. showed that the gut microbiota of mice from different vendors influenced experimental abdominal sepsis. Stool analysis revealed profound difference in bacterial composition, e.g. mice purchased from Janvier Labs showed a significant lower appearance of *S. aureus* in the intestinal microbial flora as compared to mice purchased from Charles River (Hilbert *et al*., 2017). Research from Villarino *et al.* showed that the composition of the gut microbiota in mice of different vendors has profound modulatory effects in the severity of malaria (Villarino *et al*., 2016).

Comparing mice purchased from Charles River and Janvier Labs, after the experimental induction of AAI showed no significant differences in the cellular immune response. However, mice purchased from Janvier Labs tended to yield higher concentrations of type 2-typical cytokines (IL-4, IL-5, IL-13), while the OVA-specific IgE-response was more pronounced in mice purchased form Charles River.

Because of the more pronounced cytokine response and the sufficient IgE production, mice for subsequent experiments in this thesis were purchased from Janvier Labs.

The final mouse model of AAI established for the research in this thesis showed several hallmark features important for the analyzation of AAI: A significant increase in absolute numbers of DCs, eosinophils, mast cells and M2-polarized monocytes/macrophages could be shown in the respiratory tract. Additionally, B cells, CD4+ T cells, the frequency of Th2 cells as well as their activation status was significantly increased after AAI induction. Moreover, the induction of AAI led to systemic effects which were displayed by significantly increased numbers of eosinophils and neutrophils measured in the periphery, a significant increase in the OVA-specific IgE concentration in the serum as well as significant airway hyperreactivity following methacholine challenge.

Effects of intranasal SEB on AAI:

Over the last years, research has become aware of the respiratory microbiome as a novel factor affecting allergic and inflammatory airway diseases. Today it is accepted, that colonization of the airways by microorganisms, including opportunistic pathogens, has wide implications for respiratory immunity in health and disease (Budden *et al.*, 2019). One of the most important opportunistic bacterial pathogens is *S. aureus* and nowadays it is widely

accepted that, next to frequent persistent colonization, every individual will get into contact with *S. aureus* at least once in their life (Wertheim *et al*., 2005; Hu *et al*., 1995; Gonzales-Zorn *et al*., 2005; van Belkum *et al.*, 2009). *S. aureus* colonization is associated with various allergic diseases. It has been shown that the skin of over 90 % of patients with AD is colonized by *S. aureus* and that disease severity directly correlates to its growth in biofilms (Kobayashi *et al*., 2015; Di Domenico *et al.*, 2019; Cho *et al.*, 2001; Akiyama *et al.*, 2000). Furthermore, also patients with chronic allergic rhinosinusitis are frequently colonized (Vickery *et al.*, 2019; Thunberg *et al.*, 2017) and for asthma a significant relationship between nasal *S. aureus* carriage and the disease has been recognized (Kim *et al*., 2019). However, the underlying mechanisms of these clinical associations are largely unclear.

Different *S. aureus* proteins came into focus with respect to the induction of Th2-biased immune responses. They can either act directly as targets of a specific allergic response or affect responses to common allergens. E.g., it was recently shown that serine protease like proteases (Spls) are capable of inducing a rather Th2-biased immune response than the expected Th1/Th17 response when used as the allergen/antigen in a murine model of AAI. Additionally, it was shown that there is an increased IgE binding to Spls in sera of asthmatic patients as compared to serum of healthy patients (Stentzel *et al.*, 2016; Stentzel *et al*., 2017). In clinical as well as experimental studies, also *S. aureus* toxins have been proposed to contribute to the development of allergic diseases like severe asthma, eczema and allergic rhinitis. Especially a sensitization to staphylococcal enterotoxins (SEs) is associated with allergic multimorbidity (Sorensen *et al*., 2017; Abdurrahman *et al*., 2020). In mice, previous studies have performed treatment of wild-type mice with SEB in different models of AAI and altogether demonstrated a strong immune modulatory potential of SEB (Hellings *et al*., 2006; Huvenne *et al*., 2010b; Yu *et al*., 2012). When mice were sensitized with a combination of SEB and *D. farinae via* the skin, they developed more severe skin inflammation than those treated with SEB or *D. farinae* alone (Serhan *et al.*, 2019). Moreover, combined epicutaneous SEB/OVA-sensitization before an OVA airway challenge led to increased inflammatory cells (eosinophils, neutrophils) in the lungs, increased airway mucus hyperplasia and significantly increased levels of IL-4 and IL-17A measured in the BAL as compared to mice sensitized with OVA alone (Yu *et al*., 2012). Furthermore, *i.n.* application of SEB, and not any other *S. aureus* toxin, together with OVA facilitated sensitization to OVA *via* the respiratory tract resulted in

increased serum levels of OVA-specific IgE, a significant recruitment of eosinophils and lymphocytes and increased airway hyperreactivity (Huvenne *et al*., 2010b).

In a similar approach to the one used for the experiments of this thesis, peripherally OVA-sensitized mice were treated *i.n.* with 500 ng SEB before the challenge with nebulized OVA. Here, SEB administration led to increased cell counts, especially eosinophils, in BAL cytospins as well as to enhanced bronchial mRNA expression levels of IL-5, IL-4, eotaxin-1, IL-12 p40, IFN- γ and TGF-β and increased serum concentrations of IL-4, IL-5 and IFN-γ (Hellings *et al.*, 2006). This thesis now aimed at taking these important observations further through comprehensive analyses within one model of OVA-induced AAI towards unraveling the underlying immunological mechanisms of SEB-mediated effects on AAI. For such comprehensive analyses, different doses of SEB were *i.n.* administered at different crucial time-points in the induction of AAI.

Effects of intranasal SEB administered together with the OVA-challenge on AAI:

Treatment with 50 ng SEB during the OVA-challenge in sensitized mice led to a significant increase of DCs and M2-ploarized monocytes/macrophages in the lungs. While Muraille *et al.* observed that the intravenous injection with SEB led to decreased DC numbers in the spleen (Muraille *et al*., 1997), Yoon *et al.* demonstrated that *i.p.* injected SEB is a potent activator of splenic DCs. In their study, 50 µg/kg SEB were injected *i.p.* and within 6 hours after the injection, a migration of immature splenic DCs from the marginal zone to the periarterial lymphatic sheath was induced. At the same time, the maturation of DCs was observed by upregulation of CD40, CD80 and CD86 expression (Yoon *et al*., 2001). With respect to the lung, Krysko *et al. i.n.* treated mice with OVA, SEB or a combination of both and observed that the treatment with SEB alone but particularly the combined treatment led to increased numbers of $CD11c^{+}MHCII^{high}$ and $CD11b^{+}MHCII^{+}CD103^{-}$ DC phenotypes (Krysko *et al*., 2013).

Furthermore, our data are in line with the recent observation that *S. aureus* induces type 2 polarization of monocytes/macrophages and the activation of M2-polarized macrophages through the production of enterotoxins. Such polarized macrophages show decreased phagocytosis of *S. aureus* and thereby allow its long-term survival (Flora *et al*., 2019; Krysko *et al*., 2011). Also, macrophages are potent modulators of asthma (Frickert & Gibson, 2017) and experimental studies showed that the inhibition of M2-polarization aggravates airway

hyperreactivity (Draijer *et al.*, 2018) while vice versa airway hyperreactivity can be alleviated by increased M2 polarization (Song *et al.*, 2015). As opposed to these findings, it was observed that airway hyperreactivity was not significantly affected despite significantly increased numbers of M2 macrophages in mice treated with 50 ng SEB during the allergic challenge. On the other hand, treatment with 500 ng SEB together with the allergic challenge led to significantly alleviated airway hyperreactivity while M2-polarization was not significantly altered as compared to AAI alone. In treatment with 500 ng SEB before sensitization the frequency of M2-polarized macrophages was significantly less as compared to AAI alone, while at the same time airway hyperreactivity was also not significantly affected. As development of airway hyperreactivity itself is a multifactorial process (Chapman & Irvin, 2015), one can only speculate that in the model used in this thesis alternative mechanisms overlaying the expected effects of altered M2 polarization on airway hyperreactivity are at play. Possibly also the sex of the mice used in our experiments plays a role, as generally airway hyperreactivity in response to methacholine is less pronounced in female mice (Card *et al.*, 2006; Matsubara *et al*., 2008).

With respect to the superantigenic properties of SEB (Pinchuck *et al.*, 2010; Antonsson *et al.*, 1997; Balaban, 2000) and possibly related effects on lymphocytes, in this thesis a dose-dependent increase of $CD4^+$ and $CD8^+$ T cell numbers and CD69-expression in the respiratory tract of mice with *i.n.* SEB administration during the allergic challenge was observed as compared to AAI alone. Furthermore, significantly increased numbers of CD69-expressing B cells in the lungs after *i.n.* administration of 50 ng SEB during the allergic challenge was shown. Even seven days after *i.n.* SEB treatment alone, a significant increase in activated B cell was demonstrated in this thesis. This is possibly a direct effect of SEB, as it was demonstrated that staphylococcal enterotoxins are potent B cell activators in human PBMC cultures (Stohl, 1995; Stohl & Linsley, 1994).

On the level of the respiratory cytokine response, the administration of SEB during the allergic challenge led to a dose-dependent increase of the pro-inflammatory cytokines IL-6 and TNF-α, as well as to significantly increased concentrations of IFN-γ and a trend towards increased IL-17A, which mirrors the typical antibacterial type 1 immune response after *S. aureus* infection (Pinchuk *et al.*, 2010; Archer *et al*., 2016; Watkins *et al*., 2011). Other reports showed increased levels of typical type 2 cytokines after *i.n.* treatment with SEB alone or in combination with OVA (Hellings *et al*., 2006; Huvenne *et al*., 2010; Herz *et al*., 1999; Yu *et al*.,

2012; Huvenne *et al*., 2013). In contrast, no significant changes and if so, rather decreased levels of IL-4, IL-5 and IL-13 in AAI were observed, if mice were *i.n.* administered SEB during the allergic challenge. These observations suggest that the SEB-induced Th1- and pro-inflammatory cytokine response possibly shifts the balance away from the allergy-associated Th2-respone (Eberl, 2016). This is further supported by the significantly reduced frequency of Th2 T cells, which was observed in the lungs of mice after the *i.n.* administration of 500 ng SEB during the allergic challenge as compared to AAI alone. Importantly, these changes were associated with significantly decreased airway hyperreactivity. Thereby these results demonstrate that SEB-mediated modulation of AAI can have beneficial effects on lung functional parameters. This finding is remarkable, as generally SEB has been associated with an aggravation of AAI (Hellings *et al*., 2006; Huvenne *et al*., 2010a).

Effects of intranasal SEB administered prior to sensitization in AAI:

It has been shown that a single *i.p.* injection of SEB leads to lung inflammation characterized by lung infiltration of mixed populations of granulocytes, mononuclear phagocytes and NK cells (Neumann *et al*., 1997). Additionally, *i.n.* SEB facilitates respiratory sensitization, showed by an increased DC maturation and migration to the draining lymph nodes, as well as an increased influx of eosinophils and lymphocytes to the respiratory tract (Huvenne *et al*., 2010b). Furthermore, SEB has been shown to facilitate dermal sensitization (Yu *et al.*, 2012; Serhan *et al.*, 2019). However, the question whether the *i.n.* administration of SEB would remotely affect peripheral sensitization has not been addressed so far.

Surprisingly, *i.n.* administration of SEB prior to *i.p.* sensitization indeed showed distinct effects on subsequently induced AAI. Administration of 50 ng SEB prior to sensitization apart from significantly increased basophil numbers and significantly decreased IL-5 levels led to rather subtle changes in airway inflammation and did not affect hyperreactivity. Strikingly however, these mice revealed significantly elevated levels of OVA-specific IgE in the serum, suggesting that SEB encountered *via* the respiratory tract has the potential to significantly enhance peripheral allergic sensitization. However, additional experiments showed that the antigen-specific IgG and IgE production was not significantly affected by *i.n*. SEB administration prior to the allergic sensitization alone. Analyzing OVA-specific serum IgG and IgE-levels six days after the last of three weekly *i.p.* sensitizations, no effects of SEB administration prior to

sensitization were observed. The results of these experiments led to the assumption that in this model the allergic challenge is decisive for the production of high levels of antigen-specific IgE in general and for the significantly increased OVA-specific IgE response following administration of 50 ng SEB prior to sensitization.

Clinical studies have shown that *S. aureus* colonization in AD leads to a higher degree of allergic sensitization and a higher frequency of asthma (Wichmann *et al*., 2009). Importantly, furthermore a role for SEB and also for SEB-specific IgE antibodies in allergic sensitization and in asthma pathogenesis has been proposed (Schreiber *et al*., 2019; Sintobin *et al*., 2019).

Strikingly, the *i.n.* administration of 500 ng SEB prior to sensitization led to significantly reduced total cell counts and eosinophil numbers in the lungs and BAL, significantly reduced mast cell numbers, basophil numbers and Th2 cell frequencies in the lungs as well as significantly reduced levels of IL-4, IL-5 and IL-6 in the BAL as compared to mice with induced AAI alone.

In conclusion, while the *i.n.* administration of a relatively low dose of SEB (50 ng) prior to sensitization led to a significant increase in the antigen-specific IgE-response, SEB administered at a higher dose (500 ng) before sensitization significantly affected AAI at multiple levels, resulting in an altogether ameliorated inflammatory phenotype. Based on these results, one central question is whether the amelioration of AAI observed for *i.n.* administration of 500 ng SEB before sensitization reflects long-term effects of SEB on the microenvironment of the respiratory tract or long-term, remote systemic effects. Such effects of SEB have been described, enhancement of the numbers and activity of bone marrow neutrophils *via* cytokines (Ferreira-Duartea *et al*., 2020).

Long-term effects of intranasal SEB administration on the respiratory tract immune environment:

In order to investigate, if the above described and discussed effects of *i.n.* SEB administration prior to the allergic sensitization reflected long-term imprinting effects of *i.n.* SEB administration on the respiratory tract immune environment, the accumulation of immune cells, their activation as well as cytokine production were examined over time - one, seven and fourteen days following SEB administration alone.

With respect to effects of SEB alone on the local immune network, in the literature mostly immediate effects after SEB treatment are described. Related to cellular influx to the respiratory tract, Herz *et al.* examined the effects of *i.n.* treatment with different doses of SEB alone and observed dose-dependent airway inflammation that was marked by the infiltration of lymphocytes and granulocytes 24 h after the last application (Herz *et al*., 1999). In this thesis, similar results for *i.n.* SEB-treatment alone were obtained with respect to short term effects of SEB. In addition to the unspecific, polyclonal activation of T cells, it has been shown that as additional short-term effects, SEB is capable of increasing the Ig production in B cells, enhancing the activation and survival of eosinophils, inducing chemokine production in macrophages which leads to neutrophil immigration and activating DCs *via* TLR-2 (Desouza *et al.*, 2002; Mandron *et al.*, 2006; Huvenne *et al*, 2010a). Furthermore, by MHCII binding and cross-linking, SEB can activate the immune system after contact with epithelial cells (Schulz *et al.,* 1998; O´Brien *et al.*, 2006; Huvenne *et al.*, 2013).

In this thesis, 24 h after the last *i.n.* administration of 50 ng or 500 ng SEB, cytokine production was not affected as compared to PBS-treated control mice. In contrast to these results, Herz *et al.* could show an increase of IL-4 and TNF-α concentrations in the BAL after the *i.n.* administration of different SEB doses (Herz *et al*., 1999). In line with results of this thesis, Krakauer *et al*. were not able to detect elevated concentrations of pro-inflammatory serum cytokines on several different short-term time-points (3, 4, 5, 6 and 24 hours) after *i.n.* administration of various non-lethal doses SEB (Krakauer *et al.,* 2010b). Another study by Litton *et al.* on the contrary, showed an increase of the expression of different cytokines (TNF, IL-2, IFN-γ) in isolated cells of inguinal lymph nodes 2-15 h after *i.p.* SEB injection which completely subsided by 24 h after injection (Litton *et al.*, 1994). Matching these results, Rajagopalan *et al.* showed increased concentrations of the pro-inflammatory cytokines IL-12p70, TNF-α, IFN-γ and IL-6 in the serum very shortly after the *i.n.* administration of SEB (4 h). However, 8 h after the administration the concentrations were already decreasing (Rajagopalan *et al.*, 2006).

Analysis of the respiratory immune network at later time-points following *i.n.* administration of SEB interestingly indeed showed clear sustained effects. With respect to the cellular immune response, seven days after the last *i.n.* SEB administration (50 ng SEB), absolute numbers of DCs, eosinophils, M2-polarized monocytes/macrophages and $CD4^+$ T cells were still significantly increased in the lung as compared to PBS-treated control mice. Additionally,

at this time point in the lung absolute numbers of activated B cell, $CD4^+$ T cells and Th2 cells were significantly elevated. In the BAL, absolute numbers of eosinophils and $CD4^+$ T cells were significantly increased. After an *i.n.* administration of 500 ng SEB similar results were observed, with the exception of absolute numbers of DCs, $CD4^+$ T cells as well as activated Th2 cells, which were not significantly increased as compared to the control group.

In a study of Saloga *et al.*, a single cutaneous exposure to small amounts of SEB led to a strong inflammatory response in mice which was characterized by mast cell degranulation, the induction and upregulation of adhesion molecules and the infiltration of inflammatory cells. The inflammatory response in the skin of SEB-treated mice lasted up to seven days (Saloga *et al.*, 1996). Also, other species showed long lasting effects after SEB-treatment. In rats, the intracutaneous injection of different doses of SEB led to a significant infiltration of lymphocytes in the dermis which peaked at 56 h after the injection and could still be detected seven days after the injection (Ohshima *et al.*, 2011).

Strikingly, while cytokine levels were not significantly altered at the earlier time-points, seven days after the last of the repeated *i.n.* administration of 50 ng or 500 ng SEB the concentrations of the typical type 2 cytokine IL-4, as well as concentrations of pro-inflammatory cytokines IFN-γ, IL6, TNF-α and IL-17A were significantly increased in the BAL as compared to the control group. Additionally, a trend towards increased IL-5 concentrations was observed.

An *in vitro* study, where isolated eosinophils from BAL and blood of allergic asthma patients were co-cultured with $CD4^+$ T cells and incubated with SEB for six days increased concentrations of IL-5, IL-13 and IFN-γ were measured in the supernatant of the co-culture as compared to $CD4^+$ T cells incubated with SEB alone. (Liu *et al.,* 2006). Another study by Heaton *et al.* isolated PBMCs from asthmatic patients, as well as patients with atopic eczema/dermatitis syndrome which were then *in vitro* incubated with SEB for four days. After the incubation levels of different cytokines were measured in the supernatant. Especially levels of IL-5 were significantly increased in the supernatant of cultured PBMCs from asthmatic patients and patients with atopic eczema/dermatitis syndrome compared to isolated PBMCs from non-atopic control patients (Heaton *et al.*, 2003).

Overall, the results of these experiments indicate that the *i.n.* administration of SEB indeed has sustained effects on the respiratory immune environment.

Strikingly, fourteen days after the last *i.n.* administration of 50 ng or 500 ng SEB significantly increased total cell numbers, as well as significantly increased absolute numbers of DCs (50 ng SEB) and eosinophils (500 ng SEB) in the BAL were measured as compared to controls in the experiments of this thesis. Furthermore, trends for increased absolute numbers of macrophages in the BAL, as well as $CD69^+$ B cells in the draining lymph nodes were observed. As these are all key cells involved in AAI, it is well conceivable for *i.n.* SEB to have long-term effects in subsequently induced AAI. A study showed that SEB increases the expression of T cell immunoglobulin mucin domain (TIM)4 on DCs which ligates TIM1 on $CD4^+$ T cells driving them to become Th2 cells (Liu *et al*., 2007). Other studies also show a Th2 polarization of the immune response supported by DCs after treatment with SEB (Mandron *et al.*, 2006; Yu *et al.*, 2012). Several studies have shown a rapid turnover for tracheal DCs in the lung after external stimuli, interstitial DCs however are known to be much more long-lived (Holt *et al*., 1994; von Garnier *et al*., 2005; Guerts van Kessel & Lambrecht, 2008). A long-term increase or rather the long-term survival of DCs after *i.n.* SEB administration might amplify the immune response of a following trigger, e.g. an allergic sensitization further and enhance the type 2 immune response. Eosinophils are also activated by SEB treatment and their survival is promoted (Breuer *et al.*, 2000; Hellings *et al.*, 2006, Yu *et al.*, 2012). E.g. the airway exposure to SEB activates and mobilizes eosinophil populations in the bone marrow and facilitates their migration to the blood and subsequently the lung tissue (Pinheiro-Torres *et al.*, 2021). Through their cytokine production, circulating eosinophils may also support a subsequent type 2 immune response. However, these results are contrary to those of this thesis, since experiments with administration of 500 ng SEB prior to the allergic sensitization led to a rather ameliorated AAI.

Additionally, the preliminary analysis of unsensitized controls for the pre-treatment with SEB before OVA-sensitization and challenge (SEB_{50}/a.o./OVA and SEB_{500}/a.o./OVA), yielded significantly increased total cell numbers, absolute numbers of $CD4^+$ T cells and eosinophils in the BAL on day 25 after the last *i.n.* SEB administration. These results furthermore indicate a long-term influence of *i.n.* SEB administration on the respiratory tract immune environment

For years it was assumed that immunological memory was a hallmark and exclusive function of the adaptive immune response. However, over the last years a growing amount of studies indicated that also cells of the innate immune system like DCs and monocytes/macrophages

may show adaptive characteristics and could provide better protection when it comes to reinfections, which is called trained immunity (Bowdish *et al*., 2007; Netea *et al*., 2011; Christ *et al*., 2018). The concept of trained immunity includes the long-term functional reprogramming and thereby imprinting of innate immune cells, which enables them to a more rapid response to a second, non-specific stimulus after the return to a non-active state. In contrast to adaptive memory, trained immunity is rather mediated by epigenetic reprogramming of transcriptional pathways and it is assumed that it is generally reversible and of a shorter duration than the classical adaptive memory (Dominguez-Andres & Netea, 2018; Netea *et al*., 2020). However, recent studies suggested a transgenerational effect through trained immunity (Berendsen *et al*., 2019; Moore *et al*., 2019). For monocytes/macrophages and DCs immune memory responses have been shown following bacterial, fungal and infections with parasites (Saeed *et al*., 2014; Chen *et al*., 2014; Hole *et al.*, 2019). This might indicate that innate immune cells possibly also evolve in the sense of innate memory after infections with *S. aureus* or an encounter with SEB. Ultimately, further studies such as epigenetic analyses of different innate immune cells following *i.n.* SEB administration are needed in order to test this hypothesis. Also, additional cell types next to those analyzed in the experiments of this thesis, such as ILC-2 and cells of the respiratory epithelium, will need to be incorporated in such analyses.

5.1 Conclusion

In conclusion, the results of this thesis clearly show a strong and exceptionally versatile potential of SEB to modulate AAI as well as airway hyperreactivity. While administration of SEB during the allergic challenge shifted inflammation towards a Th1- and pro-inflammatory phenotype and ameliorated airway hyperreactivity, administration of the higher dose of SEB prior to sensitization generally dampened AAI without significantly enhancing Th1- or pro-inflammatory parameters. Importantly, SEB-treatment before sensitization furthermore revealed a clear potential to boost the OVA-specific IgE response. This effect manifests most likely during the allergic challenge. Furthermore, the *i.n.* administration of SEB alone had significant long-term effects on the immune cellular composition, the activation status of lymphocytes, as well as the cytokine production in the respiratory tract and draining lymph

nodes. These results clearly suggest that SEB is able to sustainably modulate respiratory immunity, most likely with implications for inflammatory processes in AAI. Future investigations could utilize the experimental setup analyzed in this thesis and extend the analysis of SEB-mediated effects when SEB is co-administered with the allergic challenge to later time-points than the 48 h time-point analyzed here.

Based on the results of this thesis, it can be proposed that SEB rather distinctly modulates than generally aggravates allergic processes in the airways. A major finding is that the nature as well as the consequence of such SEB-mediated modulation strongly depend on the timing and the dose of SEB-encounter. Most likely also the site where SEB is applied (or produced by colonizing *S. aureus*) plays a major role.

On the basis of these findings and considerations, future experimental and clinical studies will have to disentangle the exact interplay between SEB, allergic sensitization and AAI depending on when, where and how intensely SEB is encountered. Such knowledge will importantly enable targeted strategies of prevention and therapy, especially in the light of the large number of *S. aureus* carriers and patients suffering from allergic asthma in the human population.

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7 Ehrenerklärung

Ich versichere hiermit, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; verwendete fremde und eigene Quellen sind als solche kenntlich gemacht. Ich habe insbesondere nicht wissentlich:

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Magdeburg, den 24.01.2022

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Ilka Jorde