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The aryl hydrocarbon receptor as a new potential target in allergen-specific immunotherapy

Sonja Maria Heine

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Vorsitz: Prof. Dr. Ellen Renner

Prüfer*innen der Dissertation:

1. Priv.-Doz. Dr. Simon Blank
2. Prof. Dr. Jörg Durner
3. Assoc. Prof. Dr. Sabine Flicker

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Für meine Eltern.

Lernen ist wie rudern gegen den Strom.
Sobald man damit aufhört, treibt man zurück.

- Benjamin Britten

Abstract

Allergic diseases show an increasing prevalence worldwide. They include several complex manifestations and are caused by an inappropriate reaction of the immune system towards an otherwise harmless environmental antigen. Allergen-specific immunotherapy (AIT) is currently the only available causative treatment option. The goal of AIT is to restore immune tolerance by repetitive administration of increasing doses of the disease-relevant allergen, until a constant maintenance dose is reached and administered over a course of several years. AIT has been performed successfully in many patients over the past decades. However, some patients are not or insufficiently responding to current treatment protocols, showing the need for a better understanding of underlying mechanisms and adapted treatment protocols. Besides the investigation of basic mechanisms, the identification of target structures that can help to induce the required immune tolerance and to improve the therapeutic outcome of AIT has become a focus of attention in recent years.

This study investigated the general role of the aryl hydrocarbon receptor (AhR) in AIT and its potential to improve the efficacy of AIT. The AhR is a ligand-dependent transcription factor involved in numerous biological processes. A variety of exogenous and endogenous ligands can induce the AhR and result in the expression of different target genes. With expression in multiple immune cells, an important role has also been revealed in the regulation of innate and adaptive immunity, which makes it an interesting potential target for several immune disorders. In the course of this work, AIT was successfully performed in AhR-deficient ($AhR^{-/-}$) mice in a model of ovalbumin-induced allergic asthma. First results generated in a model of house dust mite-induced allergic asthma led to the assumption that the AhR is not needed for successful AIT, independent of the choice of allergen and the route of sensitization.

The effects of AhR activation on AIT and the allergic phenotype were investigated by administration of the low-affinity ligand quercetin and the high-affinity ligand 10-Chloro-7H-benzimidazo[2,1-a]benzo[de]iso-quinolin-7-one (10-Cl-BBQ) in combination with AIT and as a single-treatment strategy. Quercetin was only able to ameliorate parameters of allergic asthma in a rather acute manner, independent of AIT and most likely by inhibition of the mammalian target

of rapamycin (mTOR) signaling pathway. An improvement of the therapeutic outcome by the combination of AIT and quercetin administration could not be shown. On the contrary, 10-Cl-BBQ was able to further improve the therapeutic outcome of AIT and resulted in ameliorated parameters of allergic asthma. The combined treatment did not only reduce total cell numbers in bronchoalveolar lavage fluid, pulmonary T helper 2 and T helper 17 cells as well as mucus hypersecretion, but also restored or maintained levels of alveolar macrophages. The absence of the observed beneficial effects in $AhR^{-/-}$ mice after treatment with 10-Cl-BBQ confirmed the dependency of 10-Cl-BBQs mode of action on the AhR.

This work could demonstrate that the AhR is not required for successful AIT. However, the herein generated results introduce the AhR as a potential therapeutic target and could build a basis for further investigations into how high-affinity AhR-ligands could be used as immunomodulators to improve the outcome of AIT.

Zusammenfassung

Allergische Erkrankungen zeigen weltweit eine steigende Prävalenz und umfassen eine Vielzahl komplexer Krankheitsbilder. Ausgelöst werden allergische Erkrankungen in der Regel durch eine überempfindliche Reaktion des Immunsystems auf ein ansonsten harmloses Umweltantigen. Die allergen-spezifische Immuntherapie (AIT) stellt zum aktuellen Zeitpunkt die einzige kausale Behandlungsmöglichkeit für allergische Erkrankungen dar. Ziel der AIT ist die Wiederherstellung der Immuntoleranz durch die wiederholte Verabreichung steigender Dosierungen des verursachenden Allergens, bis zum Erreichen einer konstanten Erhaltungsdosis, die über mehrere Jahre verabreicht wird. Trotz der erfolgreichen Behandlung zahlreicher Patienten im Verlauf der letzten Jahrzehnte treten immer wieder Fälle auf, in denen Patienten nicht oder nur unzulänglich auf die angewandten Therapieprotokolle ansprechen. Dies zeigt den nach wie vor vorhandenen Bedarf für ein besseres Verständnis der zugrunde liegenden Mechanismen und den damit verbundenen Verbesserungen existierender Therapieansätze auf. Die Identifikation geeigneter Zielstrukturen, deren Ansprechen den Erfolg der AIT verbessern könnte, ist während der letzten Jahre zusätzlich in Fokus der Aufmerksamkeit gerückt.

Das Ziel der vorliegenden Arbeit umfasste die Untersuchung der Rolle des Aryl-Hydrocarbon-Rezeptors (AhR) in der AIT, sowie seine mögliche Eignung als Zielstruktur zur Verbesserung des Therapieerfolgs. Als Liganden-abhängiger Transkriptionsfaktor ist der AhR in eine Vielzahl biologischer Prozesse involviert. Die Aktivierung kann durch eine große Vielfalt exogener und endogener Liganden erfolgen und in der Expression unterschiedlicher Zielgene resultieren. Der AhR wird auch in verschiedenen Zellen des Immunsystems exprimiert und durch seine wichtige Rolle bei der Regulierung der innaten und adaptiven Immunität zu einer interessanten, potenziellen Zielstruktur für die Therapie verschiedener Immunerkrankungen. Die erfolgreiche Durchführung der AIT in AhR-defizienten ($AhR^{-/-}$) Mäusen in einem Modell für Ovalbumin-induziertes allergisches Asthma, sowie erste Ergebnisse aus einem Modell für Hausstaubmilben-induziertes allergisches Asthma führten zu der Annahme, dass der AhR keine Voraussetzung für einen Therapieerfolg darstellt, unabhängig von der Wahl des Allergens und der Sensibilisierungsroute.

Die Auswirkungen einer gezielten Aktivierung des AhRs während der AIT wurden mit Hilfe des schwachaffinen Liganden Quercetin und des hochaffinen Liganden 10-Chloro-7H-benzimidazo[2,1-a]benzo[de]iso-quinolin-7-one (10-Cl-BBQ) untersucht. Beide Liganden wurden sowohl in Kombination mit AIT, als auch als separate Behandlungsstrategie eingesetzt. Charakteristische Parameter allergischen Asthmas zeigten nach alleiniger Quercetin-Gabe, ohne zusätzliche AIT, nur eine kurzeitige Verbesserung, die höchstwahrscheinlich durch eine Inhibition des mammalian target of rapamycin (mTOR)-Signalweges vermittelt wird. Eine Kombination aus Quercetin und AIT konnte nicht zur Verbesserung des Therapieerfolgs beitragen. Im Gegensatz dazu wirkte sich die Kombination aus AIT mit 10-Cl-BBQ positiv auf den Behandlungserfolg der AIT aus und war in der Lage, die Ausprägung charakteristischer Parameter allergischen Asthmas deutlich zu reduzieren. Die kombinierte Therapie war nicht nur in der Lage, die Gesamtzellzahl in bronchoalveolarer Flüssigkeit, die pulmonalen Typ-2-T-Helferzellen und Typ-17-T-Helferzellen sowie eine übermäßige Schleimsekretion zu vermindern, sondern resultierte zusätzlich in Prozentzahlen alveolarer Makrophagen vergleichbar zu jenen in nicht-allergischen Kontrolltieren. Das Ausbleiben der beobachteten positiven Effekte in $AhR^{-/-}$ Mäusen nach der Behandlung mit 10-Cl-BBQ bestätigte die Abhängigkeit der Wirkungsweise von 10-Cl-BBQ vom AhR.

Zusammenfassend konnte mit der vorliegenden Arbeit gezeigt werden, dass der AhR keine Voraussetzung für eine erfolgreiche AIT darstellt, eine gezielte Aktivierung des AhR aber durchaus zu einer Verbesserung des Therapieerfolgs beitragen kann. Die Ergebnisse dieser Arbeit zeigen das Potenzial des AhR als therapeutische Zielstruktur und könnten eine Grundlage für weitere Untersuchungen über den Einsatz hochaffiner AhR-Liganden zur Verbesserung des Therapieerfolgs der AIT darstellen.

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Abbreviations

10-Cl-BBQ	10-Chloro-7H-benzimidazo[2,1-a]benzo[de]iso-quinolin-7-one
4E-BP1	eukaryotic translation initiation factor 4E-binding protein
AhR	aryl hydrocarbon receptor
$AhR^{-/-}$	AhR-deficient
AhRR	AhR repressor
AIP	AhR-interacting protein
AIT	allergen-specific immunotherapy
AKT	protein kinase b
Alum	aluminium hydroxide
APC	antigen-presenting cell
ARNT	AhR nuclear translocator
BALF	bronchoalveolar lavage fluid
bHLH	basic helix-loop-helix
Bregs	regulatory B cells
BSA	bovine serum albumin
cDNA	first strand complementary DNA
CTLA-4	cytotoxic T-lymphocyte associated protein 4
CYP1A1	cytochrome P450 family-1 subfamily-A polypeptide-1
CYP1A2	cytochrome P450 family-1 subfamily-A polypeptide-2
CysLT	cysteinyl leucotrienes
DC	dendritic cell
Der f	*Dermatophagoides farinae*
Der p	*Dermatophagoides pteronyssinus*
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DRE	dioxin response element
ELISA	enzyme-linked immunosorbent assay

FcεRI	high-affinity IgE receptor
FICZ	6-Formylindolo[3,2-b]carbazole
GPx	glutathione peroxidase
GWAS	genome-wide association studies
H&E staining	hematoxylin and eosin staining
HDM	house dust mite
HLA	human leucocyte antigen
HRP	horseradish peroxidase
HSP90	heat shock protein 90
i.n.	intranasal
i.p.	intraperitoneal
I3C	indole-3-carbinole
ICZ	indolo-[3,2-b]-carbazole
IDO	2,3-dioxygenase
Ig	immunoglobulin
IL	interleukin
ILC	innate lymphoid cell
ILR	interleukin receptor
iTregs	induced regulatory T cells
mAbs	monoclonal antibodies
MHC	major histocompatibility complex
mTOR	mammalian target of rapamycin
nTregs	natural regulatory T cells
OVA	ovalbumin
PAS	PER-ARNT-SIM
PAS staining	periodic acid-Schiff staining
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD-1	programmed cell death 1
PG	prostaglandins

PI	propidium iodide
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
ROS	reactive oxygen species
RT	room temperature
s.c.	subcutaneous
scRNA-seq	single-cell RNA sequencing analysis
SNPs	single-nucleotide polymorphisms
SOD1	superoxide dismutase 1
SOD2	superoxide dismutase 2
TCDD	2,3,7,8-Tetrachlorodibenzo-p-dioxin
TDO	tryptophan 2,3-dioxygenase
Tfh cell	T follicular helper cell
TGF-β	transforming growth factor β
Th cell	T helper cell
TLR	toll-like receptor
Tr1 cells	type 1 regulatory T cells
Tregs	regulatory T cells
TSLP	thymic stromal lymphopoietin

1. Introduction

1.1 Allergic diseases

Allergic diseases include multiple complex manifestations like allergic rhinitis, food allergy, atopic eczema, anaphylaxis, and allergic asthma and represent one of the most common types of diseases[1]. Globally, 8-10 % of the population suffer from one or more allergic disorders and the prevalence is increasing in both developing and developed countries[2]. Allergic diseases manifest as the result of an inappropriate reaction of the immune systems towards an otherwise innocuous environmental antigen[3]. Being continuously exposed to various harmless environmental antigens, the immune system induces tolerance towards them[3]. Abrogation or failure of this tolerance induction results in an active immune response towards the respective antigen[3]. As complex multifactorial disorders, determined by genetic, environmental, and socioeconomic factors, allergic diseases show broad heterogeneity and lead to different phenotypes[1]. Allergic diseases are inflammatory disorders and therefore involve multiple cell types, immunoglobulins, cytokines, and soluble factors in their mechanism of action[4]. A common feature of many allergic diseases is an increase in immunoglobulin (Ig) E, leading to the two categories of IgE-mediated and non-IgE-mediated allergic diseases in clinical practice[4]. IgE-mediated allergic conditions are classified as type I hypersensitivity reactions[5]. There are three more classes of hypersensitivity reactions, namely type II, III, and IV, which are not IgE-mediated[5]. Cytotoxic reactions (type II) and immune complex reactions (type III) involve IgG, IgM and for type III reactions sometimes IgA antibodies and lead to an activation of the complement system, which can result in cell and tissue damage[5]. In type IV hypersensitivity reactions, T cells and macrophages are activated due to cytokine release, eventually resulting in tissue damage[5].

1.2 Risk factors and causes of allergic diseases

The development of allergic diseases is complex and not fully understood yet, with the involvement of both environmental and genetic components[6].

Environmental exposures can dramatically influence the phenotype of allergic diseases[7]. Changes in lifestyle and environmental exposures are induced by increasing urbanization and rapid population growth[8]. The Western lifestyle corresponds to changes characterized by increased indoor time, decreased physical activity, antibiotic usage, and obesity prevalence[5,8,9]. The hygiene hypothesis states that increased exposure to early-life infections leads to a decreased risk for the development of allergic diseases[10]. Studies have shown that children raised in rural areas had a lower prevalence of atopy and asthma, supporting a protective role for early outdoor bacterial exposure[11,12]. In contrast, increased time spent indoors comes with a higher risk of being exposed to indoor fungi and mold[13]. Fungal exposure has been associated with asthma, allergic rhinitis, and eczema in several studies[14,15]. There is an increasing concern over indoor air pollution since some societies spend up to 90 % of their time inside, exposed to pollutants from tobacco smoke, solid fuels, stoves, and construction materials[16,17]. Together with outdoor air pollution, usually occurring as a mixture of natural and human-made pollutants, air pollution acts as a risk factor for allergic diseases[16,18]. Exposure to traffic-related air pollution for example resulted in a 40 to 83 % increased risk of aeroallergen sensitization by the age of 4 and in an increased risk of food allergy by the age of 8[19,20].

Genomic components have been investigated with genome-wide association studies (GWAS), identifying several disease-associated risk loci[21]. Estimations explain 25-80 % of asthma risk and up to 90 % of allergic rhinitis risk by genetic inheritance[22,23]. The most replicated loci with genome-wide significance include orosomucoid-like 3, gasdermin B, and gasdermin A, specific to childhood-onset disease[24,25]. Also, significantly replicated are loci with several interleukin receptor (ILR) genes, including IL1RL1, the receptor for Interleukin (IL)-33, and toll-like receptors (TLRs)[26,27]. Besides a number of genes related to epithelial function, genes related to immunity were identified[21]. Single nucleotide polymorphisms (SNPs) in asthma were mostly identified in genes linked to HLA (human leucocyte antigen) regions and type 2 inflammation[21]. Several significant SNPs have been associated with major histocompatibility complex (MHC)-II antigen

(HLA-DR) genes[28]. *HLA-DR* genes play a central role in the immune system by presenting peptides derived from extracellular proteins[29]. Among the type 2 inflammation genes are besides *IL1RL1*, also thymic stromal lymphopoietin (*TSLP*) and *IL-13*[21]. Recent studies have identified 267 loci significantly associated with asthma or allergic rhinitis, and 170 protein-coding risk genes, with one third of the identified genes having an epithelial function[21]. With asthma and allergies being multifactorial disorders affected by environmental and genetic factors, the involvement of several genes, each with a minor effect, seems likely[21]. Different genetic pathways are most likely involved with varying proportions in different populations[21]. A better understanding of genetic mechanisms and their interactions with environmental factors will be an indispensable requirement to develop personalized treatment solutions to address the various endotypes of allergic diseases[6].

1.3 Mechanisms of allergic inflammation

1.3.1 Sensitization

The development of allergic diseases requires sensitization to an allergen in the first place[30]. In the context of IgE-mediated allergic diseases, sensitization reflects the ability of the allergen to induce a T helper (Th) 2 cell response[30]. The produced Th2 cytokines, mainly IL-4 and IL-13, promote an immunoglobulin class-switch recombination in B cells and drive IgE production[30]. Usually, the epithelial barrier acts as the first line of defense[31]. Key events like epithelial activation or injury result in the release of alarmins, which favor the development of a tissue microenvironment predisposed to type 2 immune responses[31]. The increased permeability makes the epithelium more susceptible to environmental triggers[32]. Interaction of environmental allergens with innate immune receptors, including TLRs and protease-activated receptors, stimulate epithelial cells to produce cytokines that drive Th2-like adaptive immune responses[32]. Allergens can cross the impaired epithelial barrier and are taken up by antigen-presenting cells (APCs)[33]. APCs, mostly dendritic cells (DC), migrate to the lymph nodes, where they present selected allergen-derived peptides on MHC-II molecules to naïve T cells, inducing Th2 and T follicular helper cell (Tfh) responses[33]. IL-33, IL-25, and TSLP produced by epithelial cells are able to stimulate type 2 innate lymphoid

cells (ILC) to produce Th2 cytokines, further adding to a Th2-promoting environment by the production of IL-5 and IL-13[34]. Th2, Th17, and Tfh cells than interact with B cells and induce IgE class-switch recombination and B cell maturation, resulting in the differentiation of B cells to allergen-specific IgE-producing plasma cells[31,33]. Subsequent binding of allergen-specific IgE on high-affinity IgE receptors (FcεRI) on the surface of mast cells and basophils completes the sensitization phase[31]. Upon a new encounter with the respective allergen, allergic inflammation occurs.

1.3.2 Early and late phase response

In allergic inflammation, different temporal phases are classified[30]. Early phase reactions (type I immediate hypersensitivity reactions) are induced within seconds or minutes after allergen exposure[30]. Recurrent contact with the allergen induces cross-linking of adjacent IgE molecules on mast cells and basophils[35,36]. Subsequent aggregation of FcεRI triggers intracellular signaling which results in the secretion of different preformed mediators and the *de novo* production of lipid mediators, certain growth factors, and cytokines[35-39]. Preformed histamine, neutral proteases, and chemotactic factors are released by the degranulation of mast cell cytoplasmic granules[40,41]. Additionally, the eicosanoid pathway is activated to produce mediators, such as cysteinyl leukotrienes (CysLT) and prostaglandins (PG)[42]. Local release of such mediators triggers acute signs and symptoms of early phase reactions that can include vasodilation, mucus hypersecretion, contraction of bronchial smooth muscle, and increased vascular permeability[43]. Therefore, a systemic release of mediators by mast cells and basophils can lead to severe anaphylaxis[43,44].

Cytokines, Chemokines, and growth factors that are newly synthesized by mast cells as a response to IgE or allergen contact are released slower than preformed mediators[45]. They have the potential to recruit and activate other immune cells[37]. Mast cells are also able to secrete products with anti-inflammatory or immuno-suppressive properties, like IL-10 or transforming growth factor β (TGF-β), and therefore are able to affect many aspects of the biology of DCs and cells of the adaptive immune system[46]. Mediator release in early phase reactions by activated mast cells, together with antigen-stimulated T cells, is thought to strongly influence late phase reactions[47,48]. Those reactions usually occur 2-6 h after allergen exposure, often peak at 6-9 h, and involve the

recruitment of leucocytes, granulocytes, and monocytes[47]. The recruitment of Th2 cells at early stages and Th1 cells at late stages can further contribute to changes in the environmental cytokine milieu at affected sites[49]. Th2 and Th17 cells are recruited and activated at the site of inflammation and release cytokines. The Th2 cytokines orchestrate allergic inflammation, mucus hypersecretion, and airway hyperreactivity, with IL-4 involved in IgE class-switch recombination in B cells, IL-4 and IL-13 as a requirement for the production of high-affinity IgE and IL-5 for recruitment of eosinophils[50]. Whereas Th2 cells are strongly involved in eosinophilic asthma, Th17 cells induce neutrophilic inflammation[34].

1.3.3 Chronic phase and allergic asthma

Usually, early and late phase reactions resolve, depending on the strength of symptoms, either with or without medical assistance and respective treatment[30]. However, when allergen exposure is repetitive or continuous inflammation persists, structural changes in cells and eventually altered function of the affected organs can be the consequence[30]. Chronic airway inflammation, mediated by Th2-cytokines, is accompanied by eosinophilic inflammation, airway remodeling, fibrosis, and airway hyperplasia[30]. In some patients, induced bronchus-associated lymphoid tissue is formed, in which antigen-specific pathogenic Th2 cells are maintained[34]. Chronic allergic inflammation is not only induced by Th2 cells but by a larger variety of Th cell subsets also including Th1, Th9, Th17, and Th22 cells[34]. One example of chronic allergic inflammatory disorders is allergic asthma[51]. Allergic asthma is ranked among the type I hypersensitivity reactions[52]. The complexity and heterogeneity of this disease result in multiple phenotypes, employing different pathophysiological pathways[31]. The most common and best characterized type so far is allergic eosinophilic asthma, which is accompanied by type 2 inflammation[53,54]. Pro-inflammatory mediators, released by eosinophils, are major contributors to inflammation, including mucus hypersecretion, airway remodeling, airway disfunction, and airway epithelial cell damage[55].

1.4 Treatment options for allergic diseases

With allergen avoidance, pharmacotherapy, and allergen-specific immunotherapy (AIT), three major therapeutic options are available to manage and treat allergic disorders[56]. In order to prevent allergic reactions, avoidance of the causing allergen is a beneficial prophylaxis[57]. This strategy is practicable for some allergic diseases, e.g. food allergies, but is not applicable for inhaled allergens, like house dust mite (HDM), that cannot be completely avoided. The complexity and heterogeneity of allergic diseases require multiple treatment strategies to address patients' needs in the best possible manner[58,59].

1.4.1 Pharmacotherapeutic management of allergic diseases

Most allergic disorders are treated with a combination of symptom-relieving and control therapies[60]. In allergic asthma, glucocorticoids, which help to suppress the Th2-mediated inflammation, and β2-adrenoreceptor agonists, dilating bronchioles, are often used to manage the disease[58,61]. Glucocorticoids are among the most efficacious drugs used for the treatment of allergic diseases[62]. The genomic mechanism of glucocorticoids activates cytosolic glucocorticoid receptors, leading to activation or repression of protein synthesis[63]. Therapeutic effects are predominantly mediated by the repression of genes encoding inflammatory mediators[56]. Inhibition of other transcription factors may be responsible for side effects that often occur with long-term glucocorticoid treatments[56].

β2-adrenoreceptor agonists are used to cause smooth muscle relaxation, resulting in the dilation of bronchial passages[56]. Classified into short-, long-, and ultra-long-acting groups, they are used to treat asthma and other chronic pulmonary diseases, often in combination with corticosteroids[56,64].

In addition, therapies inhibiting or antagonizing released mediators (H_1-antihistamines, blocking monoclonal antibodies) are investigated and applied[60]. Histamine release and binding to H_1-receptors is linked to increased vascular permeability and smooth muscle contraction[65]. H_1-receptor antagonists are substances that competitively downregulate the activity of H_1-receptors[65]. Whereas they have strong control of the receptor activation, the release of histamine is not significantly affected by H_1-receptor antagonists, making anti-histamines less effective once

histamine has been released[56]. Some H_1-receptor antagonists of the second generation provide options to block the histamine receptor and also inhibit the degranulation of mast cells[56]. In allergic rhinitis, H_1- antihistamines are the most commonly used first-line medication with further applications in asthma, urticaria, and atopic dermatitis[65].

Advances in the understanding of immunological mechanisms also allow therapeutic targeting of specific molecules with a role in allergic diseases[59]. A wide range of therapeutic, mostly monoclonal, antibodies (mAbs) has been developed, targeting either cytokines and their receptors, or soluble and membrane-bound IgE[59]. Dupilumab, a mAb directed against the IL-4 receptor subunit α has proven to be efficacious in the treatment of asthma and atopic dermatitis by blocking IL-14 and IL-13 signaling[66,67]. Similar approaches are used to interfere with the eosinophilic response by targeting IL-5[68,69] or addressing the interactions between the epithelium and immune cells by targeting IL-25, IL-33, and TSLP[70-72].

IgE plays a key role in type I hypersensitivity reactions and therefore has been a prime target for disease intervention[59]. The mAb omalizumab, licensed for the treatment of allergic asthma and chronic spontaneous urticaria, binds soluble IgE and is thereby preventing its binding to IgE receptors on effector cells[73,74]. Another strategy is the interference with the production of IgE[59]. Antibodies are designed to target the membrane-bound form of IgE, thereby targeting B cells expressing an IgE B cell receptor[59].

The herewith described pharmacotherapeutic options represent only an insight into the variety of available approaches to manage symptoms of allergic diseases. Despite a large number of available therapeutic options, none of the mentioned treatment strategies is able to modify the underlying pathology long-term[60]. Additionally, the long-term use of pharmacological therapies can be accompanied by severe side effects, affecting major organ systems[75].

1.4.2 Allergen-specific immunotherapy

AIT has the goal to induce specific immune tolerance through multiple cellular and molecular mechanisms, by repetitive administration of increasing doses of the causative allergen, until a constant maintenance dose is reached and administered over a course of several years[76]. Clinical tolerance against allergens, resulting in ameliorated symptoms, can be induced, established, and

maintained by efficacious AIT[77]. Currently, AIT is the only available causative treatment for allergic diseases[78].

Successful AIT involves several mechanisms (***Figure 1***), including early desensitization of mast cells and basophils[76]. Very early decreases in the susceptibility to degranulation and decreases in anaphylaxis are observed in patients, even after the first injection of allergens in the course of AIT[76]. While Th2 cells play a central role during allergic inflammation, the induction of peripheral T cell tolerance during AIT is characterized by the generation of allergen-specific regulatory T cells (Tregs). This is thought to be an essential step in AIT and peripheral tolerance is initiated by IL-10 and TGF-β, both produced by allergen-specific Tregs during AIT[79,80]. The association of increasing numbers of $FoxP3^+CD25^+CD3^+$ cells in the nasal mucosa during AIT with clinical efficacy and the suppression of seasonal allergic inflammation supports a putative role of Tregs in the induction of allergen-specific tolerance[81]. Tregs are best known for their production of IL-10, IL-35, and TGF-β, as well as their expression of suppressor molecules, like cytotoxic T-lymphocyte associated protein 4 (CTLA-4) and programmed cell death 1 (PD-1)[77,82]. IL-10 produced by Tregs also affects mast cells, basophils, and eosinophils. Production of IL-5 by Th2 cells is suppressed, mast cells show a reduction in pro-inflammatory cytokine release, and eosinophilic function and activity are downregulated[83]. Furthermore, direct interactions between Tregs and mast cells inhibit FcεR1-dependent mast cell degranulation[84].

Whereas T cells become fully tolerant to AIT, B cells show a decisive skew from producing IgE to the production of IgG4[85]. A transient increase in serum-specific IgE is followed by a gradual decrease during the course of treatment[86]. IgG4 shows an early and rapid increase in response to AIT, with rising concentrations during AIT[86]. IL-10 produced by Tregs is potently suppressing allergen-specific IgE while increasing IgG4 production[87]. In the course of AIT, regulatory B cells (Bregs), a heterogeneous subset with immunosuppressive properties, are involved in the suppression of antigen-specific $CD4^+$ T cell proliferation, as well as in the production of IgG4 through their high capacity of IL-10 production[88]. Br1 cells, a subset of Bregs, selectively upregulate IgG4 during their transition to plasma cells[89]. During AIT, increases of allergen-specific IgG1 and IgG4 in the range of 10- to 100-fold are detectable[90]. IgG4 is able to compete with IgE for binding on Fcε-receptors of mast cells and basophils, acting as a blocking antibody, preventing activation and degranulation of these effector cells[91].

Effects of AIT are not only induced and maintained by cells of the adaptive immune system. DCs can act either as inflammatory or as tolerogenic[77]. The outcome is determined by the environmental conditions, regardless of the antigen-uptake[77]. Inflammatory conditions lead to increased expression of MHC-II, costimulatory molecules and inflammatory cytokines, and consequently to increased APC-capacity[92]. Subsequently, the most relevant adaptive T cell response towards a particular pathogen type is initiated[92]. Tolerogenic DCs are induced during antigen uptake in the absence of inflammation, in the presence of suppressive cytokines like IL-10 and TGF-β, as well as TLR2-, TLR-7, and TLR9-triggering molecules[77,93]. The production of IL-10, TGF-β, and immunoglobulin-like transcripts-2, -3, and -4 are essential for the establishment and maintenance of immune tolerance[77,93]. Similar to DCs, ILCs and macrophages are also able to act pro- and anti-inflammatory. M2 macrophages, activated by Th2-type cytokines have been reported to exert both allergic and anti-allergic properties[94,95]. Also, regulatory ILCs have been attributed with the capacity to limit inflammation through the secretion of IL-10 and TGF-β1[96]. Together with a regulatory subset of natural killer cells, able to produce IL-10, those populations have to be addressed in further investigations to evaluate their role in tolerance induction during the course of AIT[77,97].

AIT has been successfully applied for decades, but long treatment durations, high costs, and the risk of severe adverse reactions during treatment stand as major limiting factors[77]. Heterogeneous patient-responses to AIT protocols reveal the need for biomarkers to identify the most beneficial treatment approach for each patient[98]. The combination of AIT with other strategies, like biological immune response modifiers, could also provide an option for addressing the unmet clinical needs in allergic diseases[76]. Combined strategies could be providing synergistic effects or reduce side effects of individual therapies[76]. Further treatment options can bring higher flexibility and personalized treatment solutions to address the heterogeneous types of allergic diseases[76]. The possible advantages are also accompanied by the challenge of identifying potential targets which can be addressed during AIT[76].

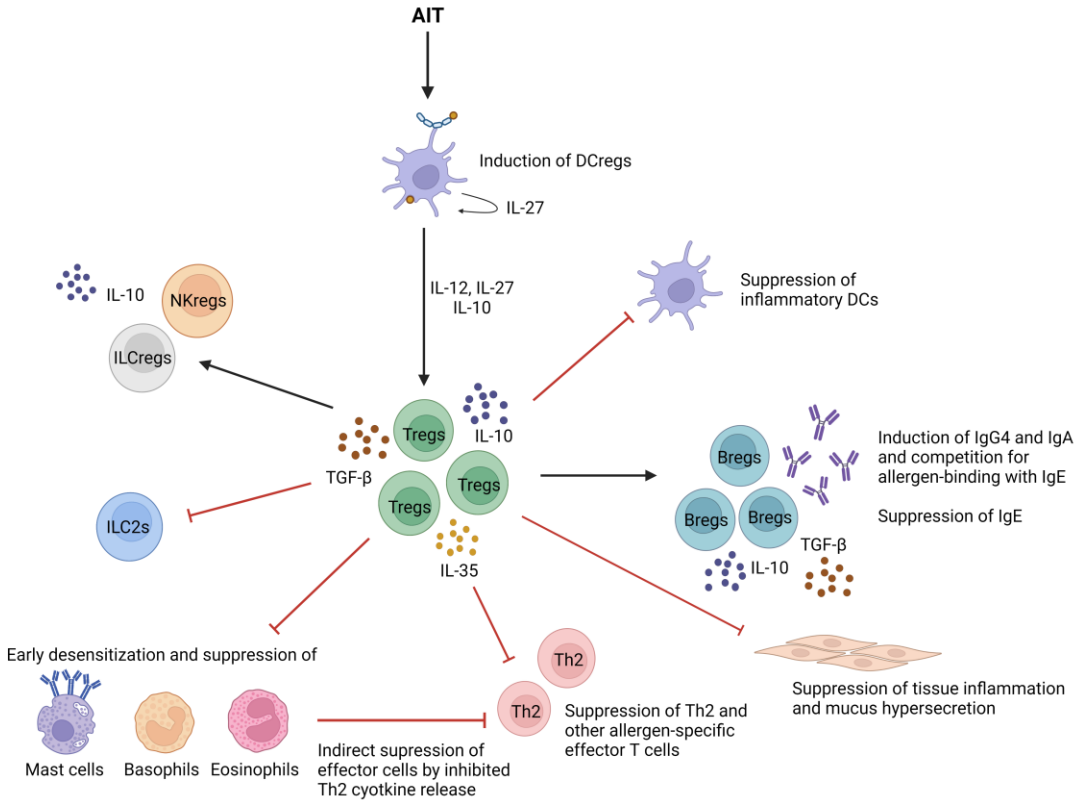

Figure 1: Mechanisms of AIT. The figure is adapted from Akdis (2012)[76], Shamji and Durham[99] (2017) and Kucuksezer et al. (2020)[77].

1.5 The aryl hydrocarbon receptor

The aryl hydrocarbon receptor is a ligand-dependent transcription factor and belongs to the family of basic helix-loop-helix (bHLH) PER-ARNT-SIM (PAS) domain transcription factors[100]. bHLH-PAS proteins are highly conserved and, as transcriptional regulators, play an important role in the regulation of numerous developmental and physiological processes[101]. They are involved in sensing environmental signals, changes in the circadian rhythm, oxygen tension, or redox potential, among others[102]. The AhR is the only known bHLH-PAS protein that is activated by small ligands, provided by diet, microorganisms, metabolism, and pollutants[103]. Being involved in the toxin metabolism, the AhR binds highly toxic ligands, like 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD)[104]. Studies of the AhR in context with TCDD, together with the description of

medical symptoms after TCDD exposure, contributed strongly to reveal the influence of the AhR on the immune system[100]. The AhR is highly expressed in multiple organs and tissues, including a number of different immune cells[103,105]. Important roles of the AhR have been revealed in the regulation of innate and adaptive immunity[106,107]. Th cell subsets show varying expression of the AhR, with Th17 cells and Tregs having the highest expression[100,108]. The AhR is also expressed in DCs, intestinal epithelial cells, macrophages, and ILCs, amongst others[109,110].

1.5.1 AhR signaling

The activity of the AhR is tightly controlled on several levels[100]. In the absence of a respective ligand, the AhR is retained in an inactive complex, located in the cytoplasm (***Figure 2***)[100]. This complex consists of the AhR associating with a dimer of the heat shock protein 90 (HSP90)[111], the AhR-interacting protein (AIP)[112], the cochaperone p23[113], and the c-SRC protein kinase[114]. The function of HSP90 is to stabilize the AhR in a conformation of high affinity for ligand binding[115], whereas AIP maintains steady state cellular levels of the AhR and prevents ubiquitination and degradation[116]. AIP is released after ligand binding and conformational changes lead to the translocation of AhR to the nucleus[117]. In course of the conformational changes, a protein kinase C target site is exposed. Phosphorylation of this site interferes with the nuclear translocation of the AhR and represents a control mechanism of the AhR[118]. The association of AhR with the AhR nuclear translocator (ARNT) in the nucleus, results in transcriptional control of multiple target genes, including several xenobiotic metabolizing enzymes, such as cytochrome P450 family-1 subfamily-A polypeptide-1 (CYP1A1) and cytochrome P450 family-1 subfamily-A polypeptide-2 (CYP1A2)[103,119]. Several mechanisms are described how the AhR exerts transcriptional control of its target genes. The canonical pathway with direct regulation of the target genes by the AhR is the most studied among them. Upon activation, the AhR binds genomic regions of the target genes, which contain the AhR binding deoxyribonucleic acid (DNA) consensus motif, named dioxin response element (DRE)[120]. Besides the recognition of DREs, the AhR/ARNT complex is also able to interact with other transcriptional regulators[121]. Furthermore, cross-talk between the AhR and other signaling mechanisms can result in the activation of non-canonical pathways[122]. The

AhR is not only able to influence chromatin remodeling[123,124] but also interacts with epigenetic regulators, resulting in genome-wide effects[110].

Interaction of the AhR with other transcription factors allows the recruitment of the AhR to DNA target sequences different from canonical DREs[103]. Murray *et al*. could show that different ligands induce the association of the AhR with different proteins[125], which could lead to AhR recruitment to different target DNA sequences and therefore trigger ligand-specific biologic responses[103]. The AhR is also able to exert regulation of gene expression through non-genomic signaling, but similarly to ligand-independent AhR activation, the relevance of these pathways is not fully evaluated yet[103].

There are not only activating but also inhibitory mechanisms that target the AhR signaling at several levels[103]. Given that the AhR is a ligand-dependent transcription factor, ligand availability and also the ligand itself influence AhR activation, because different ligands may activate the AhR differently[121]. The induction and expression of the drug-metabolizing CYP enzymes, especially CYP1A1, mediates an autoregulatory feedback loop and ligands are efficiently metabolized[126]. If ligands cannot be degraded through metabolizing enzymes, prolonged activation of the AhR is observed and different regulatory mechanisms are required[100]. In the cytoplasm, after being exported out of the nucleus, the activated AhR is degraded by the proteasome[127]. Degradation is mainly facilitated by the covalent binding of ubiquitin[127]. Another important regulatory mechanism in the genomic pathway of AhR signaling is mediated by the AhR repressor (AhRR)[128]. The AhRR protein is similar to the AhR, but due to its missing PAS B domain ligand binding is not possible[128]. A transrepression domain of the AhRR allows the binding of corepressors. AhRR acts in a negative regulatory loop and is able to suppress AhR activity by binding to ARNT and competing with AhR/ARNT for binding DRE sequences[129]. Most likely competition for DRE binding is not the only mechanism involved in the repression of AhR signaling by AhRR. These potential mechanisms are discussed extensively in respective reviews[130,131].

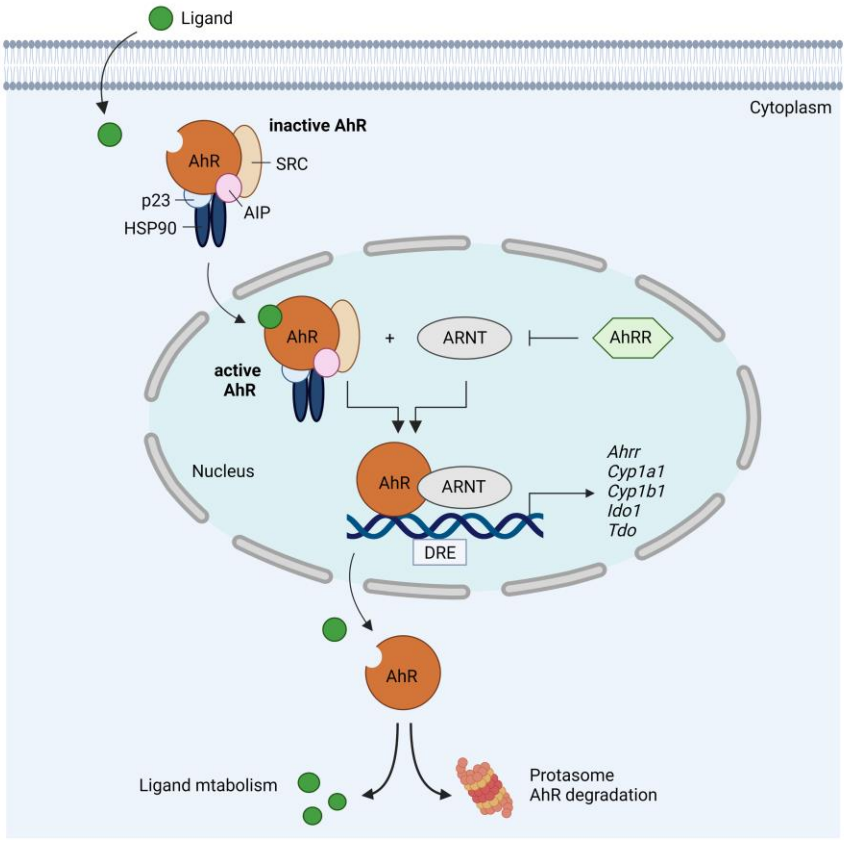

Figure 2: The canonical signaling pathway of the AhR. The figure is adapted from Rothhammer and Quintana (2019)[110].

1.5.2 AhR ligands

Initially, the AhR was recognized as a mediator of toxic effects caused by dioxins, but there is also a large variety of physiological ligands provided by the commensal flora, the diet, and the host metabolism[103]. As a ligand-activated transcription factor, ligand availability effectively controls AhR-dependent signaling[103]. In general, AhR ligands can be divided into two types, exogenous ligands from diet, diesel exhaust, and commercial products of industrial contaminations and endogenous ligands[132].

The majority of endogenous ligands are pro-ligands, which are transformed into ligands before being able to bind and activate the AhR[133]. A number of endogenous ligands is derived from the metabolism of tryptophan, an essential amino acid and precursor of many vital components[100]. The major pathway of tryptophan degradation proceeds via indoleamine 2,3-dioxygenase (IDO) and

tryptophan 2,3-dioxygenase (TDO) and generates the AhR agonist kynurenine[134]. One of the most potent AhR ligands is the tryptophan derivate 6-Formylindolo[3,2-b]carbazole (FICZ), which can activate the AhR at picomolar concentrations and has a comparable binding affinity to TCDD[100,133].

Indoles are another important group of AhR ligands[100]. They can either be generated endogenously by bacterial metabolism of tryptophan or taken up from exogenous sources by dietary intake[100]. Among dietary sources of AhR ligands are cruciferous vegetables, which are rich in glucosinolates[135,136]. Enzymatic degradation turns this compound into indole-3-carbinole (I3C), which is further converted to the high-affinity AhR ligands indolo-[3,2-b]-carbazole (ICZ) and 3,3-diindolylmethane[137]. Flavonoids, including quercetin and resveratrol, represent another group of AhR-activating molecules taken up by diet[138,139].

The most famous and best characterized exogenous high-affinity ligands are polycyclic aromatic hydrocarbons, polychlorinated biphenyls, and halogenated dioxins, including TCDD[140]. Besides having a strong affinity for the AhR, most of these pollutants cause toxic effects[141]. Acute exposure to dioxins causes skin damage, whereas long-term exposure results in developmental and reproductive abnormalities, immunotoxicity, or thyroid disruption[141]. Besides the above mentioned, a number of chemicals with striking structural diversity in comparison to each other and the structure of TCDD, is able to induce the AhR[140]. The majority of these chemicals, including benzimidazole derivates and synthetic flavonoids, act as relatively weak to moderate AhR ligands[140]. Exceptions are synthetic ligands like 10-Chloro-7H-benzimidazo[2,1-a]benzo[de]iso-quinolin-7-one (10-Cl-BBQ), discovered in a small-molecule screening by Punji *et al.*[142]. The non-toxic compound is able to induce AhR activation at nanomolar levels to a similar extent as TCDD, while being rapidly metabolized[143].

1.5.3 The AhR as a potential therapeutic target

Although the AhR is historically known for its function as an environmental sensor, attention has shifted towards elucidating the role of the AhR in immune functions[144]. AhR ligands induce a variety of cellular and epigenetic mechanisms to attenuate inflammation, which makes the AhR an interesting potential therapeutic target[144]. Mechanisms through which the AhR is able to

attenuate inflammation include thymic atrophy[145], apoptosis[146], induction of Tregs[147], induction of myeloid-derived suppressor cells[148], cytokine suppression[149], and epigenetic changes[150].

The effects observed upon AhR activation are influenced by different factors and the immunological outcome is determined by the characteristics of the ligand, the cell type that expresses AhR, and present coactivators[151]. In tumor biology, the AhR is overexpressed in different tumor types and can be a negative or positive prognostic factor[152]. Selective AhR modulators with tumor-specific AhR agonistic or antagonistic activity will be required to individually address the either pro-oncogenic or tumor suppressor-like function of the AhR, depending on the tumor type[152]. Activation of the AhR has also shown promising results in the prevention and treatment of inflammatory diseases. AhR activation by I3C was able to prevent colitis and associated dysbiosis in a murine model[153] and experimental multiple sclerosis could be suppressed by attenuating inflammation after ligand-dependent AhR activation[154]. For the inflammatory skin diseases atopic dermatitis and psoriasis a topical AhR agonist is already on the market[144]. Tapinarof has been shown to restore skin homeostasis by decreasing the production of pro-inflammatory cytokines, the reduction of oxidative stress, and an increased expression of epithelial barrier genes[155]. The discovery that the AhR is a crucial regulator of lung immune homeostasis also suggests an important role of the AhR in lung inflammation[132]. Decreased airway inflammation by regulation of Th2 cytokine production in rodent models of asthma has been reported after AhR activation[132]. On the other hand, there are reports of increased lung inflammation after AhR activation by TCDD[156]. These contrasting findings highlight the complexity of AhR biology in lung diseases[132]. AhR activation may potentiate or attenuate lung inflammation, depending on the nature of the ligands, the experimental conditions, and the disease model[132,157,158]. Whereas several studies already addressed the function of the AhR in allergic asthma, its potential role and suitability as therapeutic target in AIT has not been investigated yet.

2. Aims of the study

Further and deepened understanding of the mechanisms underlying AIT is essential to improve therapeutic options and provide help to patients who are not responding to current treatment protocols. Besides detailed mechanistic knowledge, the approach of combining AIT with further treatment options has come to the focus of attention. This study addresses the role of the AhR and its suitability as a target in AIT. Given the strong involvement of the AhR in immunological processes and its role in inflammatory diseases, the first hypothesis of this work is that a functional AhR plays an essential role in the course of AIT and may be a requirement for a successful therapeutic outcome. The second hypothesis to be tested states that a targeted activation of the AhR by specific ligands can be beneficial for the therapeutic outcome of AIT. The following study addresses those hypotheses from two angles.

The first part of this study investigates if a functional AhR is a requirement to perform successful AIT, testing the first stated hypothesis. For that, the outcome of AIT in $AhR^{-/-}$ mice is analyzed in murine models of OVA- and HDM-induced allergic asthma. Effects of AIT on parameters of allergic inflammation are compared between $AhR^{-/-}$ and wildtype mice in the OVA-model. With the AhR as a requirement for successful AIT, reduced efficiency of AIT in $AhR^{-/-}$ mice is expected.

In the second part, the AhR is targeted with the AhR agonists quercetin and 10-Cl-BBQ during the course of AIT to test the second stated hypothesis. The aim is to investigate if a combined approach of AIT and AhR ligand treatment is able to impact the outcome of AIT, ideally resulting in improved therapeutic efficiency. Anti-inflammatory responses and tolerance induction after AhR activation have been reported in literature, suggesting that AhR activation during AIT may be beneficial on the therapeutic outcome of AIT.

3. Material

3.1 Buffers and media

10x phase blotting buffer

480 mM Tris, 390 mM glycin, 0.37 % SDS, ddH_2O

10x SDS running buffer (pH 8.8)

250 mM Tris, 2 M glycin, 1 % SDS, ddH_2O

1x ACK lysis buffer (pH 7.2 – 7.4)

155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA-2Na-$2H_2O$, ddH_2O

4x stacking gel buffer

125 mM Tris/HCl, ddH_2O

5x separation gel buffer

375 mM Tris/HCl, ddH_2O

6x Laemmli buffer

1 M Tris, 3.5 M 2-Mercaptoethanol, 10 % SDS, 30 % glycerol, 0.25 % bromphenol blue, ddH_2O

CO-IP complete buffer

150 mM NaCl, 25 mM HEPES (pH 7.5), 0,2 % (w/v) NP-40, 1 mM gylcerol, 1 mM DTT, cOmplete protease inhibitors, 10 mM NaF, 8 mM β-glycerophosphate, 300 µM sodium vanadate, ddH_2O

Complete medium

RPMI 1640, 1 % L-glutamine, 1 % penicillin-streptomycin, 1 % sodium pyruvate, 1 % MEM non-essential amino acids, 50 µM 2-mercaptoethanol

Digestion medium

RPMI 1640, 1 mg/mL Collagenase A, 100 µg/mL DNase I, 2 % FCS

Ear clip lysis buffer

10 mM Tris/HCl (pH 8.0), 50 mM KCl, 0,5 % NP-40, 0,5 % Tween®80, ddH_2O

ELISA blocking buffer

50 mM Tris, 1 % BSA, ddH_2O

ELISA coating buffer

100 mM $NaHCO_3$, 35 mM Na_2CO_3, ddH_2O

ELISA stop solution

0.16 M H_2SO_4, ddH_2O

ELISA wash buffer (pH 7.4)

50 mM Tris, 0.1 % Tween®20, ddH_2O

FACS buffer

DPBS, 2 % FCS

ELISA wash buffer (pH 7.4)

50 mM Tris, 0.1 % Tween®20, ddH_2O

PBS-T

0.1 % Tween®20 in PBS

TAE buffer

40 mM Tris (pH 7.6), 20 mM $C_2H_4O_2$, 1 mM EDTA, ddH_2O

3.2 Chemicals

Table 1: Chemicals used for buffer preparation, histology and *in vivo* experiments.

Name	Manufacturer
10-Cl-BBQ	Tocris
2-Mercaptoethanol	Sigma-Aldrich
2-Propanol	VWR Chemicals
Acetic acid ($C_2H_4O_2$)	Carl Roth
Ammonium chloride (NH_4Cl)	Sigma-Aldrich
Ammonium persulfate (APS)	PanReac AppliChem
Anisole, anhydrous, 99.7%	Sigma-Aldrich
Anisole, anhydrous, 99.7%	Sigma-Aldrich
Bromphenol blue	PanReac AppliChem
Dimethyl sulfoxide (DMSO)	Thermo Fisher Scientific
Dithiothreitol (DTT)	Sigma-Aldrich
EDTA Disodium Salt 2-hydrate (EDTA-2Na-2H_2O)	PanReac AppliChem
Ethanol absolute Molecular biology grade	PanReac AppliChem
Glycerol	PanReac AppliChem
Glycine	PanReac AppliChem
HEPES	Gibco
Methanol	Merck Millipore
Nonident™ P-40 (NP-40)	PanReac AppliChem
Paraformaldehyde (PFA)	Merck Millipore
Potassium bicarbonate ($KHCO_3$)	Carl Roth
Potassium chloride (KCl)	Merck Millipore
Quercetin	Sigma-Aldrich
ROTIPHORESE®NF-Acrylamid/Bis-Lösung 30	Carl Roth

Sodium (ortho)vanadate	Sigma-Aldrich
Sodium carbonate (Na_2CO_3)	Sigma-Aldrich
Sodium chloride (NaCl)	PanReac AppliChem
Sodium dodecyl sulfate (SDS)	Merck Millipore
Sodium fluoride (NaF)	Sigma-Aldrich
Sodium hydrogen carbonate ($NaHCO_3$)	Merck Millipore
Sulfuric acid (H_2SO_4)	Merck Millipore
TEMED	PanReac AppliChem
TRIS for buffer solutions	PanReac AppliChem
TRIS Hydrochlorid (Tris HCl)	Carl Roth
Tween®20	Bernd Kraft
Tween®80	Sigma-Aldrich
Xylene	AppliChem
β-glycerophosphate disodium salt hydrate	Sigma-Aldrich

3.3 Material for animal experiments

Table 2: Reagents and equipment for *in vivo* experiments.

Name	**Manufacturer**
Albumin from chicken egg white, grade V	Sigma-Aldrich
Der f extract	Allergy Therapeutics
Der p extract	Allergy Therapeutics
Imject™ Alum	Sigma-Aldrich
Ketamine	WDT
Pariboy® inhalation device	PARI
PBS, pH 7.4	Gibco
Peanut oil	Sigma-Aldrich
Sevoflurane	Baxter
Sodium chloride solution, 0.9%	Braun
Xylacine	aniMedica

3.4 Material for molecular biology and protein chemistry

Table 3: Reagents and equipment for molecular biological analysis

Name	Manufacturer
Agarose SERVA Wide Range	SERVA
DNA AWAY	Molecular BioProducts™
EconoTaq PLUS GREEN 2X Master Mix	Lucigen
GeneRuler 50 bp DNA Ladder	Thermo Fisher Scientific
LightCycler® 480 SYBR Green I Master	Roche
Nuclease-Free Water	Qiagen
Proteinase K from *Tritirachium album*	SERVA
Quick-RNA Miniprep Plus Kit	Zymo Research
RAase AWAY	Molecular BioProducts™
RevertAid H Minus First Strand cDNA Synthesis Kit	Thermo Fisher Scientific
SERVA DNA Stain Clear G	SERVA

Table 4: Primers for genotyping of $AhR^{-/-}$ mice.

Name	Sequence (5' to 3')	Manufacturer
oIMR443	GGATTTGACTTAATTCCTTCAGCGG	Metabion International
oIMR444	TCTTGGGCTCGATCTTGTGTCAGGAACAGG	Metabion International
oIMR 8162	TGGATGTGGAATGTGTGCGAG	Metabion International

Table 5: Primers for gene expression analysis.

Name	Sequence (5' to 3')	Manufacturer
Aktinβ_F	TTCTTTGCAGCTCCTTCGTT	Metabion International
Aktinβ_R	ATGGAGGGGAATACAGCCC	Metabion International
CYP1A1_F	GACACAGTGATTGGCAGAG	Metabion International

CYP1A1_R	GAAGGTCTCCAGAATGAAGG	Metabion International
GAPDH_F	CGTCCCGTAGACAAAATGGT	Metabion International
GAPDH_R	TTGATGGCAACAATCTCCAC	Metabion International
GPx_F	CCACCGTGTATGCCTTCTCC	Metabion International
GPx_R	GATCGTGGTGCCTCAGAGAG	Metabion International
SOD1_F	CCAGTGCAGGACCTCATTTT	Metabion International
SOD1_R	CACCTTTGCCCAAGTCATCT	Metabion International
SOD2_F	GGCCAAGGGAGATGTTACAA	Metabion International
SOD2_R	GAACCTTGGACTCCCACA	Metabion International

Table 6: Reagents and equipment for western blot analysis.

Name	**Manufacturer**
20X LumiGLO® Reagent and 20X Peroxide	Cell Signaling Technology
Bovine serum albumin	Sigma-Aldrich
cOmplete™, EDTA-free Protease Inhibitor Cocktail	Roche
Cytiva Amersham™ Hyperfilm™ ECL	Cytiva
Immobilon-P Membran, PVDF, 0,45 µm	Merck Millipore
Non-fat dried milk powder	PanReac AppliChem
PageRuler™ Prestained Protein Ladder, 10 to 180 kDa	ThermoFisher Scientific
Whatman® paper	Cytiva

Table 7: Antibodies for western blot analysis.

Name	**Manufacturer**
4E-BP1 (53H11) Rabbit mAb #9644	Cell Signaling Technology
Akt Antibody #9272	Cell Signaling Technology
HRP-conjugated anti-mouse antibody, #715-035-150	Jackson ImmunoResearch
HRP-conjugated anti-rabbit antibody, #711-035-152	Jackson ImmunoResearch
Phospho-4E-BP1 (Thr37/46) (236B4) Rabbit mAb #2855	Cell Signaling Technology
Phospho-Akt (Ser473) Antibody #9271	Cell Signaling Technology
β-Actin (8H10D10) Mouse mAb #3700	Cell Signaling Technology

3.5 Material for cell culture assays and cell isolation

Table 8: Reagents and equipment for cell culture assays and cell isolation from organs.

Name	Manufacturer
Collagenase from Clostridium histolyticum, Type IA	Sigma-Aldrich
Deoxyribonuclease I from bovine pancreas	Sigma-Aldrich
DPBS, w/o calcium and magnesium	Gibco
EasyEights™ EasySep™ Magnet	STEMCELL Technologies
EasySep™ Mouse CD4+ T Cell Isolation Kit	STEMCELL Technologies
Fetal calf serum (FCS)	
L-Glutamine (200 mM)	Gibco
MEM Non-Essential Amino Acids Solution (100X)	Gibco
PBS (10X), pH 7.4	Gibco
Penicillin-Streptomycin (5,000 U/mL)	Gibco
Percoll®	Cytiva
Purified NA/LE Hamster Anti-Mouse CD28, Clone 37.51	BD Biosciences
Purified NA/LE Hamster Anti-Mouse CD3e, Clone 145-2C11	BD Biosciences
RPMI 1640 Medium	Gibco
Sodium Pyruvate (100 mM)	Gibco
UltraPure™ 0.5M EDTA, pH 8.0	Invitrogen

3.6 Antibodies and material for flow cytometry

Table 9: Antibodies for flow cytometry.

Name	Clone	Manufacturer
Alexa Fluor® 488 anti-mouse CD8a Antibody	53-6.7	BioLegend
Alexa Fluor® 700 anti-mouse CD3 Antibody	17A2	BioLegend
Alexa Fluor® 700 anti-mouse I-A/I-E Antibody	M5/114.15.2	BioLegend
Alexa Fluor® 700 Rat Anti-Mouse CD4	RM4-5	BD Biosciences
APC anti-mouse CD64 (FcγRI) Antibody	X54-5/7.1	BioLegend

Biotin Rat Anti-Mouse CD25	7D4	BD Biosciences
Brilliant Violet 421™ anti-mouse IL-33Rα (IL1RL1, ST2) Antibody	DIH9	BioLegend
Brilliant Violet 650™ anti-mouse CD4 Antibody	RM4-5	BioLegend
Brilliant Violet 650™ anti-mouse/rat XCR1 Antibody	ZET	BioLegend
Brilliant Violet 711™ anti-mouse/human CD11b Antibody	M1/70	BioLegend
Brilliant Violet 785™ anti-mouse CD11c Antibody	N418	BioLegend
BV786 Mouse Anti-Mouse RORγt	Q31-378	BD Biosciences
CD11b Monoclonal Antibody, APC-eFluor™ 780	M1/70	eBioscience
CD45 Monoclonal Antibody, APC-eFluor™ 780	30-F11	eBioscience
F4/80 Monoclonal Antibody, APC-eFluor™ 780	BM8	BioLegend
FITC anti-mouse Ly-6C Antibody	HK1.4	BioLegend
FOXP3 Monoclonal Antibody, PerCP-Cyanine5.5	FJK-16s	eBioscience
Gata-3 Monoclonal Antibody, eFluor™ 660	TWAJ	eBioscience
NK1.1 Monoclonal Antibody, APC	PK136	BioLegend
Pacific Blue™ anti-mouse CD45.2 Antibody	104	BioLegend
Pacific Blue™ anti-mouse Ly-6G Antibody	1A8	BioLegend
PE Rat Anti-Mouse Siglec-F	E50-2440	BD Biosciences
PE/Cyanine7 anti-mouse CD11c Antibody	N418	BioLegend
PE/Cyanine7 anti-mouse Ly-6G Antibody	1A8	BioLegend
PE/Dazzle™ 594 anti-mouse CD206 (MMR) Antibody	C068C2	BioLegend
PerCP/Cyanine5.5 anti-mouse CD45 Antibody	30-F11	BioLegend
ROR gamma (t) Monoclonal Antibody, PE	AFKJS-9	eBioscience

Table 10: Reagents for flow cytometry.

Name	**Manufacturer**
7-Aminoactinomycin D	Enzo Life Sciences
BD FACSFlow™	BD BioSciences
Foxp3/Transcription Factor Staining Buffer Set	eBioscience

PE/Cyanine7 Streptavidin	BioLegend
Zombie Aqua™ Fixable Viability Kit	BioLegend

3.7 Material for cytokine and immunoglobulin analysis

Table 11: Kits and reagents for cytokine and immunoglobulin assays.

Name	Manufacturer
1-Step™ Ultra TMB-ELISA Substrate Solution	ThermoFisher Scientific
Anti-Chicken Egg Albumin (Ovalbumin) antibody, Mouse monoclonal, clone OVA-14	Sigma Aldrich
Biotin Mouse Anti-Mouse IgG1[a], Clone 10.9	BD Biosciences
LEGEND MAX™ Mouse OVA Specific IgE ELISA Kit	BioLegend
LEGENDplex™ Mouse Immunoglobulin Isotyping Panel (6-plex)	BioLegend
LEGENDplex™ MU Th Cytokine Panel (12-plex)	BioLegend
Ms IgE OptEIA ELISA Set	BD Biosciences
Streptavidin, Peroxidase Conjugate	Calbiochem®

3.8 Material for histology

Table 12: Reagents and equipment for histological analysis.

Name	Manufacturer
Cover slips	Thermo Fisher Scientific
Entellan®	Merck Millipore
Eosin Y, alcoholic	Labor + Technik
Hematoxylin	Labor + Technik
low profile microtome blades	Leica Biosystems
Microscope slides	Carl Roth
Paraffin	Thermo Fisher Scientific
PAS staining kit	Merck Millipore

3.9 Consumables

Table 13: Plastic ware and consumables.

Name	Manufacturer
6-well plates	Greiner Bio-One
96-well plates, Nunc MaxiSorp™ flat-bottom	Thermo Fisher Scientific
96-well plates, U-bottom	Greiner Bio-One
96-well plates, V-bottom	Greiner Bio-One
EASYstrainer™ cells strainer, 70 µm	Greiner bio-one
FACS tubes 1.3 mL	Greiner Bio-One
FACS tubes 5mL	Fisher Scientific
LightCycler® 480 Multiwell Plate 384, white	Roche
LightCycler® 480 Sealing Foil	Roche
Microvette 500 Z-Gel	Sarstedt
Nunc™ MicroWell™ 96-Well, Nunclon Delta-Treated, Flat-Bottom Microplate	Thermo Fisher Scientific
Omnifix® Luer, 10 ml	B. Braun
Omnifix®-F Luer Solo	B. Braun
Pasteur pipettes, glass, 230 mm	Carl Roth
PCR tubes	Starlab
Pipette tips 10 µl, 200 µl, 300 µl, 1000 µl	Sarstedt
Reaction tubes 1.5 mL, 2 mL	Sarstedt
Reaction tubes 5 mL	Eppendorf
Serological pipettes 2 mL, 50 mL	Greiner Bio-One
Serological pipettes 5 mL, 10 mL, 25 mL	Sarstedt
Sterican®, Single-use hypodermic needles, G18	B. Braun
Sterican®, Single-use hypodermic needles, G26	B. Braun
Sterile syringe filter 0.22 µM	Merck Millipore
Tube 15 mL, 50 mL	Sarstedt

3.10 Instruments

Table 14: Instruments.

Name	Manufacturer
2100 Bioanalyzer Instrument	Agilent
Accuri™ C6	BD Biosciences
BioVanguard Green Line	Telstar Life Science
Centrifuge 5424 R	Eppendorf
Centrifuge 5810 R	Eppendorf
Chromium Controller	10x Genomics
Consort EV20 power supply	SCIE-PLAS
Eclipse TS100	Nikon
EG 1150 C, embedding machine	Leica Biosystems
FACS LSR II Fortessa	BD Biosciences
Gel iX Imager	Intas Science Imaging
Infinite®200 PRO	Tecan
Innova®42 Incubator Shaker	New Brunswick Scientific
LightCycler® 480 System	Roche
Mastercycler™ Nexus Gradient	Eppendorf
MilliQ1, EASYpure UV	Merck Millipore
NanoDrop™ 2000	Thermo Fisher Scientific
NovaSeq 6000 sequencing system	Illumina
Olympus BX41	Olympus
Olympus Color View III	Olympus
PB303 DeltaRange	Mettler Toledo
Peqpower, power supply	Peqlab
PerfectBlue™ gel system	Peqlab
Pipettes	Eppendorf
Pipettor	Brand
ROCKER 2D basic	IKA
Rotary Microtome HM 340E	Thermo Fisher Scientific

Scaltec SBC 31	Mettler Toledo
ThermoMixer®C	Eppendorf
TissueLyser LT	Qiagen
TP1020, tissue processing system	Leica Biosystems
TV400YK-2D-IEF-SYS, 2-D electrophoresis system	SCIE-PLAS
V20-SDB semi-dry blotter	SCIE-PLAS
Vortex genie	Scientific Industries

3.11 Software

Table 15: Software.

Name	**Manufacturer**
BD FACS Diva	BD Biosciences
BioRender	BioRender
DropletUtils	[159]
Excel 2016	Microsoft
FlowJo V10	Tree Star
GraphPad Prism 8	GraphPad Software
i-control	Tecan
LegendPlex Analysis Software	BioLegend
LightCycler® 480 Software, Version 1.5	Roche
Olympus Cell B image acquisition software	Olympus
R 4.1.1	R Core Team
RNAStarSolo	[160]
RStudio v2022.02.3+492	RStudio PBC

4. Methods

4.1 Animal procedures

4.1.1 Animals

For experiments conducted with wildtype animals, female C57BL6/J mice between the age of 5-6 weeks were purchased from Charles River (Sulzfeld, Germany) and housed under specific pathogen-free conditions with food and water *ad libitum*. $AhR^{-/-}$ mice were bred at the Helmholtz animal facility and housed under the same specific pathogen-free conditions and provided with food and water *ad libitum*. All experiments and breedings were approved by the government of the district of Upper Bavaria (ethical approvals 55.2-2532.Vet_02-17-50, 55.2-2532.Vet_02-20-098, and 55.2-2532.Vet_02-17-222) and carried out under the federal guidelines for the use and care of laboratory animals.

4.1.2 Genotyping of $AhR^{-/-}$ mice

Mice were ear-punched for identification and the surplus tissue was collected and used for genotyping.

Genomic DNA was isolated by the addition of 50 µl ear clip lysis buffer per sample and incubation for 10 min at 96 °C. After incubation, samples were vortexed and rested to cool down to RT. 20 µg proteinase K in 50 µl lysis buffer were added per sample, following incubation at 56 °C for at least 5 h. Digested ear clips were vortexed and heat-inactivated for 15 min at 96 °C. After centrifugation at 10 000 rpm for 10 min, 1 µl of supernatant was used as a DNA template for genotyping with polymerase chain reaction (PCR). PCR was performed with EconoTaq® DNA polymerase and primers recommended by The Jackson Laboratory (***Table 4***). Volumes of reagents per reaction are described in ***Table 16***. PCR was run on a thermocycler and cycling conditions were adapted from the EconoTaq® protocol (***Table 17***). PCR products were stained with DNA stain Clear G and separated on a 2 % agarose gel by electrophoresis at 120 V for 50 min in Tris-

Acetat-EDTA (TAE) buffer. Bands were visualized with a UV light imaging system and size was determined by comparison to a GeneRuler 50 bp DNA Ladder.

Table 16: Volumes of reagents for genotyping of $AhR^{-/-}$ per reaction.

Reagent	Volume
EconoTaq® DNA polymerase	10µl
Nuclease free water	5µl
oIMR443 (10µM)	1µl
oIMR444 (10µM)	2µl
oIMR8162 (10µM)	1µl
DNA template	1µl
Total Volume per reaction	20µl

Table 17: Cycling conditions for genotyping of $AhR^{-/-}$ mice.

Step	Temperature	Duration	Cycles
Incubation	95°C	3min	1x
Denaturation	94°C	30s	
Annealing	62°C	40s	32x
Elongation	72°C	40s	
Final extension	72°C	5min	1x

4.1.3 Murine model of allergen-specific immunotherapy for ovalbumin-induced allergic asthma

Mice from the non-allergic groups received 2 mg aluminium hydroxide (alum) in 200 µl phosphate-buffered saline (PBS) via intraperitoneal injection (i.p.), whereas all other groups were sensitized with 30 µg ovalbumin grade V (OVA) and 2 mg alum (i.p.) on days 0, 7 and 21. Mice in the AIT groups were treated subcutaneously (s.c.) with 500 µg OVA in 200 µl PBS on days 36, 39, and 42. All other mice received 200 µl PBS (s.c.). All groups were challenged with OVA-aerosol (1 % OVA in PBS) on days 35, 38, and 41 during the AIT phase, as well as on days 57,

60, and 63 at the end of the experiment. OVA-aerosol challenges lasted 15 min each and were performed in an acrylic glass chamber. The 1 % OVA solution in PBS was nebulized using a Pariboy® inhalation device. All mice were sacrificed on day 64 by an overdose of an i.p. injected anesthetic (10 % ketamine/ 2 % xylazine in PBS).

Blood was collected on days -1, 33, 44, and 64 either by retrobulbar bleeding under sevoflurane anesthesia or via puncture of the tail vain. The method was chosen according to the underlying ethical approval for the respective experiment. Blood samples were centrifuged (10 000 rpm, 10 min, 4 °C) and serum was stored at -80 °C for further analysis. An overview of the experimental setup is provided in ***Figure 3***.

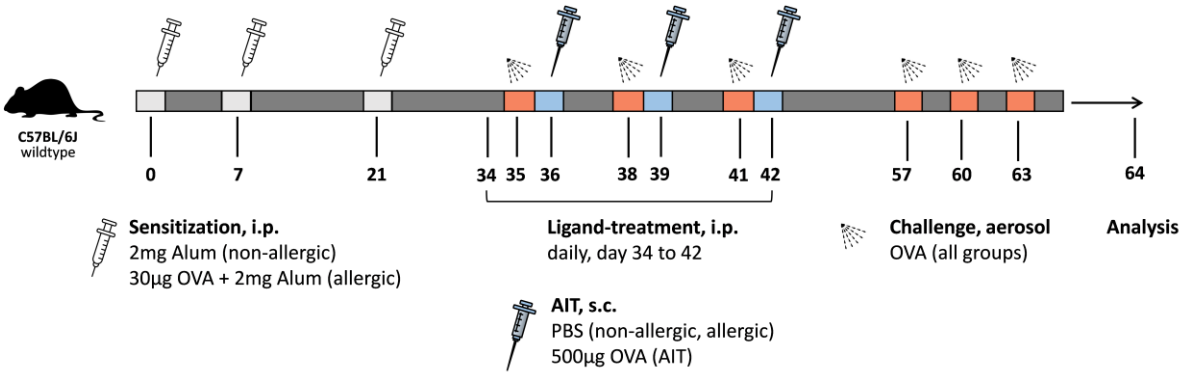

Figure 3: Experimental setup of AIT in a murine model of OVA-induced allergic asthma. Detailed descriptions regarding mouse lines, number of animals per group, and specific treatment conditions can be found in the corresponding results chapters.

4.1.4 Murine model of allergen-specific immunotherapy for house dust mite-induced allergic asthma

Mice from the non-allergic control groups received 20 µl PBS intranasally (i.n.) on days 1, 2, and 3. All other groups were sensitized with 1 µg HDM extract in 20 µl PBS (i.n.). All used HDM extracts consisted of equal parts of *Dermatophagoides pteronyssinus* (Der p) and *Dermatophagoides farinae* (Der f) extracts. Intranasal applications were always performed under sevoflurane anesthesia and 10 µl were applied per nostril. On days 8, 13 and 19 mice of the non-allergic groups received 20 µl PBS (i.n.), whereas mice of all other groups were challenged with 1 µg HDM extract in 20 µl PBS (i.n.). AIT was performed via s.c. injections of 220 µg HDM

extract on days 15, 18, and 22. Groups that did not undergo AIT were treated with 200 µl PBS (s.c.). Towards the end of the experiment, all mice were challenged with 10 µg HDM extract in 20 µl PBS (i.n.) on days 30, 31, 32, and 33. All mice were sacrificed on day 34 by an overdose of an i.p.-injected anesthetic (10 % ketamine/ 2 % xylazine in PBS).

Blood was collected on days 0, 14, 23, and 34, either by retrobulbar bleeding under sevoflurane anesthesia or via puncture of the tail vain. The method was chosen according to the underlying ethical approval for the respective experiment. Blood samples were centrifuged (10 000 rpm, 10 min, 4 °C) and serum was stored at -80 °C for further analysis. An overview of the experimental setup is provided in ***Figure 4***.

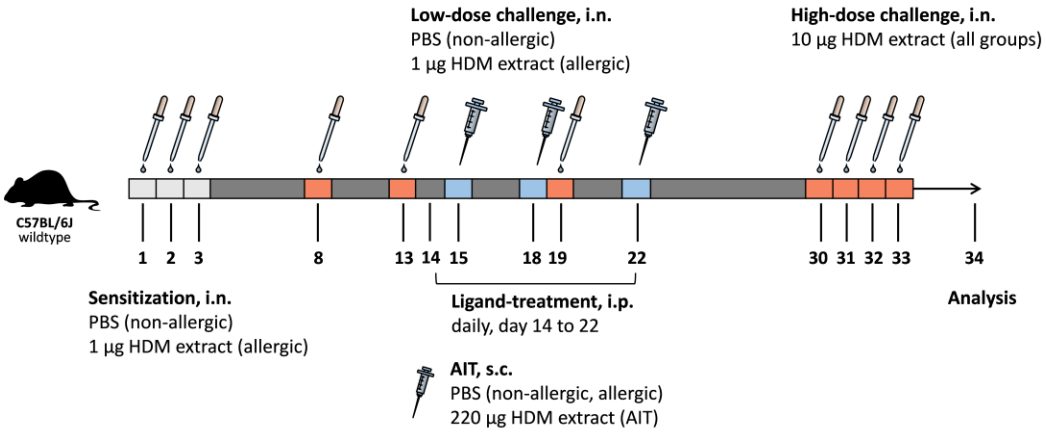

Figure 4: Experimental setup of AIT in a murine model of HDM-induced allergic asthma. Detailed descriptions regarding mouse lines, number of animals per group, and specific treatment conditions can be found in the corresponding results chapters.

4.1.5 Allergen extracts

For extract preparation, 7.5 g of raw material (>99% purity, GMP-grade) of freeze-dried Der p or Der f (bodies and faeces) were subjected to media extraction for 24 h, purified by centrifugation and clarification using a 0.2 µm syringe filter and analyzed for protein content with Bradford assay according to the manufacturer's instructions.

4.1.6 Treatment with ligands of the aryl hydrocarbon receptor during allergen-specific immunotherapy

Quercetin was dissolved in Dimethyl-sulfoxide (DMSO) and further diluted in 0.8 % Tween® 80 in PBS. Mice were injected with 8 mg/kg or 16 mg/kg quercetin (i.p.), respectively.

10-Cl-BBQ was dissolved in anisole and further diluted in peanut oil. Mice were injected with 10 mg/kg 10-Cl-BBQ (i.p.).

Ligands were injected daily between days 34 and 42 in the OVA-model (***Figure 3***) and between days 14 and 22 in the HDM-model (***Figure 4***). All groups without ligand treatment received the respective solvent (i.p.).

4.1.7 Bronchioalveolar Lavage

To perform bronchioalveolar lavage (BAL), the chest of euthanized mice was opened and the trachea was cannulated. The airways were lavaged five times with 1 mL PBS. The bronchioalveolar lavage fluid (BALF) was collected and used for further analysis. To determine the total number of cells in the collected BALF, a small fraction was stained with a fluorescence-labeled antibody against the surface marker CD45, to identify leucocytes, and the DNA-binding dye propidium iodide (PI), to exclude dead cells. Staining was performed for 5 min at 4 °C) and cells were analyzed and counted with a BD Accuri® C6 flow cytometer. To analyze the contained cells in more detail, the BALF was centrifuged (450 g, 5 min, 4 °C) and the supernatant transferred to a new tube. Cells were resuspended in buffer or medium for further flow cytometry analysis, whereas supernatant was stored at -80 °C and used for cytokine analysis at a later time point.

4.2 Cell biology methods

4.2.1 Isolation of leucocytes from lung tissue

Lungs were dissected and transferred to ice-cold RPMI in 6-well plates. After removal of the medium, lungs were chopped into small pieces (approximately 1 mm x mm) and 3 mL digestion medium were added per well. After incubation for 30 min at 37 °C and 90 rpm, digested lung pieces were resuspended with a 10 mL syringe and an 18G needle by drawing up and down. The cell suspension was transferred to a 70 µm cell strainer and mashed with a syringe piston into a 50 mL tube. The cell strainer was washed with 5 mL PBS with subsequent centrifugation (450 g, 5 min, 4 °C) and removal of supernatant. The cell pellet was resuspended in 5 mL 40 % percoll in RPMI (v/v) and transferred to a 15 mL tube. The suspension was underlayered with 5 mL 80 % percoll in RPMI (v/v) using a long glass Pasteur pipette. After centrifugation at 1600 g for 15 min at RT with acceleration and brakes set to 0, leucocytes were collected from the interphase and washed with 10 mL PBS (450 g, 5 min, 4 °C). Supernatant was removed and cells were resuspended in an appropriate medium for further analysis.

4.2.2 Enrichment of $CD4^+$ T cells from isolated lung leucocytes

$CD4^+$ T cells were enriched from pulmonary leucocytes using the EasySep™ Mouse $CD4^+$ T cell Isolation Kit and the EasyEights™ EasySep™ Magnet according to the manufacturer's instructions.

4.2.3 Flow cytometry analysis of isolated cells

All centrifugation steps were carried out at 450 g for 3 min at 4 °C. If not mentioned otherwise, staining was performed in 96-well U-bottom plates. For washing steps, cells were centrifuged and 150 µl of appropriate buffer was added per well, after removal of supernatant. After a second centrifugation step and removal of supernatant, cells were ready for the next step according to the applied protocol. All incubation steps were performed on ice and protected from light.

Depending on the cell suspension to analyze, cells were resuspended in Fc-block (anti-CD16/anti-CD32) to prevent unspecific bindings of immunoglobulins for 10 min. Live/dead staining of isolated cells was performed either with a fixable viability dye prior to surface staining or by adding a DNA-binding dye directly before flow cytometric analysis, depending on the applied staining panel. If a fixable viability dye was used, cells were washed once with PBS and stained for 10 min. Cells were washed twice with FACS buffer and incubated with antibodies against surface markers for 20 min on ice. If the applied protocol included only a surface staining, cells were washed twice with FACS buffer, resuspended in FACS buffer, and analyzed with BD LSRFortessa™ flow cytometer. If intracellular staining was performed, cells were washed twice with FACS buffer and subsequently fixed with the eBiosciene™ FoxP3/Transcription kit for 30 min. After fixation, cells were washed twice with fixation/permeabilization buffer and intracellular markers were stained for 1 h. Cells were washed once with fixation/permeabilization buffer and FACS buffer, resuspended in FACS buffer, and analyzed with a BD LSRFortessa™ flow cytometer.

4.3 Molecular biology and protein chemistry methods

4.3.1 Protein lysis of lung leucocytes

Cell concentration of isolated lung leucocytes was determined and 3 x 10^6 cells were transferred to a 1.5 mL reaction tube. All steps from hereon were carried out on ice, if not mentioned otherwise. Cells were centrifuged (350 g, 4 °C, 5 min), supernatant was removed and cells were washed with 500 µl ice-cold PBS. Cells were centrifuged (350 g, 4 °C, 5 min) and supernatant was removed. Lysis was performed by the addition of 80 µl of CO-IP complete buffer and incubation for 20 min at 4 °C on an orbital shaker. After centrifugation for 12 min at 4 °C and maximum speed the supernatant was transferred to a new tube. Laemmli buffer was added in a concentration of 1:5 and samples were incubated for 5 min at 95 °C. Samples were either stored at -20 °C or directly used for gel electrophoresis.

4.3.2 Gel electrophoresis and western blotting

Samples were run on 9 % or 12.5 % polyacrylamide gels (***Table 18***), depending on the size of the target proteins. Lysates were incubated for 2-3 min at 95 °C after thawing. 5 µl of lysates and 2 µl of pre-stained protein marker were loaded and gel electrophoresis was run at 90 V for 2 h 30 min. Separated proteins were transferred onto methanol-activated PVDF-membranes with the use of an electrophoretic semi-dry blotting system (70 mA per blot for 1 h 50 min). After transfer, membranes were blocked with 5 % milk in PBS-T for 1 h at RT. Primary antibodies were diluted 1:1000 in 2.5 % bovine serum albumin (BSA) in PBS-T and membranes were incubated overnight at 4 °C. Membranes were washed 3x for 15 min in PBS-T and incubated in horseradish peroxidase (HRP)-coupled secondary antibody solutions for 1 h at RT. Secondary antibodies were diluted 1:5000 in 1.25 % BSA in PBS-T. Membranes were washed 3x for 15 min in PBS-T and shortly washed in a. dest. 2x. The LumiGlo reagent kit was used for detection of HRP by enhanced chemiluminescence according to the manufacturer's instructions with visualization on ECL Amersham Hyperfilms.

Table 18: Reagents and volumes for preparation of polyacrylamide gels.

Reagent	**Stacking gel** (4%)	**Separation gel** (9%)	**Separation gel** (12.5%)
Acrylamide-bisacrylamide	1mL	9mL	12.5mL
4x stacking gel buffer	2.5mL	-	-
5x separation gel buffer	-	6mL	6mL
SDS 1%	1mL	3mL	3mL
ddH2O	5.4mL	11.7mL	8.2mL
APS 10%	100µl	300µl	300µl
TEMED	10µl	30µl	30µl

4.3.3 RNA extraction

Isolation of ribonucleic acid (RNA) from lung tissue or isolated lung leucocytes was performed with the Quick-RNA Minprep Plus Kit according to the manufacturer's instructions. Tissue was homogenized using the Tissue Lyser LT. Pre-cooled stainless steel beads were added to frozen tissue in DNA/RNA shield. Samples were homogenized for 3 min at 50 Hz. Quantification of isolated RNA was performed using a NanoDrop™ 2000 spectrophotometer. RNA samples were directly used for synthesis of first strand complementary DNA (cDNA) or stored at -80 °C.

4.3.4 cDNA synthesis

cDNA was prepared by reverse transcription using the RevertAid First Strand cDNA Synthesis Kit according to the manufacturer's instructions. Contamination of reagents was assessed by a no-template negative control.

4.3.5 Quantitative real-time PCR (qPCR)

Gene expression was analyzed with the LightCycler® 480 SYBR Green I Master kit on the LightCycler® 480 System according to the manufacturer's instructions. Reactions were performed in duplicates on 384-well plates. Primer sequences are listed in ***Table 5***. Detected expression levels were normalized to the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and actin, beta (ACTB), and represented as $2^{-\Delta CT}$, (ΔCT = CT gene of interest – CT house-keeping gene).

4.3.6 Single-cell RNA sequencing (scRNA-seq)

Sequencing

Sequencing was performed in the Core Facility Genomics at Helmholtz Zentrum München using the NovaSeq 6000 system. Libraries were denatured and diluted according to Illumina sequencing platform recommendations and sequenced using the following run configuration: Read 1: 28 cycles, i7 index: 10 cycles, i5 index: 10 cycles, Read 2: 90 cycles.

Data processing

Data were processed on the GalaxyEU web-based platform for reproducible computational analysis[161]. mm39 was used as the reference genome, and mapping, demultiplexing, and gene quantification were performed using RNA STARSolo[160]. The following settings were chosen: for UMI-deduplication, the CellRanger 2-4 algorithm was applied. Multimatching of cell barcodes to the whitelist was allowed for cell barcodes with N-bases. UMIs with lower-counts mapping to more than one gene were removed. Using an EmptyDrops algorithm (lower-bound threshold: 100, false discovery rate: 0.01), cell filtering was performed by DropletUtils[159].

Data analysis

The R package Seurat 4.1.0 was used for data analysis[162,163]. Droplets with fewer than 50 or more than 5500 feature RNAs were excluded. The percentage of mitochondrial RNA per droplet was not allowed to exceed 12 %. Droplets with more than 55 % ribosomal RNA were also excluded from the analyses. Before determining the 2000 most important variable features using the 'vst' selection method, the individual data sets were normalized and merged. Data were scaled and principal component analysis was performed for dimensionality reduction. For t-SNE and UMAP embeddings, only statistically significant principal components were considered (JackStraw approach). K nearest neighbors (k = 20), nearest neighbor graphs, and shared nearest neighbors were computed. Using a shared nearest neighbor modularity optimization-based clustering algorithm (resolution = 0.5), clusters were identified. The 40 most differentially expressed genes per cluster were calculated and based on this, the assignment of clusters to cell types was performed. Differentially expressed genes were calculated using the Wilcoxon rank sum test and a log fold change threshold of 0.25.

Nebulosa 1.4.0 was used to generate density plots[164].

Single cell trajectories were computed using Monocle3 1.0.0[165]. For this purpose, the Seurat object was converted into a CellDataSet and preprocessed using PCA. UMAP was used for dimensionality reduction and as a reduction method for clustering the cells. Leiden clustering was performed with k nearest neighbors (k = 20).

4.3.7 Enzyme-linked immunosorbent assay (ELISA)

Immunoglobulins were analyzed in serum samples with ELISA. All samples and standards were measured in duplicates and analyzed with an Infinite®200 PRO plate reader.

Levels of total IgG1 (tIgG1) were analyzed using the LEGENDplex™ Mouse Immunoglobulin Isotyping Panel according to the manufacturer's instructions.

For the detection of OVA-specific IgG1 (OVA-sIgG1), 96-well Nunc MaxiSorp™ flat-bottom plates were coated with 1 µg/mL OVA and incubated at 4 °C overnight. Plates were washed 3x with ELISA wash buffer and blocked with ELISA blocking buffer for 2 h at RT. A monoclonal anti-chicken egg albumin antibody was used to prepare dilutions for a standard curve. Standard and samples were diluted in blocking buffer. After incubation at 4 °C overnight, plates were washed 4x with wash buffer and OVA-sIgG1 was detected with a secondary biotinylated anti-mouse IgG1 antibody (BD Biosciences). Incubation with secondary antibody for 2 h at RT was followed by 4x washing and detection with TMB for 30 min at RT in the dark. The development process was stopped by addition of stop solution and plates were read at 450 nm and 570 nm.

Quantification of total IgE (tIgE) was performed with the BD Mouse IgE ELISA set (BD Biosciences) following the manufacturer's instructions.

OVA-specific IgE (OVA-sIgE) was analyzed using the LEGEND MAX™ Mouse OVA Specific IgE ELISA Kit (BioLegend) according to the manufacturer's instructions.

4.3.8 Measurement of cytokine levels

Cytokine levels in the BALF were measured with BioLegend's LEGENDplex™ MU Th Cytokine Panel (12-plex) according to the manufacturers' instructions and analyzed by flow cytometry with a BD LSRFortessa™ flow cytometer.

4.4 Histological analysis of lung tissue

For histological analysis, lungs were excised after bronchoalveolar lavage. Part of the left lobe was fixed in 4 % buffered formalin for at least 48 h. After fixation, tissue was moved to 70 % ethanol and dehydrated through a series of ethanol baths. Subsequently, tissue was embedded in paraffin and sections with a thickness of 4 µm were prepared using a sliding microtome and transferred to slides. Slides were incubated at 56 °C for 2 h and gradually deparaffinized by subsequent immersion in xylene. Rehydration of slides by immersion in aqueous alcoholic solutions was followed by staining with hematoxylin-eosin (H&E) to analyze cell infiltration and periodic acid Schiff (PAS) for analysis of mucus hypersecretion. The degree of inflammatory cell infiltration and mucus hypersecretion was graded on a scale from 0 to 4[166].

4.5 Data analysis and statistics

Analysis of data was performed using GraphPad Prism 8, Microsoft Excel 2016, R 4.1.1, and RStudio v2022.02.3+492. Significance of results was analyzed as outlined in the respective figures. Gaussian and non-Gaussian distributed results were analyzed by 1-way ANOVA with Tukey's test or Kruskal-Wallis test with Dunn's test, respectively. 2-way ANOVA was performed with Tukey's multiple comparisons test or Sidak's multiple comparisons test, respectively. p-values of ≤.05, ≤.01, ≤.001, and ≤.0001 are shown as *, **, ***, and ****, respectively. p-values of allergic and treatment groups compared to the non-allergic group are shown as +, ++, +++, and ++++, respectively.

5. Results

5.1 The AhR is not essential for successful AIT

The role of the AhR in AIT was first investigated in a murine model of HDM-induced allergic asthma (***Figure 5***). The efficiency of AIT was analyzed in $AhR^{-/-}$ mice in a murine model of HDM-induced allergic asthma to determine if a functional AhR is essential for successful AIT.

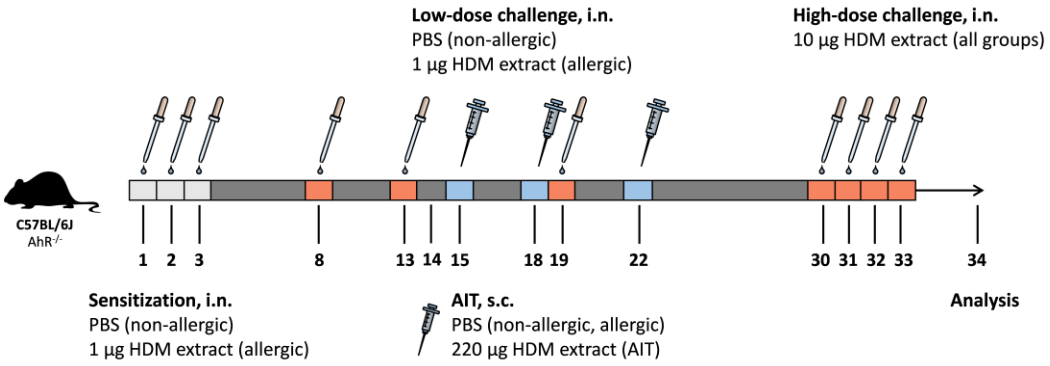

Figure 5: Experimental setup of AIT in a murine model of HDM-induced allergic asthma. Mice of the non-allergic group were treated with 20 µl PBS (i.n.) on days 1, 2, 3, 8, 13, and 19. All other groups received 1 µg HDM-extract on days 1, 2, and 3 for sensitization (i.n.) and 1 µg HDM-extract on days 8, 13, and 19 for low-dose allergen challenge (i.n.). AIT groups were treated with 220 µg HDM-extract (s.c.) on days 15, 18, and 22, all other groups received PBS (s.c.). All mice were challenged with 10 µg HDM-extract (i.n.) on days 30-33. Final analysis of all groups was performed on day 34.

Analysis of BALF cells showed an expected increase of total BALF cells in allergic mice compared to the non-allergic group. Detailed analysis of total BALF cells confirmed a significant increase of eosinophils, neutrophils, alveolar macrophages, $CD4^+$ and $CD8^+$ cells in the allergic group (***Figure 6A***). A decrease in total BALF cells as well as in analyzed subpopulations was detectable in the AIT group compared to allergic mice, even though there was a rather large variation in both groups. A similar pattern was observed in isolated pulmonary leucocytes (***Figure 6B***). Th2 cells, especially the expression of the Th2 transcription factor GATA3, showed an increase in the allergic group and a slight decrease in the AIT group. The same trends were

detectable for percentages of Th17 cells. Tregs showed a significant increase in the allergic group compared to non-allergic mice, as well as a tendency towards a reduction in the AIT group compared to allergic mice. The reduced cell infiltration in the BALF and decreased Th2 and Th17 cells after AIT pointed towards successful AIT in AhR$^{-/-}$ mice.

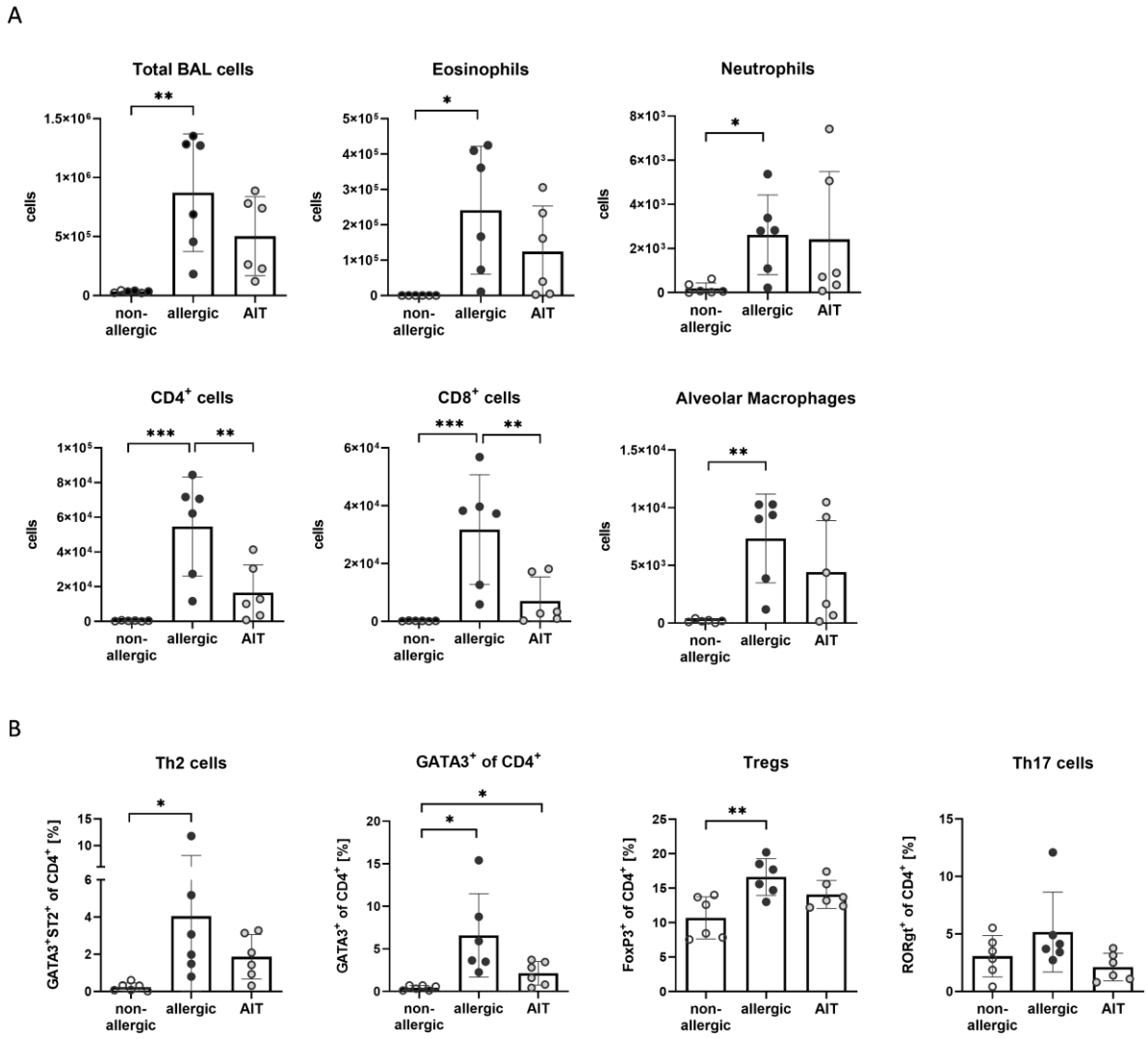

Figure 6: Effects of AIT in a model of HDM-induced murine allergic asthma in AhR$^{-/-}$ mice. (A) Total BALF cell counts and differential cell counts of eosinophils, neutrophils, CD4$^+$ cells, CD8$^+$ cells, and alveolar macrophages in BALF (n = 6). (B) Th cell populations analyzed in isolated lung leucocytes (n = 6). Gaussian and non-Gaussian distributed results were analyzed by 1-way ANOVA with Tukey's test or Kruskal-Wallis test with Dunn's test, respectively. p-values of ≤.05, ≤.01, ≤.001, and ≤.0001 are shown as *, **, ***, and ****, respectively.

These first results, obtained in the HDM AIT model, indicated that a functional AhR is no requirement for successful AIT. Nevertheless, non-allergic and allergic control groups, as well as the AIT group showed high variations in the investigated parameters. To exclude effects caused by high variations, the role of the AhR in AIT was analyzed additionally in a murine model of OVA-induced allergic asthma (***Figure 7***). This model is very well characterized and shows good reproducibility. Therefore, it is highly suitable to investigate underlying mechanisms that could play a general role in AIT. $AhR^{-/-}$ and wildtype mice were treated under the same conditions and according to the same protocol to directly compare the therapeutic outcome of AIT.

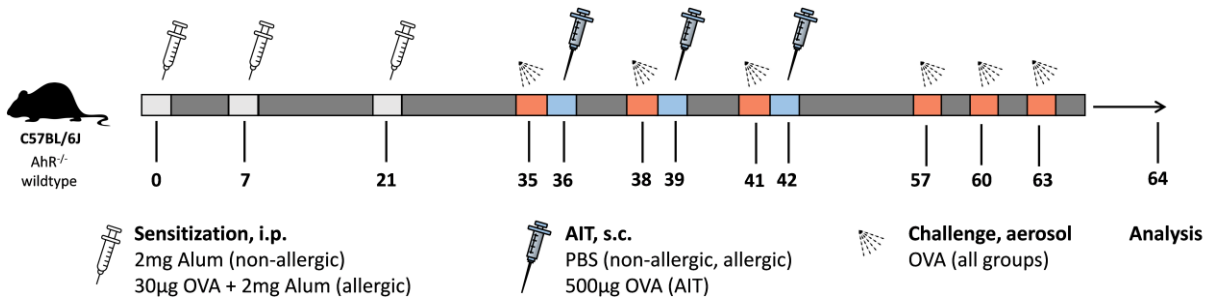

Figure 7: Schematic overview of AIT in a murine model of OVA-induced allergic asthma. Mice of the non-allergic group were treated with 2 mg alum in PBS (i.p.) on days 0, 7, and 21, all other groups were sensitized with 30 µg OVA and 2 mg alum in PBS (i.p.). AIT groups were treated with 500 µg OVA (s.c.) on days 36, 39, and 42, all other groups received PBS (s.c.). All mice were challenged with 1 % OVA aerosol for 15 min on days 35, 38, 41, 57, 60, and 63. Final analysis of all groups was performed on day 64.

Total BALF cells, as well as eosinophils, neutrophils, alveolar macrophages, $CD4^+$ and $CD8^+$ cells showed an increase in the allergic group compared to non-allergic mice for $AhR^{-/-}$ and wildtype mice (***Figure 8A***). Cell counts of the allergic groups did not differ significantly between $AhR^{-/-}$ and wildtype mice. Furthermore, the observed effects of AIT were mainly comparable between $AhR^{-/-}$ and wildtype mice. AIT comparably reduced the cell counts for both mouse lines. The only exception could be observed for $CD8^+$ cells. $AhR^{-/-}$ mice showed a higher cell count for $CD8^+$ cells, but also a strong variation within the group. Differences in cell counts between both non-allergic groups could not be detected.

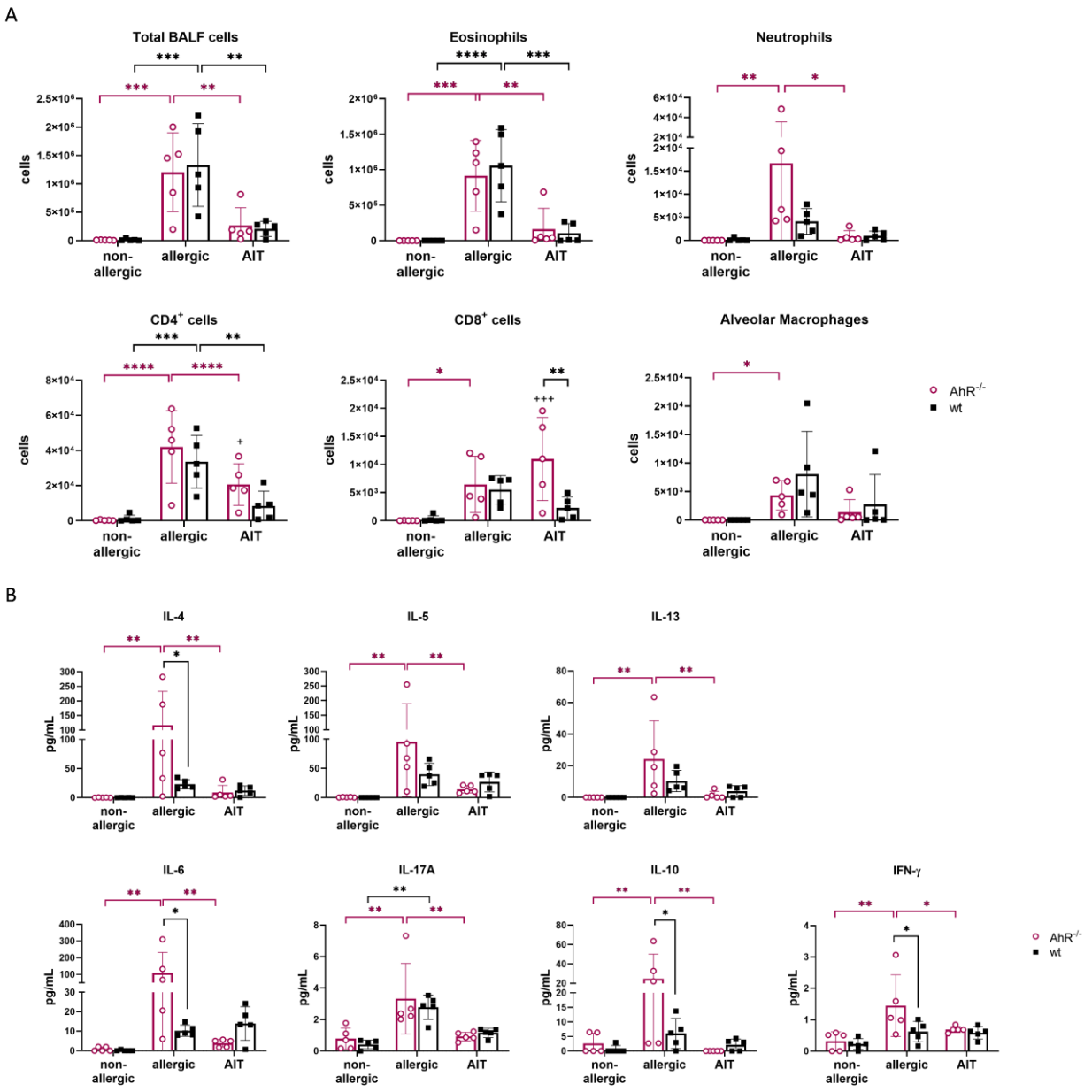

Figure 8: Comparison of the effects of AIT on BALF cells and BALF cytokines between AhR$^{-/-}$ and wildtype mice in a murine model of OVA-induced allergic asthma. (A) Total BALF cell counts and differential cell counts of eosinophils, neutrophils, CD4$^+$ cells, CD8$^+$ cells, and alveolar macrophages (n = 6). (B) Levels of IL-4, IL-5, IL-13, IL-6, IL-17A, IL-10, and IFN-γ analyzed in the BALF (n = 6). Data analysis with 2-way ANOVA with Tukey's test or Sidak's test, respectively. p-values of ≤.05, ≤.01, ≤.001, and ≤.0001 are shown as *, **, ***, and ****, respectively.

Analysis of cytokine levels in the BALF revealed significantly higher levels of IL-4, IL-6, IL-10, and IFN-γ in allergic AhR$^{-/-}$ mice compared to allergic wildtype mice (***Figure 8B***). Despite higher cytokine levels in allergic AhR$^{-/-}$ mice, AIT reduced cytokines levels significantly and in a comparable manner to AIT in wildtype mice. Measurement of cytokine levels in the non-allergic groups revealed no differences between both mouse lines

Additionally, the analysis of Th cell subsets in pulmonary leucocytes revealed no significant differences between AhR$^{-/-}$ and wildtype mice (***Figure 9A***). For Th2 cells and Tregs, an increase in allergic mice was detectable compared to the non-allergic group. Both subsets were comparably reduced after AIT in both mouse lines. For Th17 cells no significant differences were observable comparing different treatment groups and mouse lines. Eosinophils, neutrophils, and alveolar macrophages analyzed in isolated pulmonary leucocytes also showed no differences between AhR$^{-/-}$ and wildtype mice (***Figure 9B***). Eosinophils were significantly increased in allergic mice compared to the non-allergic control group. After AIT, eosinophils significantly decreased in wildtype mice. The decrease in AIT-treated AhR$^{-/-}$ was not significant compared to the respective allergic control group, but still comparable to the significant decrease in wildtype mice. Neutrophils were slightly decreased in allergic and AIT-treated mice in both mouse lines. Furthermore, alveolar macrophages showed a strong decrease comparing the allergic and AIT groups to the respective non-allergic group. All described changes in the allergic and AIT groups did not differ significantly between AhR$^{-/-}$ and wildtype mice.

Histological analysis of lung tissue confirmed the so far observed comparable effects of AIT in AhR$^{-/-}$ and wildtype mice (***Figure 9C***). Mucus hypersecretion was increased significantly in both allergic groups compared to the respective non-allergic mice. After AIT, mucus hypersecretion was reduced significantly in both mouse lines compared to the allergic control groups. A similar pattern was observable for inflammatory cell infiltration in the lung tissue, with a significant increase in cell infiltration for allergic mice and a significant decrease after AIT. Significant differences between similarly treated AhR$^{-/-}$ and wildtype mice were not detectable.

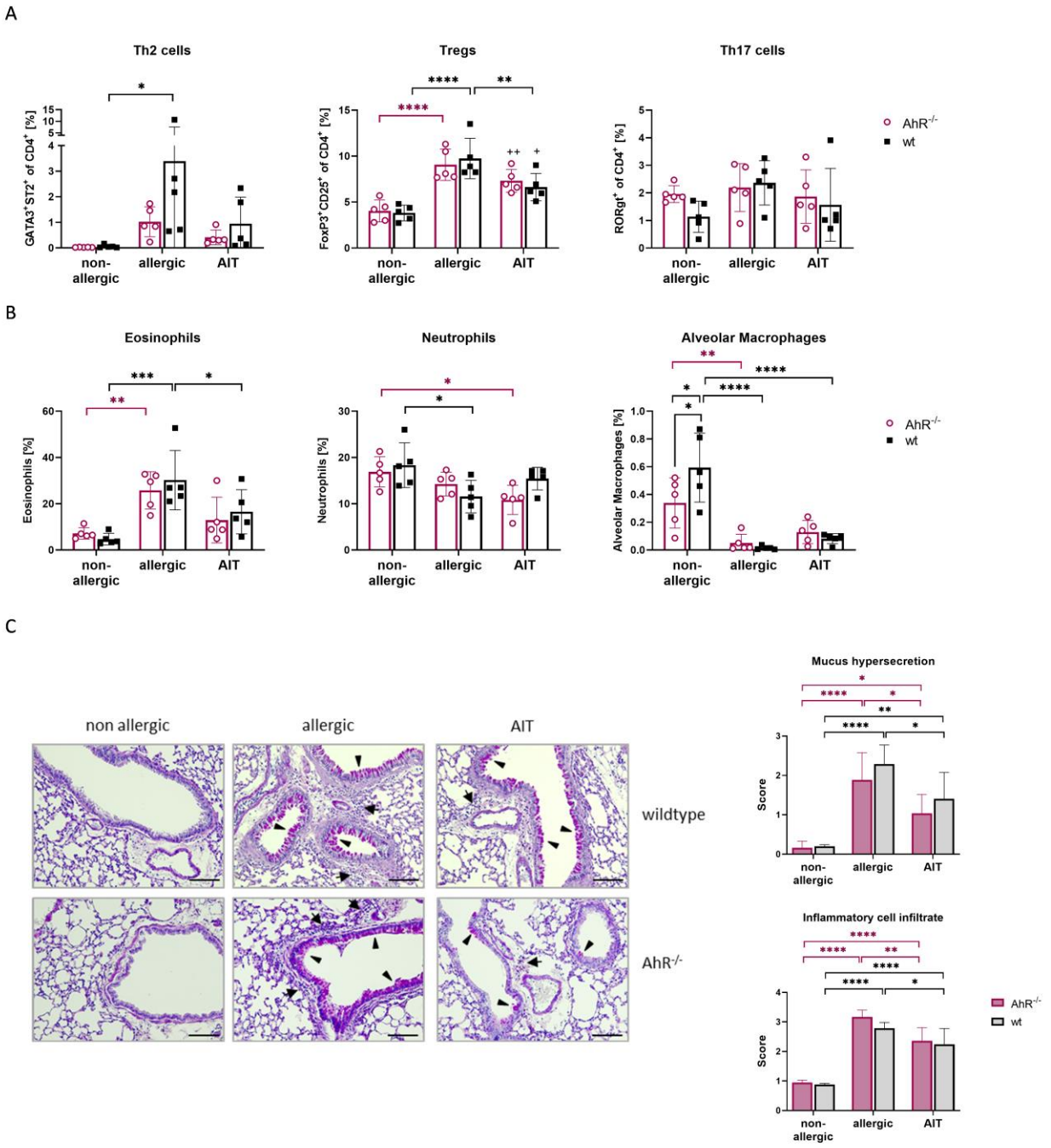

Figure 9: Comparison of cellular and histological analysis of lung tissue between AhR$^{-/-}$ and wildtype mice after AIT in a murine model of OVA-induced allergic asthma. Flow cytometric analysis of Th cell subsets in lung leucocytes (n = 6). (B) Flow cytometric analysis of eosinophils, neutrophils, and alveolar macrophages isolated from lung tissue (n = 6). (C) Histological analysis of lung tissue: representative PAS staining of lung tissue (arrowheads: mucus hypersecretion; arrows: inflammatory cell infiltrate; scale bar: 100µm) and scoring of mucus hypersecretion (n = 6) and inflammatory cell infiltrate (n = 6). Data analysis with 2-way ANOVA with Tukey's multiple comparisons test or Sidak's multiple comparisons test, respectively. P-values of ≤.05, ≤.01, ≤.001, and ≤.0001 are shown as *, **, ***, and ****, respectively.

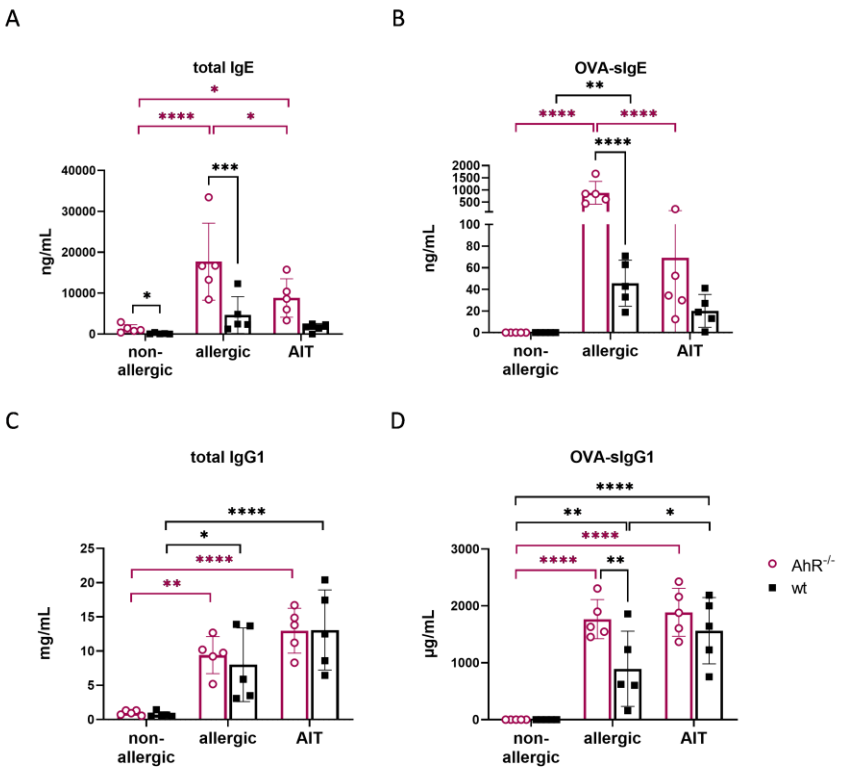

Figure 10: Comparison of immunoglobulin levels analyzed in AhR$^{-/-}$ and wildtype mice in a murine model of AIT for OVA-induced allergic asthma. Total IgE (n = 6), (B) OVA-sIgE (n = 6), (C) total IgG1 (n = 6), and (D) OVA-sIgG1 (n = 6) analyzed in serum samples collected at the end (day 64) of the experiment. Data analysis with 2-way ANOVA with Tukey's multiple comparisons test or Sidak's multiple comparisons test, respectively. P-values of ≤.05, ≤.01, ≤.001, and ≤.0001 are shown as *, **, ***, and ****, respectively.

Analysis of immunoglobulins in serum, collected at the end of the experiment (day 64), revealed significantly higher total IgE and OVA-sIgE levels in allergic AhR$^{-/-}$ mice compared to allergic wildtype mice (***Figure 10A+B***). Both parameters were significantly reduced in AhR$^{-/-}$ mice after AIT in comparison to the respective allergic group and did not differ significantly from total IgE and OVA-sIgE levels detected in AIT-treated wildtype mice. Total IgG1 did increase significantly in allergic mice and even further in the AIT-treated groups compared to non-allergic mice (***Figure 10C***). Differences between both mouse lines were not detectable. Analysis of OVA-sIgG1 in AhR$^{-/-}$ mice showed a significant and comparable increase in allergic and AIT-treated mice compared to the non-allergic group (***Figure 10D***). OVA-sIgG1 in allergic wildtype mice did also increase compared to the respective non-allergic group but was significantly lower than in allergic AhR$^{-/-}$

mice. Whereas OVA-sIgG1 in AIT-treated $AhR^{-/-}$ mice did not increase any further, a significant increase was detectable for AIT-treated wildtype mice compared to the respective allergic group.

Overall, AIT seemed to work efficiently in $AhR^{-/-}$ mice and was able to reduce characteristic parameters of allergic asthma in a comparable manner to wildtype mice.

5.2 Effects of the low-affinity AhR agonist quercetin

Experiments in murine AIT models of OVA- and HDM-induced allergic asthma could provide evidence, that the AhR is not essential to perform successful AIT. Nevertheless, to investigate if activation of the AhR is able to alter and ideally improve therapeutic outcome, further experiments with AhR agonists were performed. Therefore, AhR ligands with agonistic properties were selected and used to activate the AhR during the AIT treatment phase.

5.2.1 Effects of quercetin on AIT and allergic inflammation in HDM-induced allergic asthma

For quercetin, a flavonoid naturally occurring in some fruits and vegetables[167], several beneficial properties have been described, including strong anti-oxidant activity, anti-inflammatory and anti-allergic effects[168]. The known anti-allergic functions are mediated by the inhibition of histamine production and pro-inflammatory mediators[169,170]. In addition, quercetin is described as a low-affinity AhR agonist[138,171].

The effects of quercetin on AIT were first investigated in a murine model of HDM-induced allergic asthma, with daily i.p. injections of quercetin from day 14 to day 22 (***Figure 11***). Quercetin was administered at doses of 8 mg/kg or 16 mg/kg, either in combination with AIT or as a single-treatment strategy. Quercetin treatment as a single-treatment strategy was performed to evaluate if quercetin itself is able to modulate the allergic response without further therapeutic intervention.

Total BALF cell counts and differential counts of eosinophils, neutrophils, $CD4^+$ cells, $CD8^+$ cells, and alveolar macrophages showed an increase in the allergic group compared to non-allergic mice

(***Figure 12***). Total BALF cells, eosinophils, and $CD4^+$ T cells were decreased after AIT, but the high variation observable in the allergic group made it difficult to evaluate the effects of AIT, AIT + quercetin, and quercetin treatment properly. For neutrophils, $CD8^+$ T cells, and alveolar macrophages no reliable statement could be made regarding the effects of AIT in comparison to the allergic group. Nevertheless, the combined treatment did not improve the therapeutic efficiency of AIT. For AIT + 8 mg/kg quercetin, BALF cell counts showed large variations and were even higher than for the standard AIT treatment. The combination of AIT with 16 mg/kg quercetin led to cell counts that were comparable to the standard AIT treatment. Interestingly, the treatment with quercetin alone did have an impact on total and differential BALF cell counts. For allergic mice treated with 8 mg/kg quercetin, the numbers of total BALF cells, eosinophils, $CD4^+$ cells, and alveolar macrophages were comparable to those in the AIT group. The treatment of mice with 16 mg/kg quercetin for 9 days did reduce all analyzed cell populations and therefore also the total BALF cell counts in an even stronger manner than AIT.

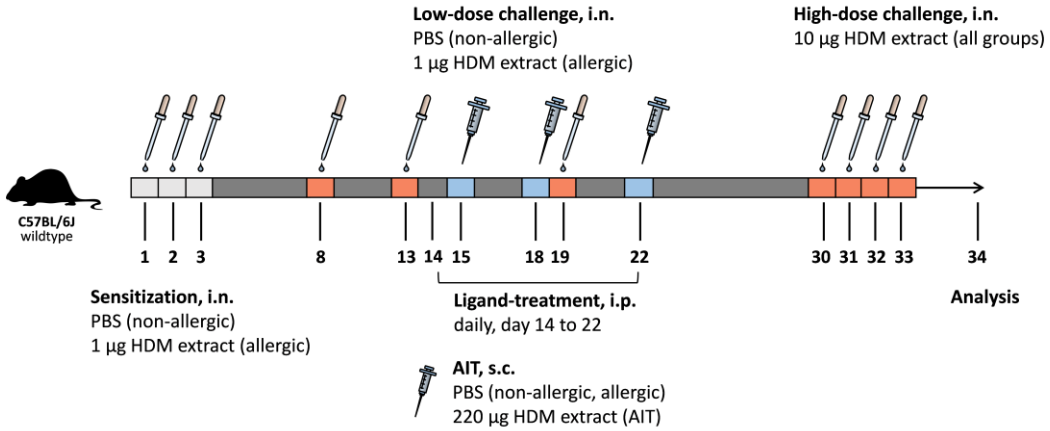

Figure 11: AhR-ligand treatment during AIT in a murine model of HDM-induced allergic asthma. Mice of the non-allergic group were treated with 20 µl PBS (i.n.) on days 1, 2, 3, 8, 13, and 19. All other groups received 1 µg HDM-extract on days 1, 2, and 3 for sensitization (i.n.) and 1 µg HDM-extract on days 8, 13, and 19 for low-dose allergen challenge (i.n.). AIT groups were treated with 220 µg HDM-extract (s.c.) on days 15, 18, and 22, all other groups received PBS (s.c.). Respective groups were treated daily with AhR-ligands or solvents (i.p.) from days 14 to 22. All mice were challenged with 10 µg HDM-extract (i.n.) on days 30-33. Final analysis of all groups was performed on day 34.

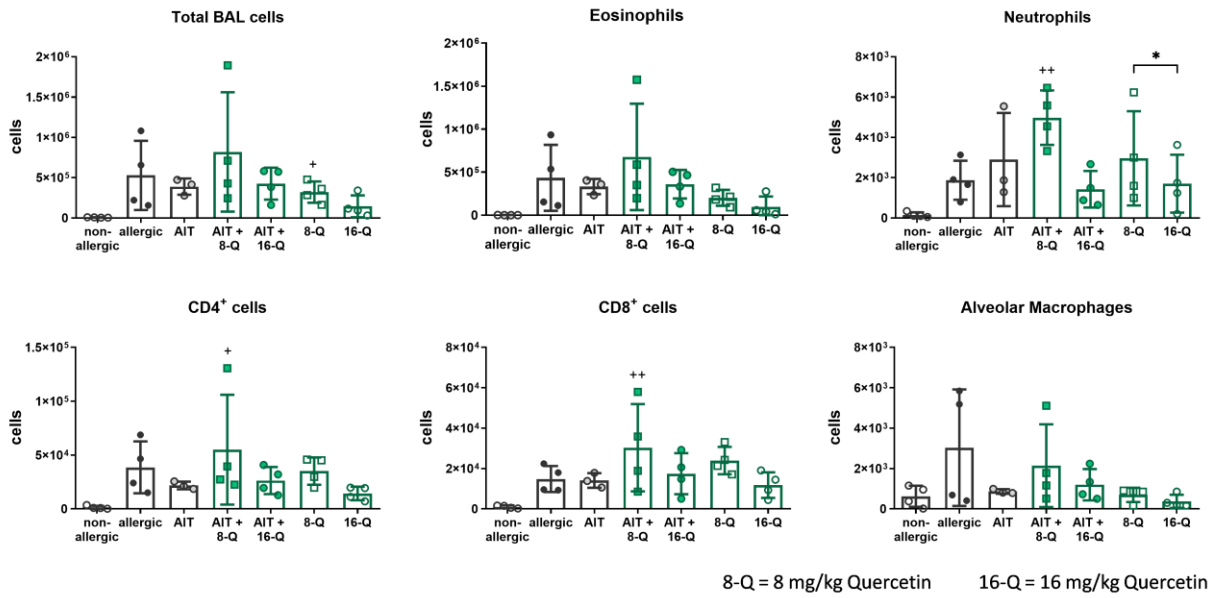

Figure 12: Effects of quercetin treatment as a single-treatment strategy or in combination with AIT on total and differential BALF cell counts. Total BALF cell counts and differential cell counts of eosinophils, neutrophils, $CD4^+$ cells, $CD8^+$ cells, and alveolar macrophages (n = 3 - 4). Gaussian and non-Gaussian distributed results were analyzed by 1-way ANOVA with Tukey's test or Kruskal-Wallis test with Dunn's test, respectively. p-values of ≤.05, ≤.01, ≤.001, and ≤.0001 are shown as *, **, ***, and ****, respectively. p-values of allergic and AIT groups compared to the non-allergic group are shown as +, ++, +++, and ++++, respectively.

Analysis of Th cell populations in pulmonary leucocytes showed an increase in Th2, Treg, and Th17 cells in the allergic group, with rather strong variations for Th2 cells, compared to non-allergic mice (***Figure 13A***). AIT decreased the percentage of all analyzed subsets in comparison to the allergic group. Similar to the effects observed in the BALF cells, the combination of AIT with quercetin did not improve the therapeutic outcome. In contrast to the combined treatment, the administration of 8 mg/kg or 16 mg/kg quercetin without AIT reduced the percentage of Th2 cells in a stronger manner than AIT. The effects of quercetin treatment on Th17 and Treg cells were comparable with the effects of AIT or the combined treatment strategies.

After repetitive treatment with 8 mg/kg or 16 mg/kg quercetin without additional AIT, Th2 cells showed a higher expression of FoxP3 compared to mice that underwent AIT or combined treatment (***Figure 13B***). The induction of this regulatory phenotype in Th2 cells was observable in a dose-dependent manner with a higher percentage for the groups treated with 16 mg/kg quercetin. A similar pattern was detectable for $RORgt^+FoxP3^+$ cells.

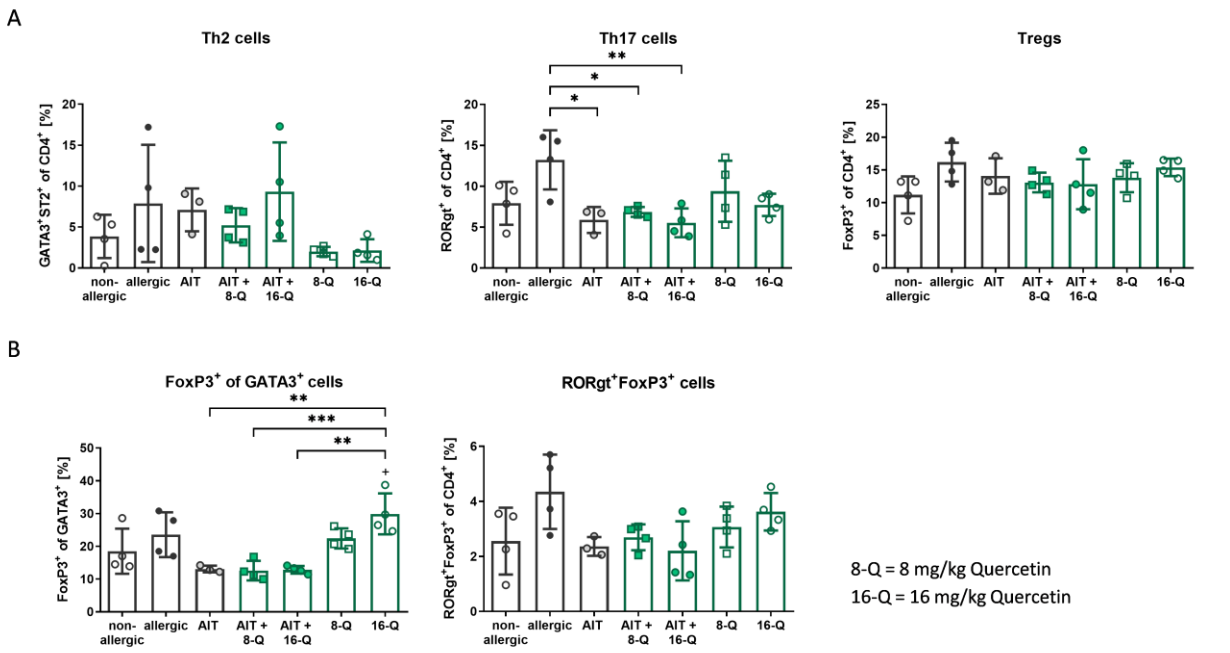

Figure 13: Effects of Quercetin treatment on Th cell subsets in an AIT model of HDM-induced murine allergic asthma. (A) Flow cytometry analysis of Th cell subsets in leucocytes isolated from lung tissue (n = 3 - 4). (B) Flow cytometry analysis of FoxP3-expressing Th2 cells and RORgt^{+}FoxP3^{+} cells in isolated lung leucocytes (n = 3 - 4). Gaussian and non-Gaussian distributed results were analyzed by 1-way ANOVA with Tukey's test or Kruskal-Wallis test with Dunn's test, respectively. p-values of ≤.05, ≤.01, ≤.001, and ≤.0001 are shown as *, **, ***, and ****, respectively. p-values of allergic and AIT groups compared to the non-allergic group are shown as +, ++, +++, and ++++, respectively.

Anti-oxidative properties of quercetin have already been described in literature[172,173]. To address the potential mechanisms that could underlay the observed effects of quercetin on Th cell subsets and BALF cells, gene expression analysis of enzymes related to oxidative stress was performed (***Figure 14A***). Glutathione peroxidase (GPx) and superoxide dismutase 1 (SOD1) and 2 (SOD2) are decreased in the presence of high reactive oxygen species (ROS) concentrations, an indicator for oxidative stress[174]. Compared to non-allergic mice all other groups showed reduced expression of GPx, SOD1, and SOD2. A slight increase was detectable for all targets after treatment with 8 mg/kg and 16 mg/kg quercetin, but the expression did not reach similar levels to non-allergic mice. Overall, fold changes in the expression of mRNA levels between all groups were rather low. Reduction of oxidative stress after quercetin treatment seemed not to be responsible for the observed effects in pulmonary leucocytes. Expression of CYP1A1, an AhR target gene, was increased in the allergic and treatment groups compared to non-allergic mice (***Figure 14B***). AIT-

treated mice with and without additional quercetin administration revealed comparable levels of CYP1A1, whereas CYP1A1 expression in the groups with 8 mg/kg or 16 mg/kg quercetin was slightly lower. An increased expression related to quercetin, a low-affinity AhR ligand, was not detectable. Quercetin was not able to alter the expression of CYP1A1 or enzymes related to anti-oxidative stress in this experimental setup. Given quercetin's half-life period of several hours[175,176], this analysis should be performed directly after the treatment phase, to properly evaluate the effects on CYP1A1 to evaluate the activation of the AhR and on gene expression levels in general.

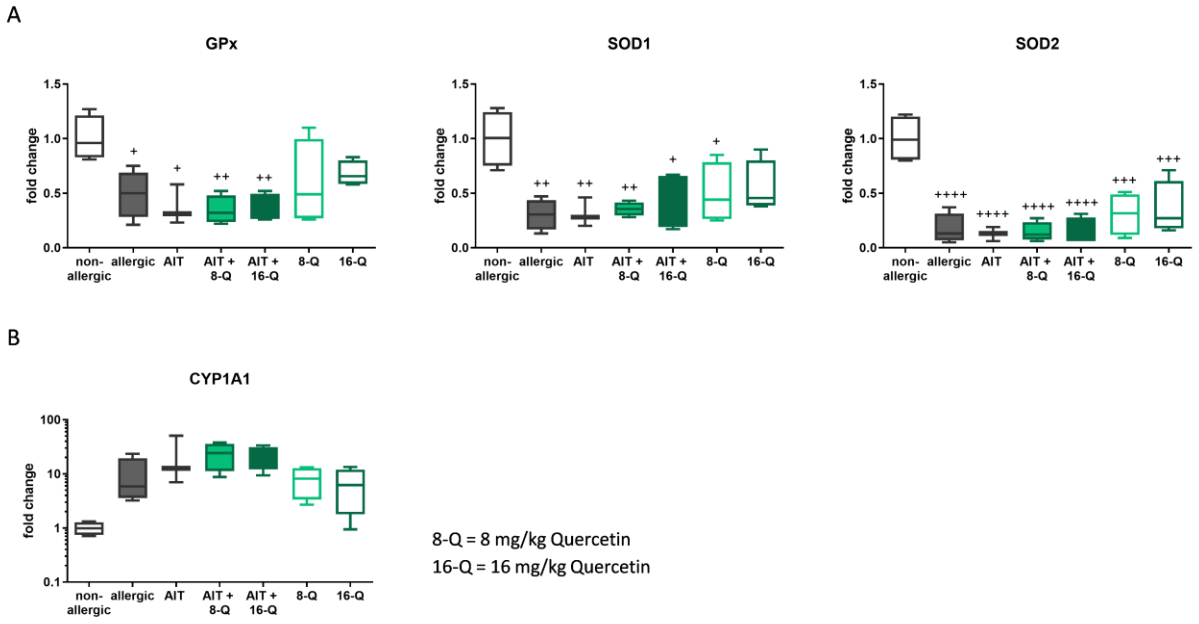

Figure 14: Effects of quercetin and AIT treatment on gene expression levels in a murine model of HDM-induced allergic asthma. (A) Gene expression levels of oxidative stress-associated genes GPx, SOD1, and SOD2 in lung tissue (n =3 - 4). (B) Gene expression levels of the AhR target gene CYP1A1 (n =3 - 4); boxplots indicate minimum, 25th percentile, median, 75th percentile, and maximum. Gaussian distributed results were analyzed by 1way-ANOVA with Tukey's test. p-values of ≤.05, ≤.01, ≤.001, and ≤.0001 are shown as *, **, ***, and ****, respectively. p-values of allergic and treatment groups compared to the non-allergic group are shown as +, ++, +++, and ++++, respectively.

5.2.2 The beneficial effects of quercetin on allergic asthma are likely mediated by the mTOR signaling pathway

The initial experiment to investigate the effects of quercetin on AIT in the HDM model revealed no benefits in combining AIT with quercetin administration. However, a decrease in Th2 and Th17 cells, as well as reduced BALF cell counts after treatment with 8 mg/kg or 16 mg/kg quercetin could be observed. The HDM model was repeated to further elucidate the observed beneficial effects, but mice were only treated with 16 mg/kg quercetin, as the high-dose treatment lead to the most pronounced effects. AIT groups were not repeated, due to the lacking beneficial impact on therapeutic outcome. Whereas the reduction of total BALF cell counts was clearly visible for all quercetin-treated mice in the initial experiment (***Figure 12***), the repetition showed highly variable responses (***Figure 15A***). This could be a first indication that the previously observed effects are either not reproducible in a reliable manner or that the applied dosage is not sufficient to efficiently maintain anti-inflammatory effects. For further analysis of potential underlying mechanisms, the quercetin-treated group was divided into non-responders, showing an allergic phenotype with high total BALF cell counts, and responders with low BALF cell numbers. All quercetin-treated mice with BALF cell counts comparable to the allergic group were defined as non-responders, whereas mice with reduced BALF cell counts after quercetin treatment were assigned to the responder group (***Figure 15A***). These groups were further investigated regarding the mechanistic target of the rapamycin (mTOR) signaling pathway. The mTOR signaling pathway has been connected to quercetin and its effects in several publications[177,178]. Protein kinase b (AKT) and eukaryotic translation initiation factor 4E-binding protein (4E-BP1), both components of the mTOR signaling pathway, were analyzed in protein lysates of pulmonary leucocytes by Western blot (***Figure 15B***). Both proteins showed increased phosphorylation in the allergic group in comparison to the non-allergic group. Phosphorylation of both proteins was also strongly increased in mice that did not respond to the quercetin treatment. In the responding animals, phosphorylation was decreased and comparable to the non-allergic group. In these mice, quercetin treatment was able to inhibit the mTOR signaling pathway.

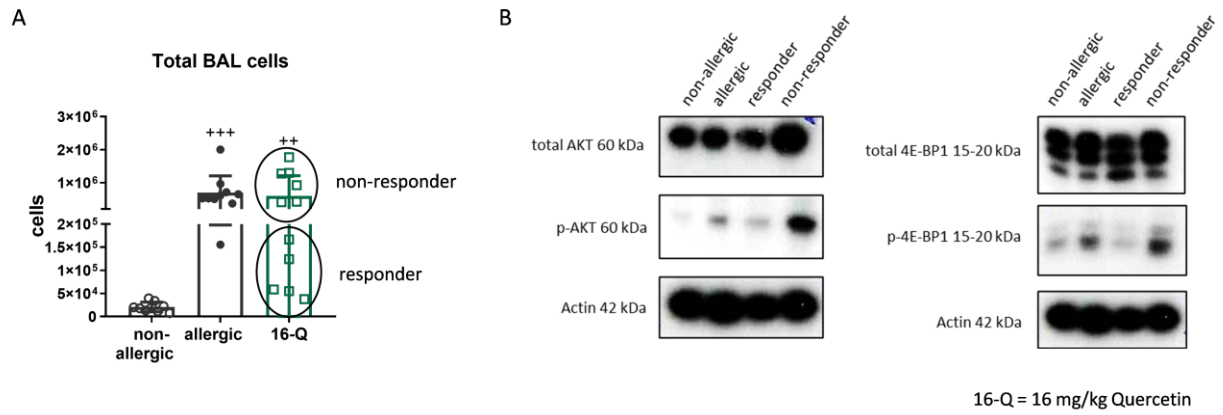

Figure 15: Effects of quercetin treatment on components of the mTOR signaling pathway. (A) Total BALF cell counts of non-allergic, allergic, and quercetin-treated mice in a murine model of HDM-induced allergic asthma. (n = 10). (B) Western blot analysis of total AKT, p-AKT, total 4E-BP1, p-4E-BP1, and Aktin as a loading control in isolated lung leucocytes. Gaussian distributed results were analyzed by 1-way ANOVA with Tukey's test. p-values of ≤.05, ≤.01, ≤.001, and ≤.0001 are shown as *, **, ***, and ****, respectively. p-values of allergic and treatment groups compared to the non-allergic group are shown as +, ++, +++, and ++++, respectively.

5.2.3 Quercetin shows no effects on AIT and allergic inflammation in a murine model of OVA-induced allergic asthma

In the HDM model, quercetin reduced BALF cell counts and percentages of Th2 and Th17 in pulmonary leucocytes and induced a regulatory phenotype in Th2 cells. Unfortunately, these effects could not be reproduced reliably. To investigate if the observed effects are rather short-term and the period between final quercetin administration and analysis is too long to maintain the beneficial effects, quercetin was also tested in a model of OVA-induced allergic asthma (***Figure 16***). With 22 days in between the last treatment with quercetin and the final analysis, this model should answer the question if quercetin is able to alleviate the characteristics of allergic inflammation long-term.

Additionally, the OVA model allows to investigate the effects of quercetin in a stable and well characterized setup, whereas the HDM model showed large variations in several groups, which makes it difficult to evaluate the effects of quercetin reliably.

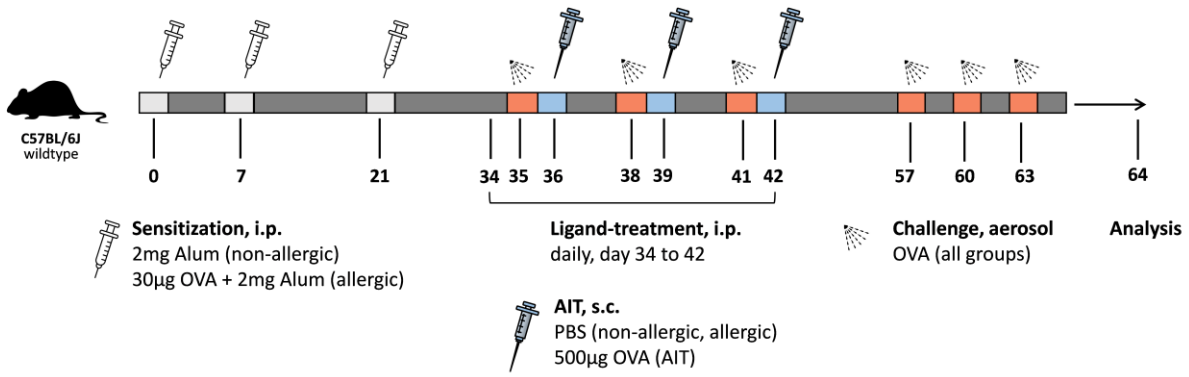

Figure 16: AhR-ligand treatment during AIT in a murine model of HDM-induced allergic asthma. Mice of the non-allergic group were treated with 2 mg alum in PBS (i.p.) on days 0, 7, and 21, all other groups were sensitized with 30 µg OVA and 2 mg alum in PBS (i.p.). AIT groups were treated with 500 µg OVA (s.c.) on days 36, 39, and 42, all other groups received PBS (s.c.). Respective groups were treated daily with AhR-ligands or solvents (i.p.) from days 34 to 42. All mice were challenged with 1 % OVA aerosol for 15 min on days 35, 38, 41, 57, 60, and 63. Final analysis of all groups was performed on day 64.

In the OVA model, mice of the allergic group showed an increase in total BALF cell counts, as well as in differential counts of eosinophils, neutrophils, $CD4^+$ cells, $CD8^+$ cells, and alveolar macrophages compared to the non-allergic group (***Figure 17***). AIT and the combination of AIT + 16 mg/kg quercetin lead to a reduction of all analyzed cell populations and the total BALF cell counts. No differences were detectable between the AIT group and the group that received the combined treatment. The administration of quercetin without additional AIT did not reduce any of the analyzed cell populations. Total and differential BALF cell counts of mice in the low-dose group, receiving 8 mg/kg quercetin, as well as mice in the high-dose group with 16 mg/kg quercetin were comparable to cell counts of allergic mice.

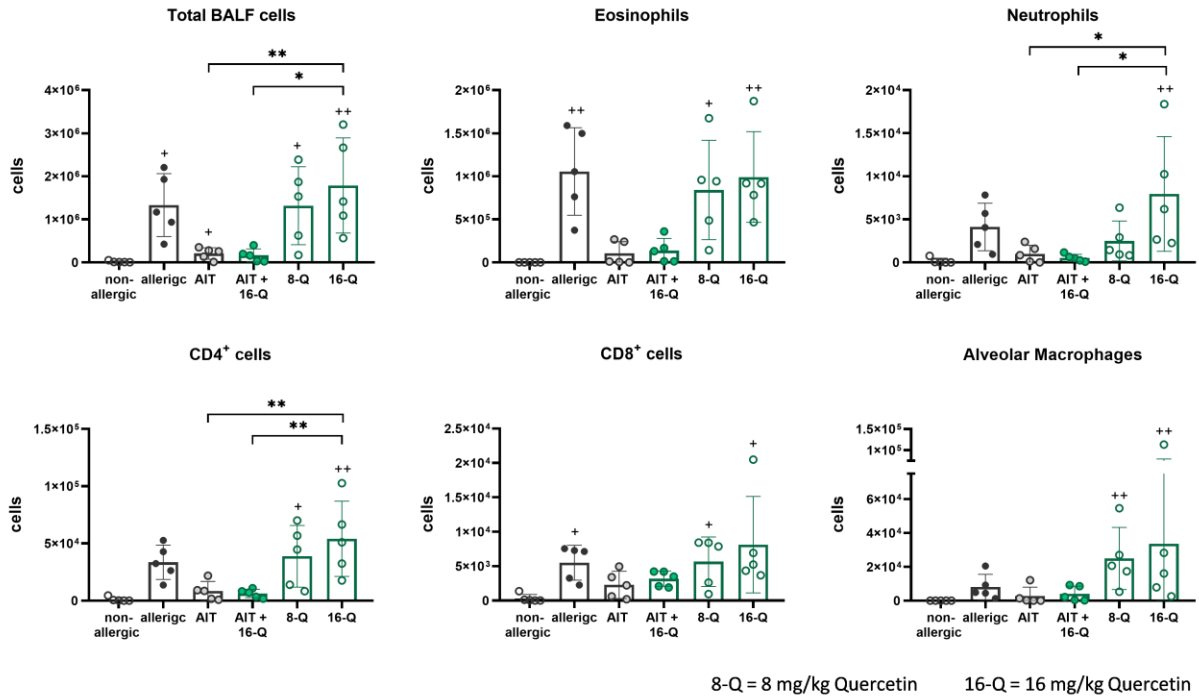

Figure 17: Influence of quercetin administration in combination with AIT treatment or as a single-treatment strategy on BALF cells in a model of OVA-induced allergic asthma. Total BALF cell count and differential count of eosinophils, neutrophils, $CD4^+$ cells, $CD8^+$ cells, and alveolar macrophages (n = 5). Gaussian and non-Gaussian distributed results were analyzed by 1-way ANOVA with Tukey's test or Kruskal-Wallis test with Dunn's test, respectively. p-values of ≤.05, ≤.01, ≤.001, and ≤.0001 are shown as *, **, ***, and ****, respectively. p-values of allergic and treatment groups compared to the non-allergic group are shown as +, ++, +++, and ++++, respectively.

A similar pattern could be observed in Th cell populations analyzed in pulmonary leucocytes (***Figure 18***). Allergic mice showed an increase in Th2 cells, Th17 cells, and Tregs compared to non-allergic mice. All analyzed populations were reduced comparably after AIT and AIT combined with daily administration of 16 mg/kg quercetin. The additional administration of quercetin was not beneficial in comparison to standard AIT treatment. The treatment with 8 mg/kg or 16 mg/kg quercetin without additional AIT did not affect Th2 cells and Tregs in comparison to the allergic group. The only effect of single treatment with quercetin was detectable in Th17 cells. Quercetin administration alone reduced Th17 cells in a comparable manner to AIT and AIT + 16 mg/kg quercetin. The strong effects of quercetin on BALF cells and Th2 cells observed in the HDM model could not be confirmed in the OVA model.

Quercetin was able to ameliorate characteristics of allergic asthma only short-term in the HDM model, most likely due to inhibition of the mTOR signaling pathway, but the observed effects were

not reproducible in a reliable manner. Additionally, the OVA model confirmed that quercetin acts rather in an acute manner. With a longer period between the last administration and the final analysis, beneficial effects were no longer observable. The therapeutic outcome of AIT could not be improved or altered by adding quercetin treatment to standard AIT.

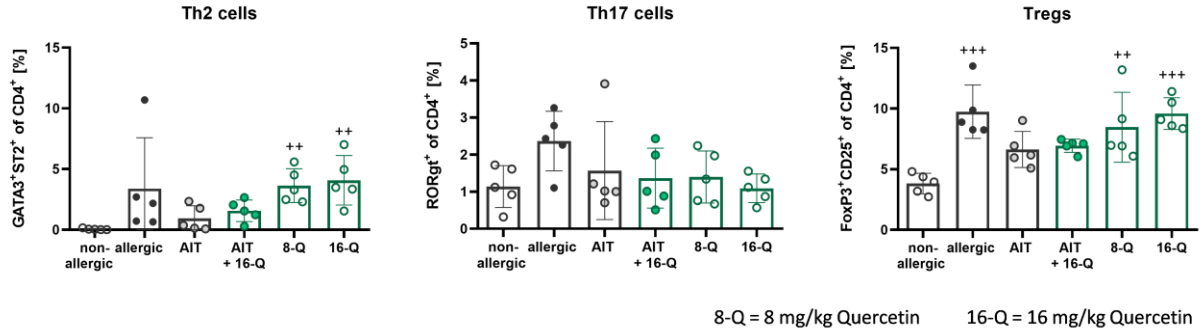

Figure 18: Influence of quercetin administration in combination with AIT standard treatment or as a single-treatment strategy on Th cell subsets of isolated lung leucocytes in a model of OVA-induced allergic asthma. Percentage of Th2 cells, Th17 cells, and Tregs of $CD4^+$ T cells (n = 5). Gaussian and non-Gaussian distributed results were analyzed by 1-way ANOVA with Tukey's test or Kruskal-Wallis test with Dunn's test, respectively. p-values of ≤.05, ≤.01, ≤.001, and ≤.0001 are shown as *, **, ***, and ****, respectively. p-values of allergic and treatment groups compared to the non-allergic group are shown as +, ++, +++, and ++++, respectively.

5.3 Effects of the high-affinity AhR agonist 10-Cl-BBQ on AIT

The combination of AIT with quercetin treatments did not improve the therapeutic outcome of AIT, neither in the HDM nor in the OVA model. Quercetin is known for its strong anti-inflammatory and anti-oxidative properties[172]. In the context of AhR activation, quercetin is described as a rather weak AhR agonist, with low binding affinity[139]. To ensure strong activation of the AhR during AIT, the high-affinity AhR agonist 10-Cl-BBQ was tested for its capacity to influence the therapeutic outcome of AIT.

5.3.1 The high-affinity AhR agonist 10-Cl-BBQ reduces characteristics of allergic inflammation in an acute manner

Initial testing of the effects of 10-Cl-BBQ on AIT was performed in an acute model of OVA-induced allergic asthma (***Figure 19***). Mice were sensitized three times on days 0, 14, and 21 and challenged with OVA-aerosol on days 35, 38, and 41. AIT was performed on days 36, 39, and 42. To investigate the effects of 10-Cl-BBQ, one group did receive daily injections of 10 mg/kg (i.p.) from days 34 to 42 in addition to AIT. For evaluation of the effects of 10-Cl-BBQ on the phenotype of allergic mice without additional AIT, mice were treated with 10-Cl-BBQ only. Both treatment strategies were compared to standard AIT, allergic and non-allergic mice. The final analysis was performed on day 43, directly after the AIT and 10-Cl-BBQ treatment phase to address the acute effects of 10-Cl-BBQ and to verify the activation of the AhR by 10-Cl-BBQ.

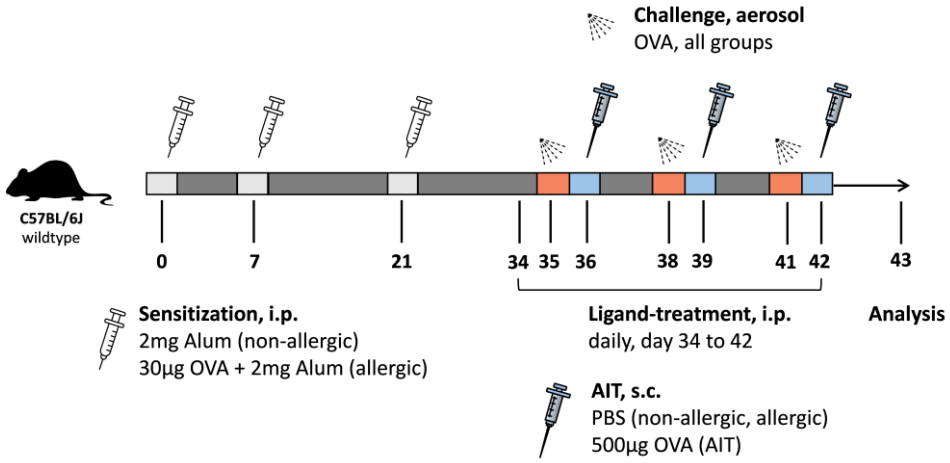

Figure 19: Experimental setup to investigate the acute effects of 10-Cl-BBQ in a murine model of allergic asthma. Acute effects of 10-Cl-BBQ + AIT and 10-Cl-BBQ were assessed in comparison to standard AIT treatment. Mice of the non-allergic group were treated with 2 mg alum in PBS (i.p.) on days 0, 7, and 21, all other groups were sensitized with 30 µg OVA and 2 mg alum in PBS (i.p.). AIT groups were treated with 500 µg OVA (s.c.) on days 36, 39, and 42, all other groups received PBS (s.c.). 10-Cl-BBQ or vehicle control was administered daily from days 34 to 42 (i.p.) All mice were challenged with 1 % OVA aerosol for 15 min on days 35, 38, and 41. Final analysis of all groups was performed on day 43.

Total BALF cell counts as well as differential counts of eosinophils, neutrophils, $CD4^+$ cells, $CD8^+$ cells, and alveolar macrophages were significantly increased in allergic mice on day 43 in comparison to the non-allergic group (***Figure 20A***). Whereas AIT showed no significant reduction of total and differential cell counts compared to the allergic group, treatment with 10-Cl-BBQ reduced total BALF cells, eosinophils, neutrophils, and $CD4^+$ cells significantly. A reduction of $CD8^+$ and alveolar macrophages compared to the allergic group was also detectable, but not in a significant manner. Acute effects of 10-Cl-BBQ analyzed on day 43 did not show differences between the AIT + 10-Cl-BBQ and the 10-Cl-BBQ group. All analyzed cell populations were reduced significantly and comparably for both groups treated with 10-Cl-BBQ.

Th2 cytokines analyzed in the BALF followed a similar pattern to total and differential BALF cell counts (***Figure 20B***). IL-4, IL-5, and IL-13 were reduced in both groups treated with 10-Cl-BBQ compared to allergic and AIT-treated mice. Standard AIT treatment did not affect levels of Th2 cytokines compared to the allergic group. Analysis of IL-6 led to similar results with a detectable reduction after treatment with 10-Cl-BBQ compared to the allergic and AIT groups. IL-17A and INF-γ were increased for all groups compared to the non-allergic control group but did not differ significantly or by trend between each other.

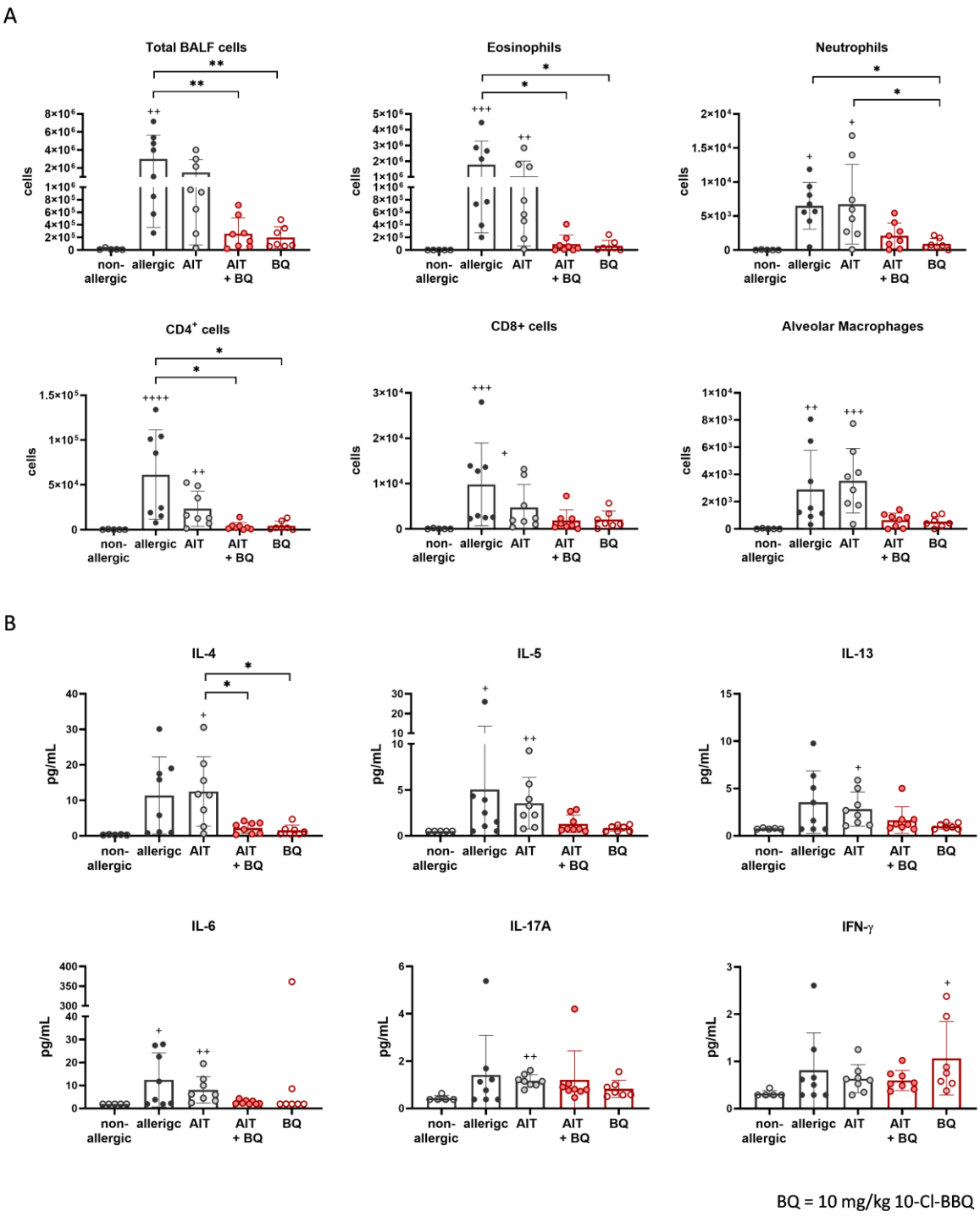

Figure 20: Acute effects of 10-Cl-BBQ as a single-treatment strategy or in combination with AIT on BALF cells and cytokines in a murine model of OVA-induced allergic asthma. (A) Total BALF cell counts and differential counts of eosinophils, neutrophils, $CD4^+$ cells, $CD8^+$ cells, and alveolar macrophages at day 43 of the experiment (n = 5 - 8). (B) Cytokine analysis in BALF at day 43 of the experiment. Gaussian and non-Gaussian distributed results were analyzed by 1-way ANOVA with Tukey's test or Kruskal-Wallis test with Dunn's test, respectively. p-values of ≤.05, ≤.01, ≤.001, and ≤.0001 are shown as *, **, ***, and ****, respectively. p-values of allergic and treatment groups compared to the non-allergic group are shown as +, ++, +++, and ++++, respectively.

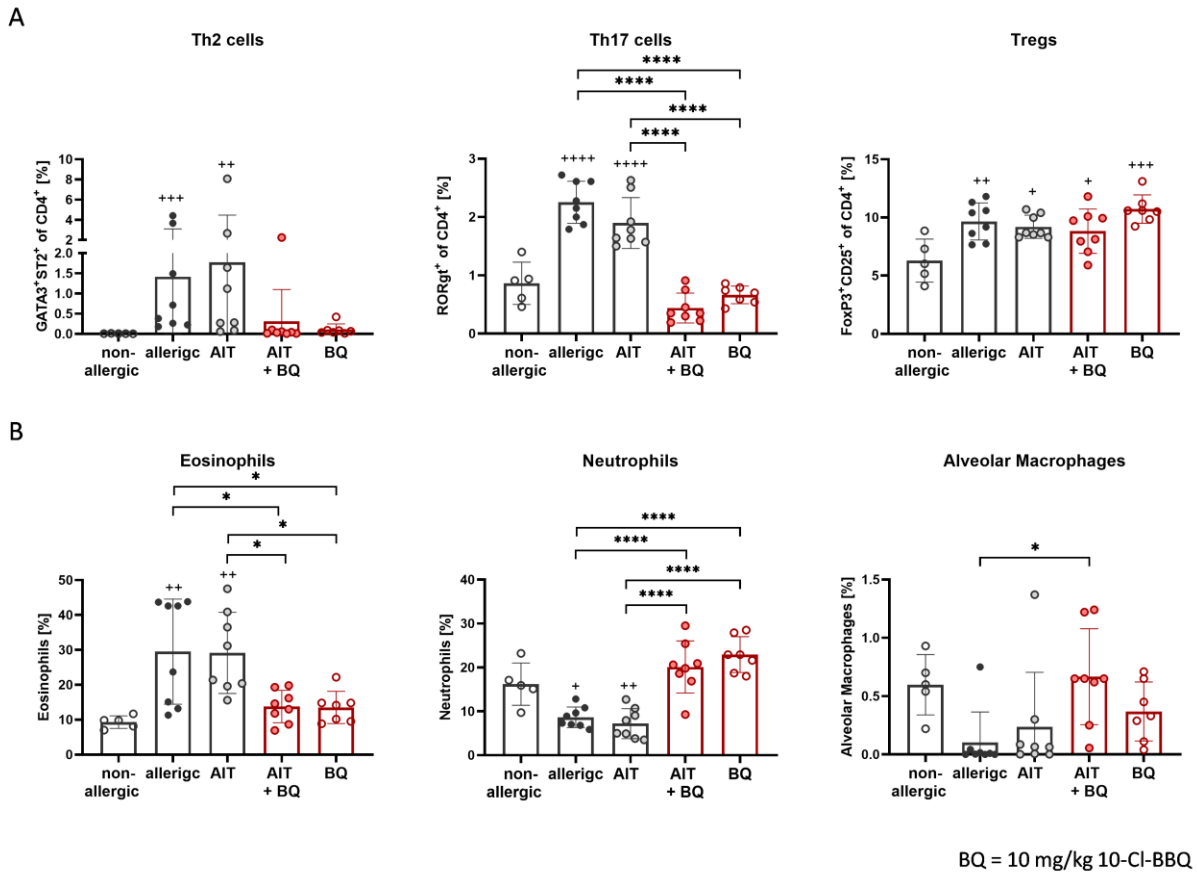

Figure 21: Acute effects of 10-Cl-BBQ as a single-treatment strategy or in combination with AIT on lung leucocytes in a murine model of OVA-induced allergic asthma. (A) Flow cytometric analysis of Th cell subsets at day 43 of the experiment (n = 5 - 8). (B) Flow cytometric analysis of eosinophils, neutrophils, and alveolar macrophages isolated from lung tissue at day 43 of the experiment (n = 5-8). Gaussian and non-Gaussian distributed results were analyzed by 1-way ANOVA with Tukey's test or Kruskal-Wallis test with Dunn's test, respectively. p-values of ≤.05, ≤.01, ≤.001, and ≤.0001 are shown as *, **, ***, and ****, respectively. p-values of allergic and treatment groups compared to the non-allergic group are shown as +, ++, +++, and ++++, respectively.

Th cell subsets in lung leucocytes showed a significant increase in percentages of Th2 cells, Th17 cells, and Tregs in allergic and AIT-treated mice compared to the non-allergic group (***Figure 21A***). 10-Cl-BBQ-treatment reduced Th2 cells by trend and Th17 cells in a highly significant manner for both ligand-treated groups. No significant differences could be detected between the AIT + 10-Cl-BBQ- and 10-Cl-BBQ-treated groups. Percentages of Tregs did not differ significantly between the allergic and the treatment groups. A slight trend towards increased Tregs could be observed for 10-Cl-BBQ-treated mice in comparison to the combined treatment strategy. Eosinophils were significantly reduced after treatment with 10-Cl-BBQ compared to allergic and AIT-treated mice,

whereas neutrophils were significantly increased ***(Figure 21B***). A significant difference between AIT + 10-Cl-BBQ and 10-Cl-BBQ treatment could not be detected. For both groups, percentages of eosinophils and neutrophils were on a comparable level as for mice in the non-allergic group. Alveolar macrophages were decreased in allergic and AIT-treated mice compared to the non-allergic group. Percentages of alveolar macrophages were elevated after treatment with 10-Cl-BBQ in both groups, but the combination with AIT increased alveolar macrophages even further and percentages were comparable to those of the non-allergic group. Overall, in Th cell subsets as well as in eosinophils, neutrophils, and alveolar macrophages, treatment with 10-Cl-BBQ induced a phenotype that closely resembles the phenotype of non-allergic mice.

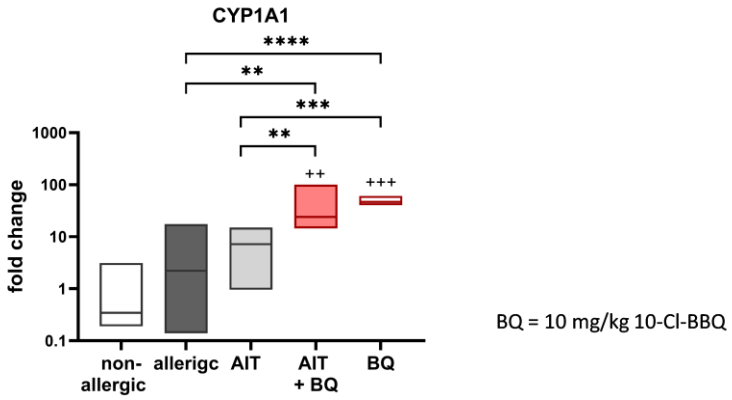

Figure 22: CYP1A1 expression after AIT- and 10-Cl-BBQ-treatment at day 43 in a murine model of OVA-induced allergic asthma. RNA was isolated from lung leucocytes and expression analysis of CYP1A1 was performed with qPCR (n = 5 - 8). Data of allergic, AIT, AIT + 10-Cl-BBQ, and 10-Cl-BBQ groups are shown as fold change compared to the non-allergic group. Boxplots indicate 25th percentile, median and 75th percentile. Gaussian distributed results were analyzed by 1-way ANOVA with Tukey's test.

Acute effects of 10-Cl-BBQ were analyzed directly after the treatment and AIT phase on day 43 to investigate the impact of repetitive 10-Cl-BBQ administration on the activation of the AhR. CYP1A1 expression was significantly increased in the AIT + 10-Cl-BBQ- and 10-Cl-BBQ-treated groups compared to non-allergic, allergic, and AIT-treated mice (***Figure 22***). This indicates a strong activation of the AhR by the administration of 10-Cl-BBQ. Allergic and AIT-treated mice did not differ significantly from the non-allergic control group.

Taken together, ligand treatment, either in addition to AIT or as a single-treatment strategy, did effectively reduce characteristics of allergic asthma in an acute manner, indicated by a decrease in BALF cell counts, Th2 cytokines in the BALF, as well as pulmonary Th2 and Th17 cells in comparison to allergic mice. The significant induction of CYP1A1 expression confirmed the activation of the AhR after treatment with 10-Cl-BBQ.

5.3.2 The combination of AIT with 10-Cl-BBQ-treatment increases therapeutic efficacy of AIT

Acute effects of 10-Cl-BBQ showed a reduction of characteristic parameters of allergic asthma in an acute manner. To address the impact of 10-Cl-BBQ on the outcome of AIT, treatment was repeated in a model of OVA-induced allergic asthma, which includes a final challenge phase after AIT and ligand treatment (***Figure 16***). Mice in the AIT + 10-Cl-BBQ and the 10-Cl-BBQ groups received daily applications of 10 mg/kg 10-Cl-BBQ (i.p.) and all other groups received respective amounts of solvent (i.p.).

Total BALF cell counts and counts of eosinophils, neutrophils, $CD4^+$ cells, $CD8^+$ cells, and alveolar macrophages were significantly increased in allergic mice compared to the non-allergic group (***Figure 23A***). AIT significantly reduced the number of total BALF cells, neutrophils, and $CD4^+$ cells in comparison to the allergic group. Cell counts of eosinophils, $CD8^+$ cells, and alveolar macrophages were decreased by trend. The combination of AIT and 10-Cl-BBQ reduced cell numbers in a highly significant manner for all analyzed cell populations, except for alveolar macrophages, which showed only a reduction by trend. However, the combined treatment strategy did reduce all analyzed cell populations further than AIT alone. A reduction in total BALF cells, even if not significant, could also be observed for treatment with 10-Cl-BBQ alone. Differential cell counts revealed that this effect is caused by a reduction of eosinophils only. Numbers of neutrophils, $CD4^+$ cells, $CD8^+$ cells, and alveolar macrophages were not affected by 10-Cl-BBQ without additional AIT. Cytokine analysis in the BALF revealed a significant reduction of IL-4 after the combined treatment strategy compared to the allergic group (***Figure 23B***). A similar, but not significant reduction could also be detected for the Th2 cytokines IL-5 and IL-13. Unexpectedly, Th2 cytokines were not much affected by standard AIT treatment. Treatment with

10-Cl-BBQ alone had no effects on Th2 cytokines and detected levels were similar to the allergic group. The Th17-associated cytokine IL-6 was reduced in both groups that were treated with 10-Cl-BBQ, whereas AIT led to an increase of IL-6. IL-17A seemed to be slightly reduced after AIT, with comparable results for the combined treatment strategy and variable levels of IL-17A for treatment with 10-Cl-BBQ alone. IFN-γ was slightly increased in the AIT-group, but not much affected in the other treatment groups. Levels of IL-10 in the analyzed BALF were very low and could not be detected reliably.

Th cell subsets analyzed in lung leucocytes revealed a visible, but not significant reduction of Th2 cells after AIT and an even stronger, significant reduction after combined treatment (***Figure 24A***). Th2 cells of the 10-Cl-BBQ-treated group were reduced comparably to those after combined treatment. Th17 cells were affected similarly, with a significant reduction after AIT compared to the allergic group and an even stronger, significant reduction after AIT + 10-Cl-BBQ and 10-Cl-BBQ treatment. Tregs showed an increase in all groups compared to non-allergic mice, but allergic and treatment groups did not differ significantly.

Eosinophils in isolated pulmonary leucocytes were significantly increased in allergic mice compared to the non-allergic group and slightly decreased after AIT (***Figure 24B***). Administration of 10-Cl-BBQ also induced only a slight reduction, but combined treatment significantly reduced eosinophils in comparison to the allergic group. Neutrophils showed a significant decrease for all groups, except for the combined treatment in comparison to non-allergic mice. Percentages of neutrophils after combined treatment were comparable to the non-allergic group. In addition, percentages of alveolar macrophages were comparable between the non-allergic and the AIT + 10-Cl-BBQ groups, whereas alveolar macrophages were significantly reduced in all other groups. Overall, the combined treatment seemed to restore levels of eosinophils and alveolar macrophages comparable to levels in non-allergic mice.

Histological analysis confirmed the strong impact of AIT + 10-Cl-BBQ on characteristics of allergic asthma (***Figure 24C***). For the AIT + 10-Cl-BBQ group, mucus hypersecretion and inflammatory cell infiltration were significantly reduced compared to the allergic and the AIT group. Standard AIT, as well as treatment with 10-Cl-BBQ slightly reduced mucus hypersecretion and inflammatory cell infiltration compared to the allergic group.

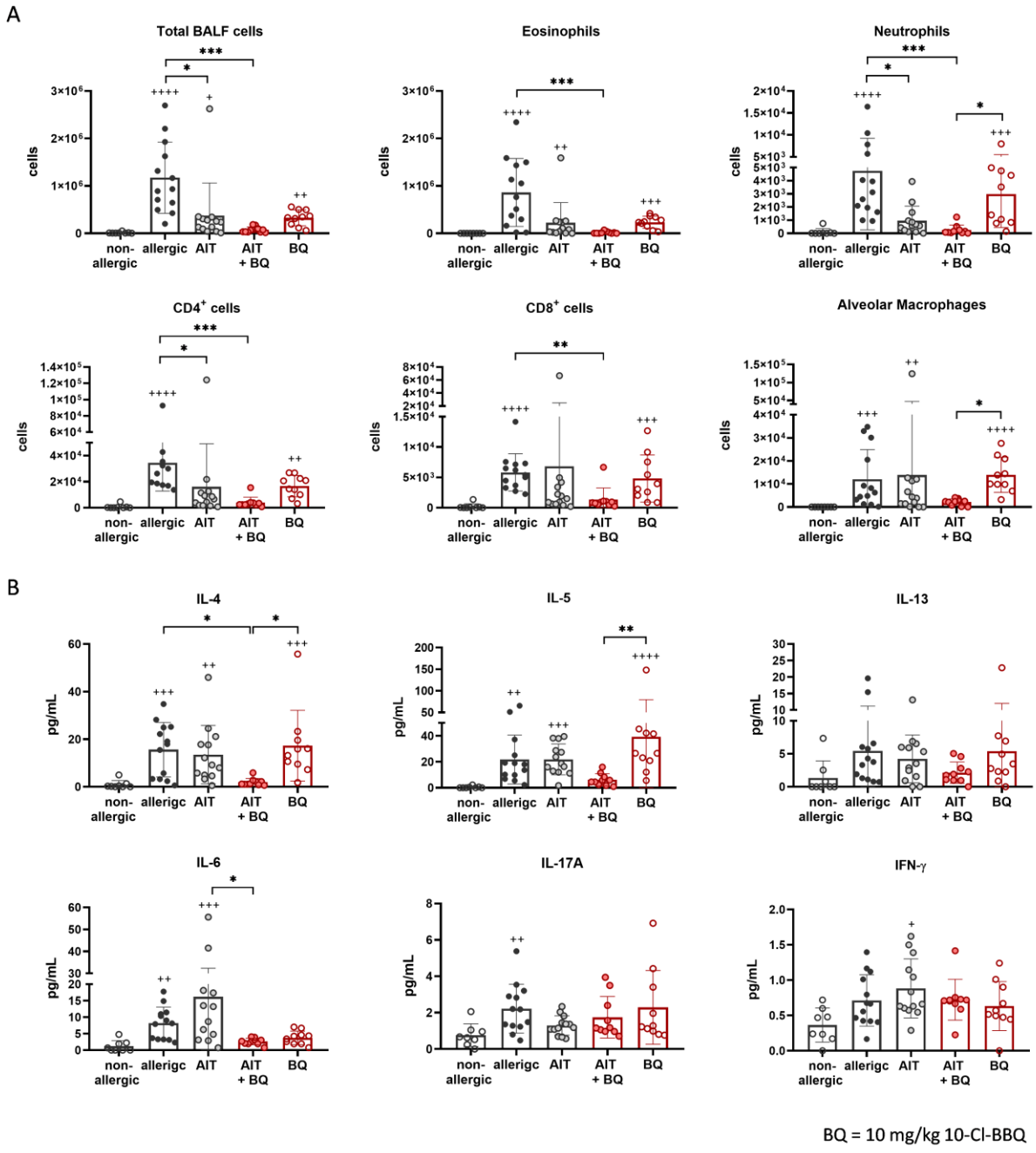

Figure 23: Effects of 10-Cl-BBQ in combination with AIT standard treatment or as a single-treatment strategy on BALF cells and cytokines in a murine model of OVA-induced allergic asthma. (A) Total and differential BALF cell counts analyzed at the end (day 64) of the experiment (n = 8 - 13). (B) Cytokine analysis in the BALF at the end (day 64) of the experiment (n = 8 - 13). Gaussian and non-Gaussian distributed results were analyzed by 1-way ANOVA with Tukey's test or Kruskal-Wallis test with Dunn's test, respectively. p-values of ≤.05, ≤.01, ≤.001, and ≤.0001 are shown as *, **, ***, and ****, respectively. p-values of allergic and treatment groups compared to the non-allergic group are shown as +, ++, +++, and ++++, respectively.

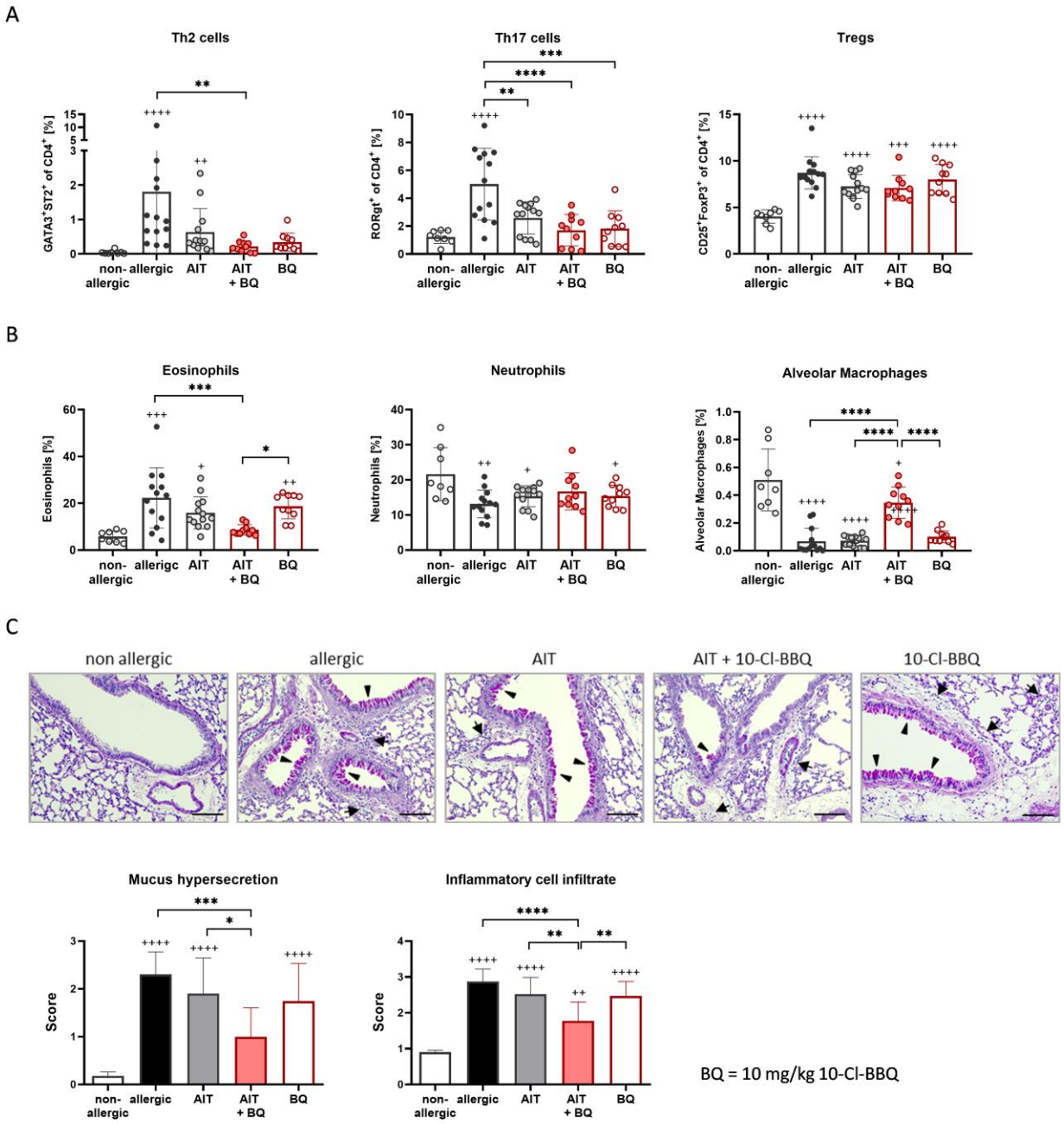

Figure 24: Effects of 10-Cl-BBQ in combination with AIT standard treatment or as a single-treatment strategy in lung leucocytes and lung tissue. (A) Flow cytometric analysis of Th2 cells, Tregs, Th17 cells, eosinophils, neutrophils, and alveolar macrophages in isolated lung leucocytes at the end (day 64) of the experiment (n = 8 - 13). (B) Histological analysis of lung tissue: representative PAS staining of lung tissue (arrowheads: mucus hypersecretion; arrows: inflammatory cell infiltrate; scale bar: 100µm) and scoring of mucus hypersecretion (n = 8 - 13) and inflammatory cell infiltrate (n = 8 - 13). Gaussian and non-Gaussian distributed results were analyzed by 1-way ANOVA with Tukey's test or Kruskal-Wallis test with Dunn's test, respectively. p-values of ≤.05, ≤.01, ≤.001, and ≤.0001 are shown as *, **, ***, and ****, respectively. p-values of allergic and treatment groups compared to the non-allergic group are shown as +, ++, +++, and ++++, respectively.

Immunoglobulins were analyzed in serum samples collected at the end (day 64) of the experiment. Total IgE was increased in all groups compared to the non-allergic group (***Figure 25A***). Levels of total IgE did not differ significantly between the allergic and treatment groups. OVA-sIgE was significantly increased in allergic, AIT- and 10-Cl-BBQ-treated mice, whereas the AIT + 10-Cl-BBQ-treatment showed only a slight and not significant increase (***Figure 25B***). Slight but not significant reductions in OVA-sIgE could be observed for the AIT and AIT + 10-Cl-BBQ groups in comparison to allergic mice.

Detected levels of total IgG1 were significantly increased in all groups compared to the non-allergic group (***Figure 25C***). The strongest increase was observable for AIT and AIT + 10-Cl-BBQ treated mice. Levels of total IgG1 measured in those groups were significantly higher than in allergic and 10-Cl-BBQ-treated mice. A similar pattern was detectable for OVA-sIgG1 (***Figure 25D***). Detected levels were significantly increased for all groups compared to non-allergic mice. AIT- and AIT + 10-Cl-BBQ-treated mice showed even higher levels that were significantly increased compared to allergic and 10-Cl-BBQ-treated groups.

The combined treatment strategy showed a stronger impact on OVA-sIgE, but total IgE, total IgG1, and OVA-sIgG1 were not influenced differently than after standard AIT.

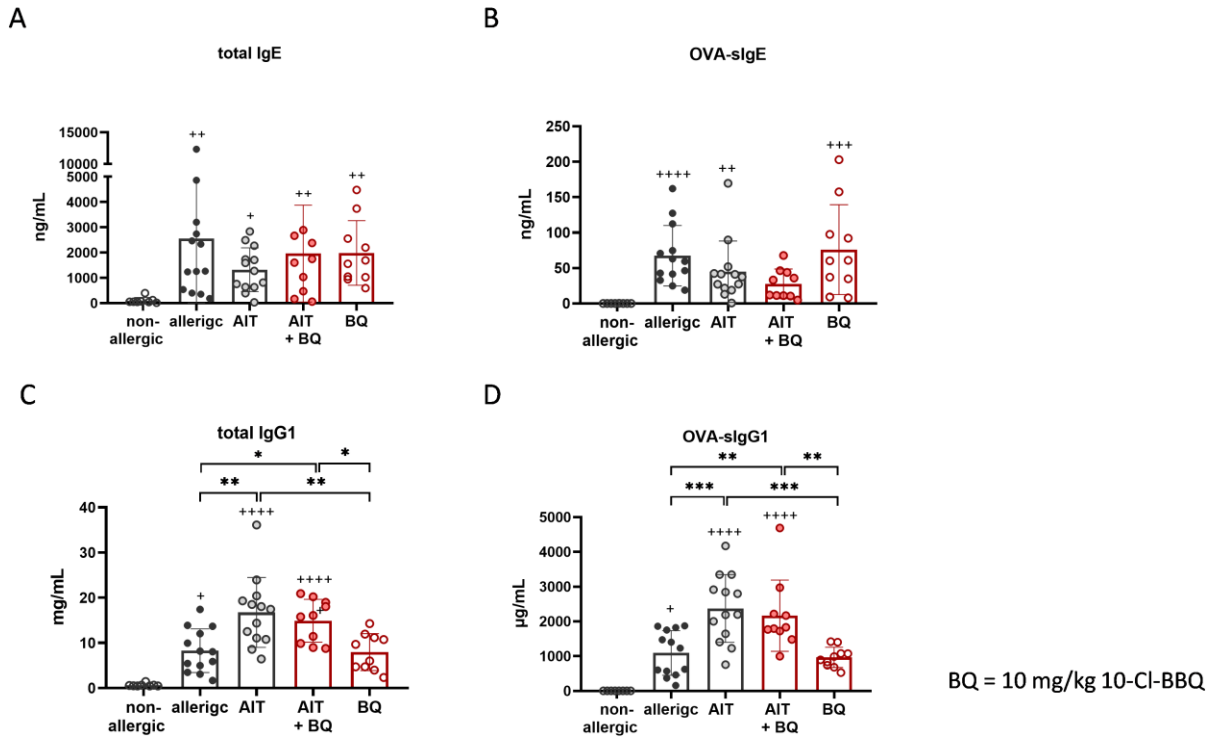

Figure 25: Analysis of the effects of AIT and 10-Cl-BBQ-treatment on immunoglobulins in a murine model of OVA-induced allergic asthma. Serum samples were collected at the end (day 64) of the experiment. (A) Analysis of total IgE, (B) OVA-sIgE, (C) total IgG1, and (D) OVA-sIgG1 (n = 8 - 13). Gaussian and non-Gaussian distributed results were analyzed by 1-way ANOVA with Tukey's test or Kruskal-Wallis test with Dunn's test, respectively. p-values of ≤.05, ≤.01, ≤.001, and ≤.0001 are shown as *, **, ***, and ****, respectively. p-values of allergic and treatment groups compared to the non-allergic group are shown as +, ++, +++, and ++++, respectively.

An impact of 10-Cl-BBQ treatment on the expression of CYP1A1 was not detectable at the end (day 64) of the experiment (***Figure 26***). Analysis of CYP1A1 mRNA expression in isolated lung leucocytes did not reveal any significant differences between any of the analyzed groups.

The combination of AIT with 10-Cl-BBQ treatment did improve the effects of AIT, detectable by a more pronounced reduction of total and differential BALF cell counts, Th2 cytokines in the BALF, as well as pulmonary eosinophils, Th2, and Th17 cells. In addition, alveolar macrophages showed comparable percentages as in non-allergic mice. Significantly reduced mucus hypersecretion, inflammatory cell infiltration, and reduced OVA-sIgE after combined treatment compared to AIT complement the observed beneficial effects.

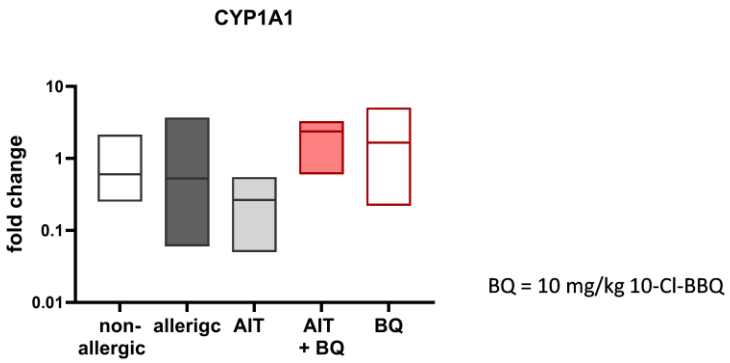

Figure 26: Expression levels of CYP1A1 in lung leucocytes after AIT and 10-Cl-BBQ treatment in a murine model of OVA-induced allergic asthma. RNA was isolated from lung leucocytes at the end (day 64) of the experiment. Expression analysis of CYP1A1 was performed with qPCR (n = 8 - 13). Data of allergic, AIT, AIT + 10-Cl-BBQ, and 10-Cl-BBQ groups are shown as fold change compared to the non-allergic group. Boxplots indicate 25th percentile, median and 75th percentile. Gaussian distributed results were analyzed by 1-way ANOVA with Tukey's test.

5.3.3 The transcriptional profile of pulmonary CD4$^+$ T cells is not changed by 10-Cl-BBQ treatment

Treatment of mice with 10-Cl-BBQ during AIT showed strong effects on eosinophils in the BALF, on pulmonary Th2 and Th17 cells, as well as on alveolar macrophages. To address potential mechanisms that are targeted and induced by activation of the AhR through 10-Cl-BBQ, pulmonary CD4$^+$ T cells were further analyzed by scRNA-seq. Cluster analysis, percentages of the identified clusters, and differences in the transcription profiles were compared between AIT-, AIT + 10-Cl-BBQ-, and 10-Cl-BBQ-treated mice. CD4$^+$ T cells were chosen because 10-Cl-BBQ was reported in literature to directly bind to the AhR in those cells[142,143]. Nevertheless, eosinophils and alveolar macrophages remain interesting targets to further evaluate the mechanism of action of 10-Cl-BBQ in the context of AIT.

Cluster analysis with tSNE embedding was performed for the groups treated with AIT, AIT + 10-Cl-BBQ, and 10-Cl-BBQ. Clusters were assigned to respective cell populations by analysis of the most differentially expressed genes. Pulmonary CD4$^+$ T cells were not sorted but enriched with a bead-based isolation kit. Contamination with other cell populations to some extent is expected and clusters representing innate lymphoid, cytotoxic, and natural killer T cells were not investigated any further. Overall, the clustering was comparable between all three treatment strategies (***Figure***

27), indicating that 10-Cl-BBQ did not induce CD4$^+$ T cell populations that are not present in AIT-treated mice. Clusters of interest were further investigated regarding changes in percentage between the different treatment groups (***Table 19***) as well as changes in their transcription profile. The most striking differences in percentage were observable for Th2 cells (cluster 2) and Tregs (cluster 3). The combined treatment strategy strongly reduced the numbers of Th2 cells compared to standard AIT and to a smaller extent also to 10-Cl-BBQ treatment. Tregs were reduced only after combined treatment, 10-Cl-BBQ alone did not affect the percentage of Tregs compared to standard AIT treatment. Naïve CD4$^+$ T cells (cluster 0) were decreased after administration of 10-Cl-BBQ, whereas a higher percentage of differentiating CD4$^+$ T cells (cluster 1) was detectable, compared to AIT and AIT + 10-Cl-BBQ. Whereas naïve CD4$^+$ T cells did not differ in percentage between AIT and AIT + 10-Cl-BBQ, the population of differentiating CD4$^+$ T cells was increased after combined treatment compared to AIT. Cluster 5, representing naïve CD4$^+$ T cells with upregulated INF-response genes and cluster 6, consisting of CD4$^+$ T cells with upregulated regulatory genes, were not affected by 10-Cl-BBQ or the combination of both treatment strategies. The most abundant difference in percentage in Th2 cells confirmed the results obtained in the flow cytometric analysis, showing a stronger reduction of Th2 cells when AIT is combined with the administration of 10-Cl-BBQ.

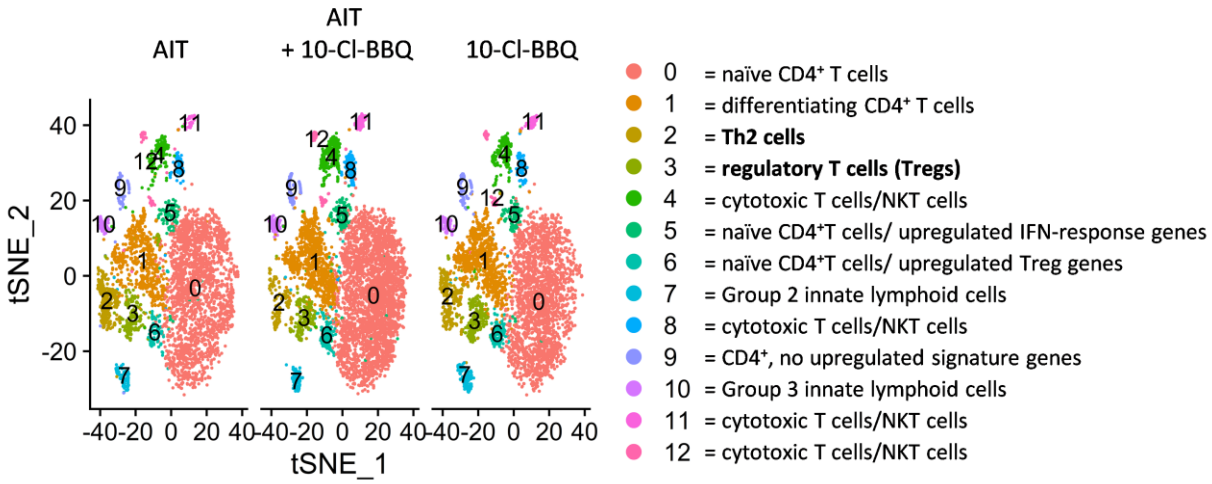

Figure 27: scRNA-seq analysis of enriched pulmonary CD4$^+$ T cells in a murine model of OVA-induced allergic asthma. Clusters in tSNE embedding for mice treated with AIT, AIT + 10-Cl-BBQ, and 10-Cl-BBQ. Clusters were allocated to cell populations according to their most differentially expressed genes.

Table 19: Percentages of clusters of interest.

		Percentage [%]		
Cluster	**Population of CD4⁺ cells**	**AIT**	**AIT + 10-Cl-BBQ**	**10-Cl-BBQ**
0	naive	53.4	54.3	49.1
1	differentiating	14.0	16.9	19.1
2	Th2	8.9	2.4	6.3
3	Tregs	5.7	3.8	5.4
5	naive/IFN-response genes	2.7	2.9	2.5
6	naive/Treg genes	2.1	2.9	2.6

5.3.4 Effects of 10-Cl-BBQ treatment in AhR$^{-/-}$ mice

The significantly increased expression levels of CYP1A1 in lung leucocytes directly after 10-Cl-BBQ treatment on day 43 (***Figure 22***) indicated a strong activation of the AhR. To further verify that the AhR is not only targeted by 10-Cl-BBQ, but responsible for the observed effects on a cellular level, sensitized wildtype and AhR$^{-/-}$ mice received daily applications of 10 mg/kg 10-Cl-BBQ (i.p.) between days 34 to 42 (***Figure 28***). Effects were compared to non-allergic mice as well as to allergic mice without additional 10-Cl-BBQ treatment, which received respective amounts of solvent (i.p.) between days 34 to 42.

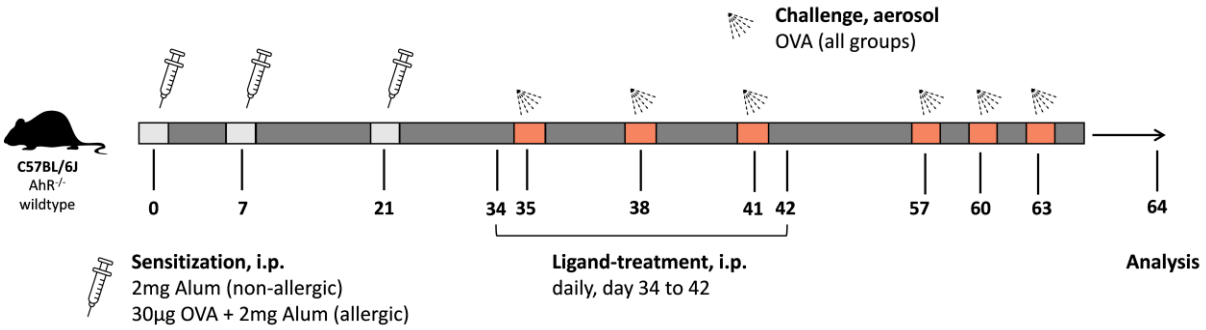

Figure 28: Effects of 10-Cl-BBQ-treatment in AhR$^{-/-}$ mice in a murine model of OVA-induced allergic asthma. Mice of the non-allergic group were treated with 2 mg alum in PBS (i.p.) on days 0, 7, and 21, all other groups were sensitized with 30 µg OVA and 2 mg alum in PBS (i.p.). Respective groups were treated daily with AhR-ligands or solvents (i.p.) from days 34 to 42. All mice were challenged with 1 % OVA aerosol for 15 min on days 35, 38, 41, 57, 60, and 63. Final analysis of all groups was performed on day 64.

Total and differential BALF cell counts were increased in the allergic groups of AhR$^{-/-}$ and wildtype mice compared to the respective non-allergic groups (***Figure 29***). A significant increase in total BALF cell counts, eosinophils, CD4$^{+,}$ and CD8^{+} cell counts was detectable for both mouse lines, whereas a significant increase in neutrophils was only detectable in AhR$^{-/-}$ mice and alveolar macrophages increased only significantly in wildtype mice. An increase was also detectable for neutrophils in wildtype and for alveolar macrophages in AhR$^{-/-}$ mice, but not in a significant manner. Cell counts were comparable for the non-allergic groups of both mouse lines, as well as for the allergic groups with no significant differences. Total BALF cell counts showed a significant reduction in wiltype mice after treatment with 10-Cl-BBQ compared to allergic wildtype mice. On the contrary, no significant difference was detectable for 10-Cl-BBQ-treated AhR$^{-/-}$ mice in comparison to the respective allergic control group. Total BALF cell counts of 10-Cl-BBQ-treated wildtype and AhR$^{-/-}$ mice differed significantly from each other. A similar picture could be observed for the counts of eosinophils, with a significant reduction in 10-Cl-BBQ-treated wildtype mice compared to the allergic group and no decreasing effects of 10-Cl-BBQ on eosinophilic counts of AhR$^{-/-}$ mice. For differential counts of neutrophils, CD4^{+} cells, CD8^{+} cells, and alveolar macrophages, no significant differences were detectable between 10-Cl-BBQ-treated AhR$^{-/-}$ and wildtype mice.

Striking differences between 10-Cl-BBQ-treated AhR$^{-/-}$ and wildtype mice could be detected in the flow cytometric analysis of Th cell subsets in pulmonary leucocytes (***Figure 30A***). Th2 cells increased significantly in allergic AhR$^{-/-}$ mice compared to the non-allergic control group. Allergic wildtype mice showed a comparable, although not significant increase in Th2 cells in comparison to the respective non-allergic group. Whereas 10-Cl-BBQ treatment reduced Th2 cells in wildtype mice, no reduction could be observed in AhR$^{-/-}$ mice in comparison to the allergic group. Th2 cells were significantly increased in AhR$^{-/-}$ mice after administration of 10-Cl-BBQ. Percentages of Th2 cells differed significantly between 10-Cl-BBQ-treated AhR$^{-/-}$ and wildtype mice. In non-allergic mice, Th17 cells were significantly increased in AhR$^{-/-}$ mice compared to wildtype mice. No differences could be observed between non-allergic, allergic, and 10-Cl-BBQ-treated AhR$^{-/-}$ mice. On the contrary, Th17 cells in wildtype mice increased significantly in the allergic group in comparison to non-allergic mice. 10-Cl-BBQ reduced percentages significantly compared to the allergic group. Percentages of Th17 cells differed significantly between AhR$^{-/-}$ and wildtype mice

after treatment with 10-Cl-BBQ. Treg cells revealed no differences comparing AhR$^{-/-}$ to wildtype mice. An increase was observable for the allergic and the 10-Cl-BBQ-treated groups in comparison to the respective control groups for both mouse lines.

Eosinophils in isolated pulmonary leucocytes were increased significantly in allergic AhR$^{-/-}$ mice and to a comparable, but not significant extent in allergic wildtype mice (***Figure 30B***). 10-Cl-BBQ treatment slightly reduced percentages of eosinophils in wildtype mice, but not in AhR$^{-/-}$ mice. Significantly higher percentages of eosinophils were detected in AhR$^{-/-}$ mice after 10-Cl-BBQ treatment in comparison to 10-Cl-BBQ-treated wildtype mice. Neutrophils were reduced slightly in allergic AhR$^{-/-}$ and significantly in 10-Cl-BBQ-treated AhR$^{-/-}$ mice compared to the respective non-allergic control group. In non-allergic wildtype mice, percentages of neutrophils were significantly increased compared to non-allergic AhR$^{-/-}$ mice. Neutrophils were significantly reduced in allergic and 10-Cl-BBQ-treated wildtype mice. After 10-Cl-BBQ treatment, percentages of neutrophils were significantly higher in wildtype mice. Alveolar macrophages did not differ significantly between AhR$^{-/-}$ and wildtyp mice. Percentages were significantly decreased in allergic and 10-Cl-BBQ-treated groups in both mouse lines.

Histological analysis of lung tissue revealed no significant differences between AhR$^{-/-}$ and wildtype mice regarding mucus hypersecretion (***Figure 30C***). The score was increased for both allergic groups in comparison to the non-allergic control groups. But whereas wildtype mice showed a significant reduction after treatment with 10-Cl-BBQ compared to the allergic group, the scores were comparable for allergic and 10-Cl-BBQ-treated AhR$^{-/-}$ mice. Both mouse lines showed a significant increase in inflammatory cell infiltrate in the allergic groups compared to the respective control groups. Infiltration of inflammatory cells was significantly reduced in 10-Cl-BBQ treated wildtype mice compared to the allergic control group. In AhR$^{-/-}$ mice treatment with 10-Cl-BBQ showed no effect on inflammatory cell infiltration and scoring was comparable to the allergic group.

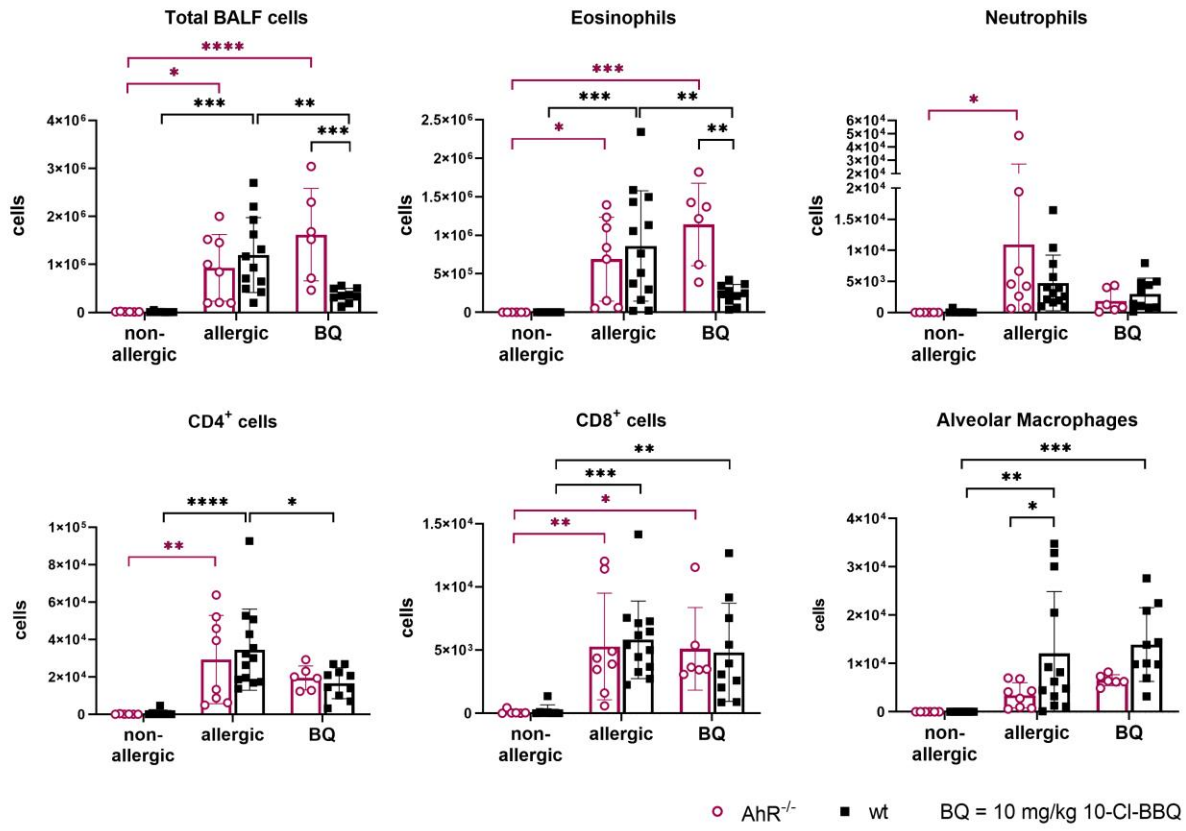

Figure 29: Total and differential BALF cell counts of non-allergic, allergic, and 10-Cl-BBQ-treated mice in a murine model of OVA-induced allergic asthma. Total BALF cell counts and differential counts of eosinophils, neutrophils, $CD4^+$ cells, $CD8^+$ cells, and alveolar macrophages were analyzed at the end (day 64) of the experiment (n = 8 - 13). Data analysis with 2-way ANOVA with Tukey's multiple comparisons test or Sidak's multiple comparisons test, respectively. p-values of ≤.05, ≤.01, ≤.001, and ≤.0001 are shown as *, **, ***, and ****, respectively.

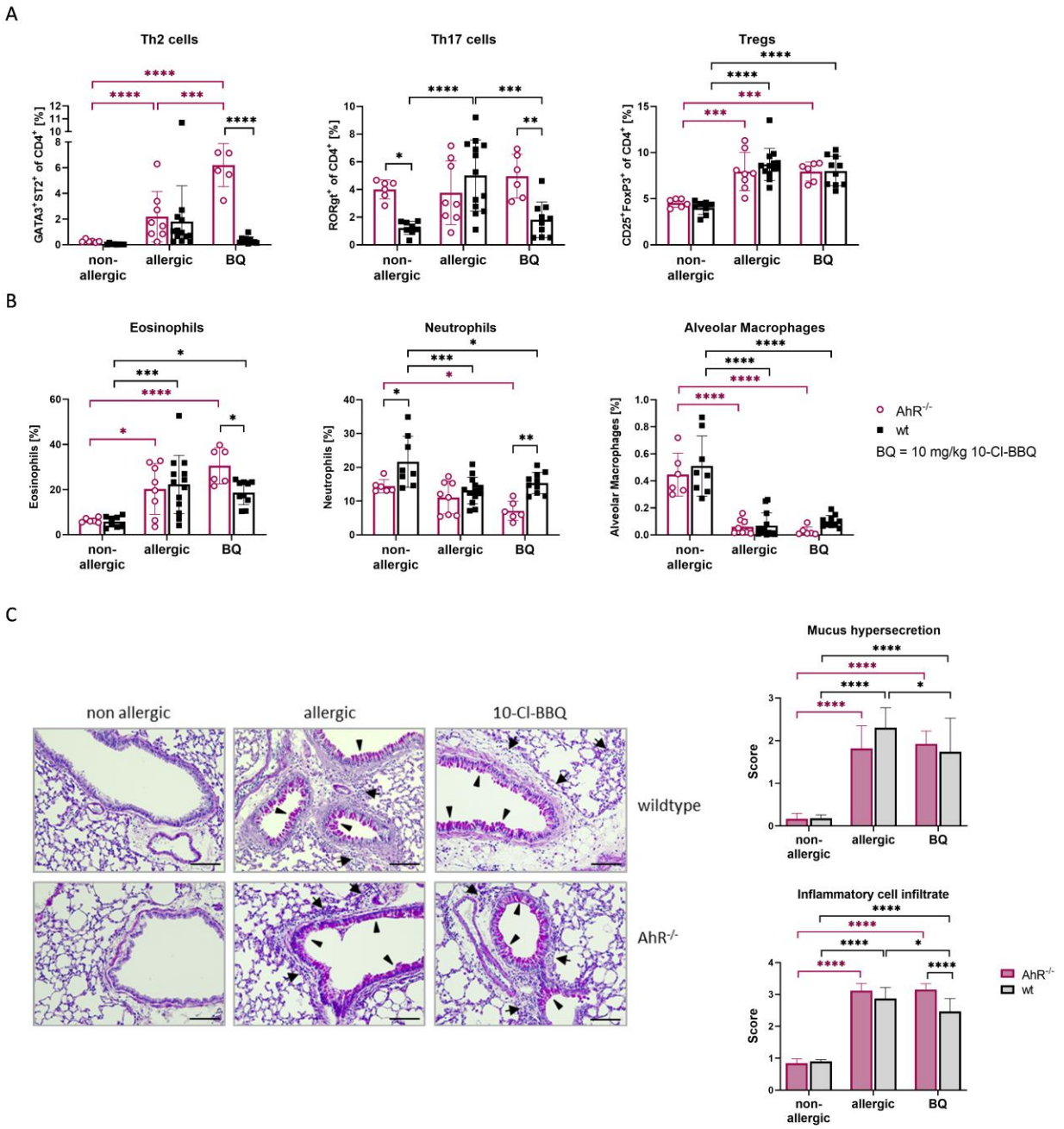

Figure 30: Comparison of the effects of 10-Cl-BBQ treatment on lung leucocytes and lung histology between AhR$^{-/-}$ and wildtype mice in a murine model of OVA-induced allergic asthma. (A) Flow cytometric analysis of Th cell subsets at the end (day 64) of the experiment (n = 8 - 13). (B) Flow cytometric analysis of eosinophils, neutrophils, and alveolar macrophages isolated from lung tissue at the end (day 64) of the experiment (n = 8 - 13). (C) Histological analysis of lung tissue: representative PAS staining of lung tissue (arrowheads: mucus hypersecretion; arrows: inflammatory cell infiltrate; scale bar: 100µm) and scoring of mucus hypersecretion (n = 6) and inflammatory cell infiltrate (n = 6). Data analysis with 2-way ANOVA with Tukey's multiple comparisons test or Sidak's multiple comparisons test, respectively. p-values of ≤.05, ≤.01, ≤.001, and ≤.0001 are shown as *, **, ***, and ****, respectively.

Analysis of immunoglobulins in serum collected at the end of the experiment did show an increase of all analyzed parameters in the allergic and the 10-Cl-BBQ-treated groups compared to non-allergic mice. Levels of total IgE (***Figure 31A***) and OVA-sIgE (***Figure 31B***) were significantly elevated in allergic and 10-Cl-BBQ-treated $AhR^{-/-}$ mice compared to respectively treated wildtype mice. Total IgG1 was comparable between allergic wildtype and allergic $AhR^{-/-}$ mice (***Figure 31C***) and significantly elevated after treatment with 10-Cl-BBQ in $AhR^{-/-}$ compared to ligand-treated wildtype mice. OVA-sIgG1 showed significantly elevated levels in allergic and 10-Cl-BBQ-treated $AhR^{-/-}$ mice compared to similarly treated wildtype mice (***Figure 31D***).

In summary, treatment of $AhR^{-/-}$ mice with 10-Cl-BBQ could demonstrate that the AhR is responsible for the previously observed effects. Whereas wildtype mice revealed decreased numbers of total BALF cells and eosinophils, as well as pulmonary Th2 and Th17 cells after ligand treatment, those parameters were not affected in $AhR^{-/-}$ mice. Also, mucus hypersecretion and inflammatory cell infiltration were only affected in wildtype mice. Immunoglobulins in serum were not affected by ligand treatment. The increased levels in $AhR^{-/-}$ mice are in line with a more pronounced allergic phenotype observable in $AhR^{-/-}$ mice[179,180].

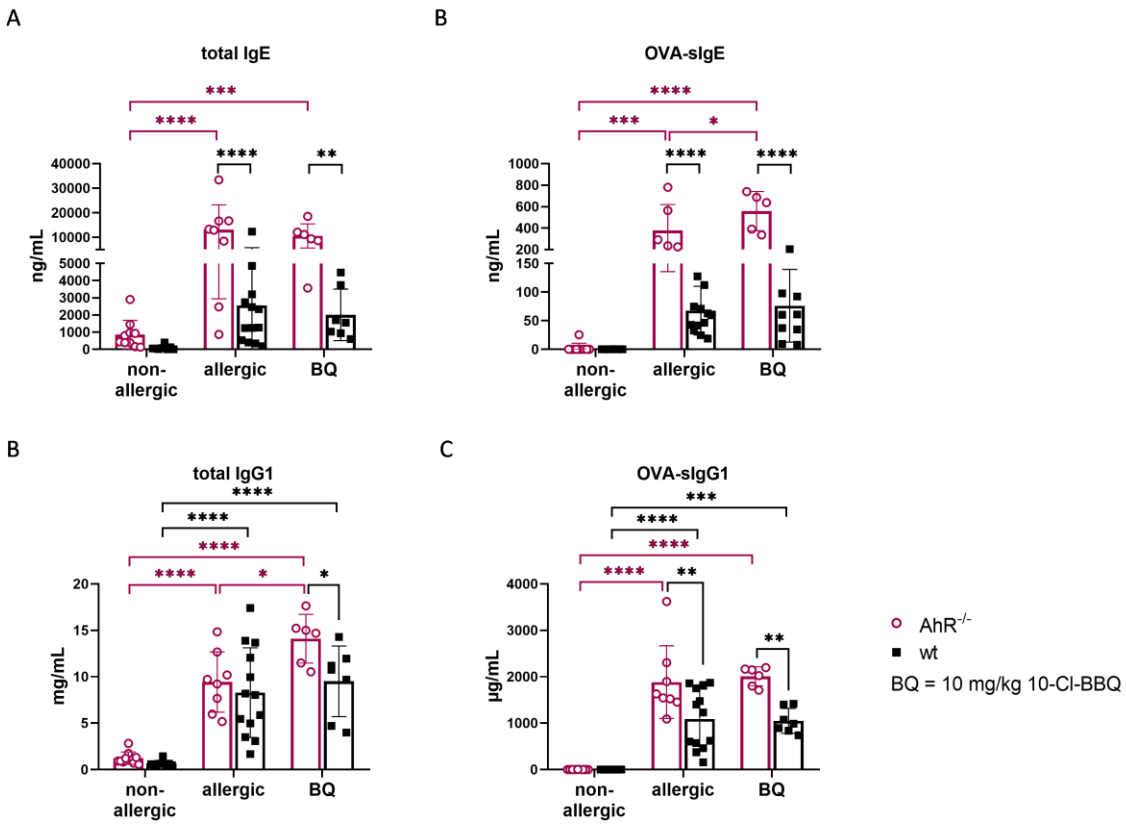

Figure 31: Analysis of immunoglobulins levels after 10-Cl-BBQ treatment in a murine model of OVA-induced allergic asthma. Serum samples were collected at the end (day 64) of the experiment. (A) Analysis of total IgE and (B) OVA-sIgE (n = 8 - 13). (C) Analysis of total IgG1 and (D) OVA-sIgG1 (n = 8 - 13). Data analysis with 2-way ANOVA with Tukey's multiple comparisons test or Sidak's multiple comparisons test, respectively. p-values of ≤.05, ≤.01, ≤.001, and ≤.0001 are shown as *, **, ***, and ****, respectively.

6. Discussion

This study addresses the general role of the AhR in AIT and further elucidates the impact of AhR activation on AIT. The hypothesized importance of the AhR for the successful outcome of AIT was investigated in models of OVA- and HDM-induced allergic asthma. Different outcomes after AhR activation with the low-affinity ligand quercetin and the high-affinity ligand 10-Cl-BBQ highlight that the choice of the ligand is essential in shaping the therapeutic outcome of AIT.

6.1 General role of the AhR in AIT

The performance of AIT in murine models of allergic asthma using $AhR^{-/-}$ mice did show that involvement of the AhR is no mandatory requirement for a successful therapeutic outcome. The direct comparison of the therapeutic effects between $AhR^{-/-}$ and wildtype mice in the OVA model demonstrated that AIT was not only working in $AhR^{-/-}$ mice, but that the overall outcome was comparable to wildtype mice. The more pronounced allergic phenotype presented by allergic $AhR^{-/-}$ mice, with elevated Th2 cytokines in BALF, total IgE, and OVA-sIgE compared to allergic wildtype mice, did not attenuate the effects of AIT in the OVA model. In addition, this confirms that the OVA AIT protocol is working efficiently and stably, also for mice with a more pronounced allergic phenotype. Reinforced allergic phenotypes for $AhR^{-/-}$ mice have already been described in literature, with an even stronger expression of the characteristics of allergic asthma than observed in this study[179,180]. The differences in outcome are most likely attributable to the choice of the allergy model. In a model of OVA-induced allergic asthma with sensitizations on days 1, 4, and 7, followed by challenges for four weeks, $AhR^{-/-}$ mice did not only reveal elevated Th2 cytokines and IgE levels but also significantly increased BALF cell counts, mucus production, airway hyperresponsiveness, and airway remodeling compared to wildtype mice[179]. A rather short model of OVA-induced allergic asthma with just one sensitization and three aerosol challenges after two weeks also resulted in increased BALF cell counts, with increased eosinophilia and lymphocyte influx into the airways in $AhR^{-/-}$ mice compared to wildtype mice, but no differences in airway hyperresponsiveness and airway remodeling[180]. The period between sensitization and

challenge phase, but mainly the number of challenges seem to highly affect the manifestation of murine allergic asthma in $AhR^{-/-}$ mice. These observations do not affect the investigation regarding the necessity of the AhR in AIT in the first place. However, it is worth mentioning that existing protocols of AIT could need adaption to meet therapeutic needs in highly allergic individuals if standard treatment shows not to be sufficient.

In addition to the results generated in the OVA model, successful AIT in $AhR^{-/-}$ mice could be also demonstrated in a model of HDM-induced allergic asthma to a certain extent. BALF cell counts, BALF cytokines, and pulmonary Th2 and Th17 cells did decrease after AIT, but variation in cell numbers and percentages for both allergic and AIT-treated mice allowed only a careful statement regarding successful therapy. For a reliable statement if AIT works in $AhR^{-/-}$ mice, independent of the choice of the causing allergen and the respective model, the experiment needs to be repeated in a stable experimental setup. A suitable experimental setup requires a treatment protocol that reliably induces a detectable increase in the characteristic parameters of allergic asthma in the allergic control group. This is an essential requirement to properly evaluate the effects of AIT. In addition, respective control groups with wildtype mice will be needed to evaluate the efficacy of AIT in HDM-induced allergic asthma for $AhR^{-/-}$ mice. In contrast to OVA, which represents only a single allergen, the HDM-extract consists of a mixture of various allergens. Subsequently, the immune response in the HDM model is directed against several allergens, likely resulting in a weaker allergic response, which complicates the establishment of a reliable model of HDM-induced allergic asthma. Nevertheless, first results of this pilot study in the HDM model point in the direction of successful AIT in $AhR^{-/-}$ mice, indicating that successful therapy is not restricted to the OVA model and therefore not limited to a specific allergen.

The AhR is involved in various biological processes including numerous mechanisms related to immune functions. Immunoregulatory roles of the AhR have already been described for various immune cell types, including T cells, macrophages, and DCs[181].

DCs serve as professional APCs that induce the differentiation of naïve T cells into effector T cells[182]. Specialized subsets of DCs, so called regulatory DCs (DCregs), can further be divided into immunogenic and tolerogenic DCs[183]. In the context of T cell activation, their function is

mainly mediated by regulatory factors, like IL-10 and IDO[184]. IDO is an immunosuppressive enzyme that catalyzes the essential amino acid tryptophan into kynurenine and AhR signaling has been identified as a requirement for IDO expression in DCs[185]. Nguyen *et al*. investigated a role of the AhR in modulating the function of DCregs by induction of naïve T cell differentiation into Tregs and Th17 cells in the presence of lipopolysaccharides (LPS) and CpG[181]. In $AhR^{-/-}$ mice secretion of IL-10 was impaired, likely affecting tolerance negatively and shifting towards a pro-inflammatory state[181]. In the absence of AhR and IDO expression in bone marrow-derived DCs, naïve T cell differentiation skewed away from a Treg toward a Th17 fate[181].

Cui *et al*. could demonstrate that AhR activation in DCs resulted in a lower expression of the costimulatory surface markers CD80, CD83, and CD86 involved in T cell activation, reduced production of inflammatory cytokines, and an increase in anti-inflammatory cytokine production[186]. The AhR-activated tolerogenic DCs were able to promote differentiation of Tregs and transfer of these tolerogenic DCs to a colitis mouse model significantly alleviated the severity of inflammation[186].

AhR-deficient murine DCs display no impaired development *in vivo* or when differentiated *in vitro* from bone marrow[181,187]. However, the results of the above mentioned studies show an important influence of AhR signaling on the differentiation of DC phenotypes. The importance of AhR activation in DCs to promote Treg differentiation has been addressed extensively[93,188,189] and is in line with the observation that AhR-deficient DCs are not able to promote Treg differentiation *in vitro* and boost the generation of Th17 cells instead[181,190].

The AhR is not only able to modulate T cell differentiation and function indirectly via DCs but also through direct transactivation and induction of epigenetic modifications[103]. It has been shown that the AhR plays an important role in the development and function of natural $FoxP3^+$ Tregs (nTregs), generated in the thymus, and in induced $FoxP3^+$ Tregs (iTregs), generated in the periphery[191]. The AhR has a large impact on Tregs by direct induction of FoxP3[192] and by control of its epigenetic status, making it more accessible to the transcription machinery[149]. Furthermore, AhR increases the expression of SMAD1, which stabilizes the FoxP3 expression[193] and limits STAT1 activation, which is known to inhibit Treg differentiation[194]. In addition, a function of the AhR has not only been associated with $FoxP3^+$ Tregs but also with the differentiation of $FoxP3^-$ $IL10^+CD4^+$ regulatory T cells, so called type 1 regulatory T (Tr1) cells.

Besides playing an important role in Treg differentiation, the AhR is also highly expressed in Th17 cells[192]. Activation of the AhR by the endogenous ligand FICZ has been shown to enhance Th17 differentiation and promote IL-22 production[108,195]. The AhR is most likely involved in the recruitment of the transcription factor RORγt to the IL-22 promotor[108,192,195]. But AhR activation does not only enhance Th17 differentiation. IL-2, as well as activation of STAT5 and STAT1, have been shown to inhibit Th17 differentiation[196,197]. The AhR is able to induce the expression of the transcription factor Aiolos, which silences *il2* expression[197] and inhibits the activation of STAT5 and STAT1[194]. Therefore, in this context AhR deficiency promotes Th17 differentiation. This could explain the higher percentages of Th17 cells in non-allergic $AhR^{-/-}$ mice compared to non-allergic wildtype mice observed in this study. However, the comparison of allergic and AIT-treated groups lead to comparable percentages of Th17 cells.

The above mentioned studies describe only a few mechanisms in which the AhR is critically involved. However, they already highlight the importance of the AhR for the induction of regulatory immune cell subsets. The principle of AIT is based on tolerance induction towards the causing allergen. Induction of regulatory phenotypes of immune cells is a central element of a successful therapeutic outcome. Especially tolerogenic DCs and Tregs hold important functions in this process. Therefore, it was rather unexpected that AhR-deficiency did not impact and diminish the therapeutic outcome of AIT. Therefore, the first hypothesis of this work, stating that the AhR is likely a requirement for successful AIT, could not be confirmed but was experimentally disproven. Nevertheless, a closer look at Treg and DC phenotypes could still be interesting and could address the question if tolerogenic subsets differ in the absence of AhR signaling and what signaling pathways are involved alternatively to establish the required tolerance. In addition, it should be kept in mind that the effects of the AhR on $FoxP3^{+}$ Tregs and other subsets of immune cells may also be influenced by the experimental model, most likely reflecting tissue- or ligand-specific effects[103,198].

6.2 Impact of AhR activation on AIT - a matter of affinity?

The AhR has shown to be dispensable for successful AIT in a murine model of OVA-induced allergic asthma. However, the effects of AhR activation on various immune cells, especially regarding tolerance induction, could still be a useful tool to improve therapeutic outcomes. With quercetin and 10-Cl-BBQ, two AhR ligands with rather distinct properties were chosen to further investigate the impact of AhR activation on AIT and address the hypothesis that AhR activation can exert anti-inflammatory or tolerogenic effects, that may benefit the therapeutic outcome.

Quercetin was not able to improve the therapeutic outcome of AIT, neither in the OVA nor in the HDM model. Surprisingly, treatment of allergic mice with quercetin without additional AIT ameliorated allergic parameters in the HDM model even further than AIT treatment. In the initial experiment total and differential BALF cell counts were decreased in a comparable or even stronger manner after treatment with 8 mg/kg and 16 mg/kg quercetin than after standard AIT treatment. A very strong impact was also detectable on Th2 cells, which were decreased even further than after AIT treatment. Interestingly, Th2 cells after quercetin treatment showed a significant and even dose-dependent increase in the co-expression of FoxP3. This regulatory phenotype could also be observed in Th17 cells by the co-expression of RORgt and FoxP3. If these regulatory phenotypes of Th2 and Th17 cells are due to a direct effect of quercetin on Th cells or a consequence of altered DC phenotypes needs further investigation. DCs have an important role in the regulation of the immune response. They are responsible for the initiation of antigen-specific immune responses but also for the balance of the immune response by the induction of tolerance[183,199]. Furthermore, DCs show high AhR expression levels[181,200]. Subsequently, they are able to induce immunomodulatory effects after exposure to AhR-activating compounds and AhR signaling has been shown to exert tolerogenic effects in DCs[201,202]. Michalski *et al*. showed that AhR activation in human monocyte-derived DCs by quercetin impaired their capability to activate T cells[203]. Furthermore, they could show that treatment of LPS-matured DCs with quercetin increased immunoregulatory molecules[203]. Mice treated with quercetin in the HDM model of this study showed decreased numbers of Th2 and Th17 cells compared to allergic mice, but no measurable changes in Treg numbers. For further insight into the underlying mechanisms, a thorough analysis of DC phenotypes could be interesting at different time points during quercetin

treatment. In an acute model of OVA-induced allergic asthma, Park *et al*. demonstrated that quercetin administration influenced the Th1/Th2 balance[170]. In quercetin-treated mice decreased levels of IL-4 and increased levels of IFN-γ were detectable[170]. Additionally, they could observe a significant inhibition of all analyzed asthmatic reactions, including eosinophils in the BALF and Th2 cells in lung tissue[170]. However, they did not further address the question if quercetin directly acts on Th1 and Th2 cells or if the effects observed in the Th1/Th2 balance are due to an impact of quercetin on APCs. Yu *et al*. demonstrated direct effects of quercetin on Th cells, with quercetin being able to suppress INF-γ production by the modulation of T-bet expression and to repress IL-2 production by a T-bet independent mechanism[204]. On the contrary, Michalski *et al*. excluded direct effects of quercetin on T cells, since they could not detect any suppression of proliferation induced by anti-CD3/anti-CD28 stimulation after exposure to quercetin[203]. These contradictory findings highlight the need for further investigations to figure out how quercetin shapes Th cell responses.

Besides anti-inflammatory and anti-allergic properties, quercetin is also known to have strong anti-oxidative characteristics[172]. Lung inflammation is accompanied by oxidative stress[205] and therefore the expression levels of enzymes related to oxidative stress were analyzed in lung tissue at the end of the experiment, to check if quercetin treatment reduced oxidative stress. Analyzed enzymes included SOD1, SOD2, and GPx. SODs constitute an important antioxidant defense against ROS-mediated injury, by controlling the levels of various ROS, limiting their potential toxicity[206]. GPx is a major peroxide-scavenging enzyme and decreased activity has been reported in tissue where stress occurs in several pathological animal models[174]. In human keratinocytes, quercetin did strongly enhance the expression of SOD1, SOD2, and GPx[173]. With regard to this study, a reduction of oxidative stress in any of the treatment groups after quercetin administration should result in an increased expression level of the analyzed enzymes. However, neither SOD1, SOD2, nor GPx were significantly altered after quercetin treatment in comparison to the allergic and AIT groups. Therefore, the beneficial effects of quercetin observed at the end of the experiment are not directly related to a reduction of oxidative stress. The question if quercetin is able to reduce oxidative stress in allergic asthma in an acute manner would have to be addressed in a different experimental model, with quercetin administration during the challenge phase. In an

OVA model of allergic rhinitis, Edo *et al*. could already show that quercetin treatment during the challenge phase significantly inhibited nasal symptoms[207].

Additionally, the expression of CYP1A1 was measured at the end of the experiment to check if quercetin was able to activate the AhR and therefore induce the expression of its target gene CYP1A1. Expression levels did not differ significantly after treatment with quercetin, independent of the chosen dosage. With a half-life period of only several hours[175,176] and 9 days between the last administration of quercetin and the final analysis, quercetin should already be cleared out of the system. Prolonged activation of the AhR after treatment with quercetin could not be detected, but a statement whether quercetin is able to activate the AhR in general in this experimental setup cannot be made relying on these data. Analysis of CYP1A1 expression would have to take place directly after the administration phase to reliably answer this question.

Verification of quercetin's diminishing effects on allergic asthma in the HDM model led to results with unexpectedly high variations. Whereas the allergic control group showed consistently increased BALF cell counts, the group treated with 16 mg/kg quercetin showed highly variable numbers of total BALF cell counts. One part of the group did not respond at all, resulting in high BALF cell counts and the other part responded quite well with corresponding low BALF cell counts. To address potential underlying mechanisms, the quercetin-treated group was divided into non-responders and responders for further analysis. An interesting signaling pathway that might be involved in quercetin's ability to ameliorate the characteristics of allergic asthma is the mTOR signaling pathway. The evolutionary conserved serine/threonine kinase mTOR is a central regulator of cell metabolism, growth, proliferation, and survival[208]. The mTOR signaling pathway involves several components, including AKT and 4E-BP1 which were analyzed during this study. mTOR itself is central to two structurally and functionally distinct complexes, mTOR complex I (mTORC1) and mTOR complex II (mTORC2)[209]. The serine/threonine kinase AKT is functionally connected with both complexes[210]. mTORC1 facilitates ribosome biogenesis[211], protein translation via phosphorylation of 4E-BP1, and inhibition of autophagy[212]. The key function of mTORC2 is the modulation of actin dynamics[212]. In the context of asthma, elevated activation of the mTOR signaling pathway together with increased Th17 cells and IL-4 production, as well as decreased Tregs and INF-γ production was observed in patients experiencing an asthma attack[213]. In murine models of OVA-induced allergic asthma, the mTOR signaling pathway was activated in asthmatic

mice and inhibition of mTOR signaling resulted in an alleviation of asthmatic markers[213]. Consequently, inhibition of the mTOR signaling pathway in murine models of allergic asthma has shown to attenuate asthma pathology[213,214]. In studies investigating cancer treatment options, quercetin has been shown to inhibit mTOR signaling, which is often hyperactivated in cancer, attributing quercetin with anticancer activity[177,178]. The reduced phosphorylation of AKT and 4E-BP1 in the quercetin-responding group compared to the allergic and the non-responding group revealed an inhibition of mTOR signaling. With 4E-BP1 being a downstream component of mTORC1 an inhibition of this part of mTOR signaling by quercetin can be shown reliably. To verify the inhibition of mTORC2, additional proteins of this signaling pathway need to be analyzed, because AKT is involved in signaling via mTORC1 and mTORC2. With reduced phosphorylation of AKT a statement regarding the involved complex cannot be made. The fact that quercetin-responders in the HDM model showed ameliorated characteristics of allergic asthma, even after quercetin was cleared out of the system already for several days, introduces the possibility that quercetin is not only acting in an acute manner but could be able to induce long-term changes. One possible explanation could lay in the potential of quercetin to induce epigenetic changes. Quercetin belongs to the groups of flavonoids and these phenol compounds have been shown to affect the two best characterized epigenetic mechanisms, modulation of the DNA methylation status and histone acetylation[215]. In cancer research, recent reports are indicating a role for flavonoids in the reinstatement of standard epigenetic marks which are changed during carcinogenesis[216,217]. The epigenetic modulatory activities of flavonoids, resulting in anti-tumor activity, are often induced by the alteration of epigenetic enzymes[218-220]. If the anti-allergic effects of quercetin are mediated by epigenetic modulations has not been addressed in detail yet. It could be interesting to further elucidate the impact of quercetin on epigenetic mechanisms also in the context of the observed inhibited mTORC1 signaling. The mTORC1 pathway has already been shown to interact with epigenetic regulators required for the modification of chromatin structure and function to control gene expression[221].

Given the highly variable outcome of the second experiment in the HDM model after treatment with 16 mg/kg quercetin, the effects of quercetin on allergic asthma were also investigated in the OVA model. With 22 days between the last quercetin administration and the final analysis, the time period was long enough to analyze if changes induced by quercetin are maintained longer

than a few days. Analysis of the OVA model verified the first results of the HDM model, proofing that a combination of AIT and quercetin is not superior to standard AIT treatment. The reduction of BALF cell counts as well as of Th2 and Th17 cells was no longer observable after 22 days, indicating that quercetin cannot ameliorate allergic asthma long-term. An explanation for the variable effects observed in the HDM model after quercetin treatment may be that the beneficial effects of quercetin are already wearing off in some of the mice and are maintained a bit longer in others. If these effects are due to epigenetic changes and if these changes affect the mTOR signaling pathway would have to be addressed further in additional studies. These questions, together with the question if quercetin is able to induce AhR activation, were not followed up in this study because quercetin was not able to improve AIT or ameliorate allergic asthma long-term. Nevertheless, the results of this study are in line with several reports attributing quercetin's anti-allergic and anti-inflammatory properties.

The application of quercetin in the HDM and OVA model of allergic asthma did not properly answer the question if the AhR can be targeted to alter and ideally improve therapeutic outcome of AIT. To investigate the hypothesis of this study, that AhR activation may benefit the outcome of AIT, the effects of the high-affinity AhR agonist 10-Cl-BBQ were analyzed in combination with AIT or as a single treatment strategy, not only at the end of the experiment but also directly after the administration phase. Significantly induced CYP1A1 expression in both 10-Cl-BBQ-treated groups confirmed the ligand-dependent activation of the AhR. A very strong impact was also detectable on BALF cells and cytokines with a measurable reduction of cell counts and Th2 cytokines after ligand treatment, with no differences between the AIT + 10-Cl-BBQ- and the 10-Cl-BBQ-treated groups. Interestingly, pulmonary Th2 cells, Th17 cells, and eosinophils were considerably reduced, whereas percentages were restored to a level comparable to non-allergic mice for neutrophils and macrophages after ligand treatment. These results indicate very strong acute effects of 10-Cl-BBQ on characteristic parameters of lung inflammation and allergic asthma, underlining the anti-inflammatory and immunomodulatory properties that can be induced by activation of the AhR signaling with respective ligands. At this early analysis time point, only one day after the last AIT injection, cell counts, cytokine levels, and percentages of cell populations analyzed in lung tissue were overall comparable between the allergic group and AIT-treated mice.

The goal of AIT is to restore immune tolerance towards the causing allergen by modulation of underlying cellular responses. Therefore, it is not surprising that effects are not detectable in an acute manner. This was already shown by Russkamp *et al*. in a comparable murine model of AIT in OVA-induced allergic asthma[222] and confirmed in this study by a significant decrease of total BALF cells, eosinophils, and neutrophils, as well as reduced pulmonary Th2 and Th17 cells when analyzed after the final challenge phase on day 64. On the other hand, the expected reduction of Th2 cytokines after AIT compared to the allergic group was not detectable and mucus hypersecretion and inflammatory cell infiltration in the histological analysis showed only a slight reduction. Studies in similar models have shown to be effective in the reduction of these parameters[222,223]. The parameters analyzed during this study could not provide a reasonable explanation, without repeating the experiment and addressing the issue further if it shows to be consistent. Nevertheless, 10-Cl-BBQ was not only able to reduce parameters of allergic asthma in an acute manner but did improve therapeutic effects of AIT even further than standard AIT protocol. Total and differential BALF cell counts were significantly decreased after the combined treatment strategy. Interestingly, treatment with 10-Cl-BBQ alone did only affect eosinophils, but no other analyzed cell populations in the BALF at the end of the experiment, whereas acute effects were detectable in all populations. The acute anti-inflammatory effects seem to be tied to ligand availability and activation of the AhR, wearing off after clearance of 10-Cl-BBQ. 10-Cl-BBQ has only a short half-life period of several hours and CYP1A1 expression analysis at day 64 confirmed that there is no prolonged AhR activation. A hypothesis regarding the mechanisms that improve therapeutic outcome of AIT by activation of the AhR with 10-Cl-BBQ can only be speculative at this stage of knowledge.

The significantly reduced type 2 cytokines in the BALF can not only be explained by a reduction of eosinophils in the BALF. Whereas eosinophils are reduced in the AIT + 10-Cl-BBQ and the 10-Cl-BBQ groups, type 2 cytokines are only reduced after combined treatment. The cytokines analyzed in the BALF are not only produced by cells that are present in the BALF, but also by cells located in the lung tissue. The strong decrease in pulmonary Th2 cells could provide an explanation, but the reduction is comparable between the AIT + 10-Cl-BBQ and the 10-Cl-BBQ groups, whereas cytokine levels are only reduced after combined treatment. However, a significant difference could be found in the pulmonary eosinophils, which were only significantly reduced

after the combined treatment. Eosinophils are recruited in large numbers to sites of allergic inflammation and contribute to the cellular microenvironment through secretion of cytokines and chemokines[224,225]. Increased expression of IL-4 and IL-5 in tissue eosinophils of patients with allergic diseases has been detected in several studies[226-228]. Eosinophil-released cytokines, including IL-13, have been identified as contributors to tissue remodeling in chronic inflammation[225,229]. It was previously shown by Diny *et al*. that intestinal eosinophils were specifically adapted to their environment and that they underwent substantial transcriptomic changes[230]. Eosinophils from $AhR^{-/-}$ mice failed to express the intestinal gene expression program and lack of AhR resulted in wider effects on the intestinal immune system and tissue adaption, also affecting the T cell compartment[230]. In the context of AIT, the models within this study did not reveal differences in eosinophil numbers in the BALF or percentages in pulmonary eosinophils between wildtype and $AhR^{-/-}$ mice. However, AhR activation during AIT led to a significant decrease of pulmonary eosinophils. If this decrease is a direct effect of AhR activation during AIT in eosinophils with subsequent effects on gene expression and other immune cells will have to be further elucidated and remains speculative at the moment.

In addition to pulmonary eosinophils, also alveolar macrophages showed percentages comparable to those of non-allergic mice after AIT + 10-Cl-BBQ treatment. Given that neither AIT nor 10-Cl-BBQ alone induced similar effects, it is a close assumption that activation of the AhR during AIT mediates the level of alveolar macrophages in lung tissue. If alveolar macrophages in mice that underwent the combined treatment still show the same or an altered phenotype as in non-allergic mice will have to be addressed further to categorize the meaning and importance of this finding. Alveolar macrophages arise from circulating blood monocytes or interstitial macrophages, which serve as an intermediate between alveolar macrophages and monocytes[231]. The AhR has been assigned an important role as a molecular switch for the fate of monocytes[232]. Different expression levels of mRNA and protein levels have been detected in monocyte-derived DCs and macrophages[232]. In allergic asthma, alveolar macrophages have been found to regulate pro- and anti-inflammatory responses in the airways and links have been established between lung macrophages, airway remodeling, and eosinophilic inflammation[233]. Especially the link between pulmonary macrophages and eosinophils could be interesting, given that both populations are impacted by the treatment with AIT + 10-Cl-BBQ. The data obtained in this study give only a hint

that it could be promising to dive deeper into AhRs function in macrophages in the context of allergic asthma and AIT. This study did only address alveolar macrophages in lung tissue but not interstitial and other types of macrophages or their polarization status. To fully elucidate the impact of AhR activation during AIT on macrophages, extensive characterization including transcriptional analysis and cytokine profiles would be required. The impaired IL-10 production in $AhR^{-/-}$ macrophages points towards the regulatory potential of AhR signaling in macrophages[234,235].

In lung tissue, 10-Cl-BBQ treatment strongly reduced Th2 and Th17 cells independent of combining the treatment with AIT, whereas Tregs remained rather unchanged. 10-Cl-BBQ is reported to influence the expression of CD25 and induce an AhR-dependent Treg phenotype[142], but in this study, no changes or further increase could be detected for either FoxP3 or CD25 expression compared to the allergic and AIT groups. AhR activation by high-affinity ligands mediates immunosuppression and is often associated with an increase of Tregs[143]. However, in a non-obese diabetic mouse model of type 1 diabetes, 10-Cl-BBQ almost completely prevented the development of insulitis, also in the absence of $FoxP3^+$ Tregs[143]. The observed inhibition of T effector cell development did not rely on $FoxP3^+$ Tregs[143]. Regarding the model of AIT in murine allergic asthma, it has to be kept in mind that Tregs are already significantly elevated in these groups compared to non-allergic mice and adding 10-Cl-BBQ may just not be able to increase percentages even further. What needs to be addressed further is if there is a change in the Treg-phenotypes after administration of 10-Cl-BBQ. For example, a subpopulation of $FoxP3^+$ Tregs characterized by high levels of membrane T cell immunoreceptor with Ig and ITIM domains expresses high levels of AhR[191]. This population is specialized in the suppression of effector T cell responses[191]. The underlying mechanism involves the expression of the suppressive molecule Fgl2 under the control of the transcription factor CCAAT/enhancer-binding protein α[191]. Although the relationship between the AhR and the CEBPα/Fgl2 axis is still unclear, data from the underlying study suggest that the AhR is disproportionately associated with specific subpopulations of regulatory T cells[191].

scRNA-seq analysis did not reveal significantly changed phenotypes of $CD4^+$ T cells when comparing AIT with AIT + 10-Cl-BBQ and 10-Cl-BBQ. $CD4^+$ T cells were chosen for a scRNA-seq analysis based on reports that 10-Cl-BBQ can act directly on the AhR in these cells[142,143]. The

rather similar transcription profiles of the analyzed clusters did not point in that direction in the context of AIT in allergic asthma. Differently regulated genes between the analyzed groups could not be assigned to certain phenotypes of e.g. Tregs or Th2 cells. To go further into subpopulations of e.g. Tregs and verify if AhR activation leads to certain phenotypes, it would probably be necessary to repeat scRNA-seq with pre-enriched Tregs to ensure sufficient cell numbers for a reliable clustering of several and distinct Treg populations. The decreased numbers of Th17 and Th2 cells are more likely a consequence of changed DC phenotypes. But this needs to be further addressed in follow-up studies together with the role of macrophages.

The significant induction of CYP1A1 expression after the administration of 10-Cl-BBQ confirmed activation of the AhR. To further verify that the activation and resulting changes in analyzed parameters are related to the AhR, allergic $AhR^{-/-}$ mice were treated with 10-Cl-BBQ. Compared to 10-Cl-BBQ-treated wildtype mice no reduction of total BALF cells and eosinophils was detectable. The missing significant reduction of pulmonary Th2 and Th17 cells, as well as the unchanged scoring in mucus hypersecretion and inflammatory cell infiltration confirmed the dependency of the AhR for the mode of action of 10-Cl-BBQ. Elevated levels of total and OVA-sIgE in comparison to wildtype verified the enhanced allergic phenotype in $AhR^{-/-}$ mice. Taken together, those observations confirmed that 10-Cl-BBQ mediates its beneficial effects through the AhR. In this study, $AhR^{-/-}$ mice were only treated with 10-Cl-BBQ but the combined treatment was not performed and compared with the outcome in wildtype mice. Even if these first results confirmed the AhR-dependency of the observed effects, this would be the final step to verify the benefits of 10-Cl-BBQ on the therapeutic outcome of AIT and to conclude proof-of-principle for the data collected so far. Due to limited capacity regarding the time- and resource-consuming breeding of $AhR^{-/-}$ mice, this issue was not addressed so far. The focus of this study was a first investigation and evaluation of the potential of 10-Cl-BBQ in AIT and may provide a good basis for further research in this direction. Nevertheless, this study provides a first experimental confirmation of the stated hypothesis, that AhR activation during AIT can benefit the therapeutic outcome.

6.3 The AhR as a therapeutic target in AIT - Potential and limitations

This study could show for the first time that the AhR is no fundamental requirement for successful AIT in murine models of allergic asthma. Nevertheless, targeting the AhR with the high-affinity agonist 10-Cl-BBQ led to a measurable improvement of several parameters that are used to define the severity of allergic asthma in murine models. The AhR is already a highly interesting target in several inflammatory conditions and these findings highlight the potential to improve AIT by addressing the AhR. To fully assess the beneficial potential of 10-Cl-BBQ on AIT, further experiments should not only include the measurement of cellular and molecular biological parameters but also address the effect on the symptoms of allergic asthma. In murine models, this would consist of an analysis of the lung function, namely measuring dynamic compliance after metacholine challenge. The already promising results of the combined treatment strategy with AIT + 10-Cl-BBQ treatment would be further strengthened by an improved symptomatic score.

The potential of the AhR to modulate innate and adaptive immune responses has made it an attractive drug target. However, the strong involvement in the immune response and other important biological processes also presents an obstruction. Effects of 10-Cl-BBQ have shown to be beneficial for the outcome of AIT, but the impact of prolonged activated AhR signaling on other organs will have to be thoroughly investigated before even considering a transfer from animal models to clinical trials. Through its ubiquitous cell and tissue distribution, alterations in AhR-dependent signaling may cause different developmental and homeostatic disorders[236]. To limit the changes induced by AhR activation to those participants that are involved in the beneficial response, it is indispensable to acquire detailed and thorough knowledge of how 10-Cl-BBQ modulates cellular and molecular responses. In more detail, which cells are initially responsible for the beneficial effects observed at the end of the experiment, and how they influence other immune cells and signaling pathways. In this context, the administration route of 10-Cl-BBQ during AIT could also be a subject of interest. In this study, 10-Cl-BBQ was administered i.p. to ensure systemic availability of the ligand, but it could also be of interest to test oral or even local administration to deliver 10-Cl-BBQ in a targeted manner and therefore avoid unnecessary burdens for other organs. In the context of AIT in the herein applied murine model of OVA-induced allergic asthma, an option would be to test s.c. delivery of 10-Cl-BBQ near the draining

lymph nodes and, if successful, even combine AIT injections with ligand treatment to reduce the number of necessary injections.

AhR-ligands exhibit tissue-specific agonistic and antagonistic activity due to multiple factors including ligand-induced conformational changes of the AhR and subsequent changes in the interactions with co-activators and co-repressors[236,237]. Different ligands are able to induce association of the AhR with different proteins, resulting in recruitment of the activated AhR-complex to different DNA target sequences[151,238]. It will be interesting to investigate in detail which AhR-signaling pathways are activated by the binding of 10-Cl-BBQ and what target genes are induced as a consequence. This study investigated and compared the effects of the low-affinity ligand quercetin and the high-affinity ligand 10-Cl-BBQ on AIT. The direct comparison supposed that high-binding affinity is necessary to exert beneficial effects, given that only 10-Cl-BBQ improved the outcome of AIT. If this is also the case for other high-affinity ligands or if 10-Cl-BBQ is able to induce a specific set of target genes that are beneficial for AIT will have to be addressed further by the application of different ligands in the same experimental setup.

In summary, this thesis did not only address the general role of the AhR in AIT for the first time but additionally introduced the AhR as a potential target to improve therapeutic efficiency. The acute effects of many compounds of both natural and synthetic origin on allergic asthma have been investigated over the past decades, but very few studies addressed the impact of immunomodulators on the course of AIT. Therefore, this study provides new insights into the mechanisms of AIT, which could be used for improvements of existing therapeutic strategies. With applicable murine follow-up studies and further experiments addressing the transferability of the obtained results from animal studies into the clinics, the AhR could be an interesting and promising target to address unmet needs in AIT.

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