# Preclinical evaluation of the *Helicobacter pylori* molecule vacuolating cytotoxin (VacA) as a therapeutic approach for allergic airway disease

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# Zusammenfassung

Die vorliegende Arbeit befasst sich mit der präklinischen Testung der therapeutischen Wirksamkeit des *Helicobacter pylori* Proteins vakuolisierendes Zytotoxin A (VacA) in der Behandlung von allergischen Atemwegserkrankungen. Die Ergebnisse sind in folgenden Veröffentlichungen erschienen:

- **Treatment with *Helicobacter pylori*-derived VacA attenuates allergic airway disease.**

  Reuter S, **Raspe J**, Uebner H, Contoyannis A, Pastille E, Westendorf AM, Caso GC, Cover TL, Müller A and Taube C

  Front. Immunol. 14:1092801. doi: 10.3389/fimmu.2023.1092801 (2023)

- **Therapeutic properties of *Helicobacter pylori*-derived vacuolating cytotoxin A in an animal model of chronic allergic airway disease.**

  **Raspe J**, Schmitz MS, Barbet K, Caso GC, Cover TL, Müller A, Taube C and Reuter S

  Respir Res 24, 178. doi: 10.1186/s12931-023-02484-5 (2023)

In Vorarbeiten konnte gezeigt werden, dass neonatale Infektionen mit *Helicobcter pylori* (*H. pylori*) im späteren Leben die Entwicklung von Asthma supprimieren können. Mittels spezifischer Depletionsmutanten konnte zudem demonstriert werden, das unter anderem das Protein VacA eine zentrale Rolle für die vorteilhaften Effekte des Bakteriums hat. In weiteren prophylaktischen Modellen konnte aufgezeigt werden, dass vergleichbar zur Infektion mit *H. Pylori*, auch eine Behandlung kurz nach der Geburt mit bakteriellem Extrakt sowie mit VacA die Entstehung einer allergischen Atemwegserkrankung im späteren Leben abmildern kann.

Ziel der Arbeiten der vorliegenden Doktorarbeit war es aufzuzeigen, dass sich VacA nicht nur prophylaktisch, sondern auch therapeutisch zur Behandlung von Asthma einsetzen lässt. In unterschiedlichen experimentellen präklinischen Ansätzen soll hierbei die Eignung des Moleküls, als potentielles neues Therapeutikum eruiert werden, das zukünftig bestehende Therapieformen für Asthma ergänzen oder sogar ablösen könnte.

In den Studien wurde mit Hilfe von drei unterschiedlichen murinen Modellen der allergischen Atemwegserkrankung und murinen/humanen *in vitro* Zellkulturen die therapeutische Effizienz

von VacA untersucht, der zugrundeliegende Wirkmechanismus analysiert und erste Rückschlüsse über dessen Wirksamkeit im Menschen gewonnen.

Dafür wurde ein akutes, ein therapeutisches und ein chronisches Modell der Lungenerkrankung etabliert, mit deren Hilfe die Wirkung von VacA in adulten Tieren auf die Entstehung, die Therapie und den chronischen Verlauf der Erkrankung untersucht werden konnte. Ausgangspunkt der Studien war das akute Modell, in dem Tiere über die Atemwege mit den humanrelevanten Allergenen der Haustaubmilbe (HDM) sensibilisiert und anschließend durch wiederholte inhalative Allergenprovokation die allergische Atemwegserkrankung induziert wurden.

Basierend aus Erfahrungswerten der Vorarbeiten wurden Tiere zunächst intraperitoneal (i.p.) während der Provokationsphase mit VacA behandelt und anschließend der Asthma- und Immunphänotyp untersucht.

Die Behandlung mit VacA führte zu einer deutlichen Reduktion klassischer Charakteristika einer asthmatischen Atemwegserkrankung. Atemwegsüberempfindlichkeit nach Metacholinprovokation, Gesamtzellzahl von Entzündungszellen (Lymphozyten, Neutrophile und Eosinophile) in der bronchoalveolären Lavage (BAL), Ausprägung von Entzündungsreaktionen in der Lunge sowie Anzahl von Mukus-sekretierenden Becherzellen in den Atemwegen waren nach Gabe von VacA deutlich schwächer ausgeprägt. Im Vergleich zu unbehandelten Tieren konnte außerdem ein Shift der HDM-spezifischen Immunglobuline (Ig) vom Typ IgG1 hin zum Typ IgG2b beobachtet werden.

Der abgeschwächte Asthmaphänotyp, war von einer generell verminderten Immunreaktion begleitet. So konnte in VacA behandelten Tieren eine geringere Anzahl von Immunzellen (dendritischen Zellen (DC), B Zellen und T Zellen) im drainierenden Lymphknoten, ein geringerer Aktivierungsgrad der DC und eine Induktion von regulatorischen T Zellen (Tregs) am VacA Applikationsort, dem mesenterialen Lymphknoten und der Milz, detektiert werden.

Um zu untersuchen, ob VacA auch über Applikationsrouten wirksam ist, die bei potentiellen Patienten eine höhere Akzeptanz aufweisen, wurde die Behandlung auch über die inhalative und orale Route im akuten Modell durchgeführt.

Vergleichbar zur i.p. Behandlung konnte auch durch die alternativen Verabreichungsarten erfolgreich die asthmatische Reaktion abgemildert werden. Interessanterweise, konnte wieder eine Induktion von Tregs am Applikationsort des VacA beobachtet werden. Diesmal im Fall der peroralen (p.o.) Gabe in den mesenterialen Lymphknoten und nach der intratrachealen (i.t.) Applikation in der Lunge.

Um zu untersuchen, ob die Induktion von Tregs auch ein Wirkmechanismus der VacA vermittelten Immunsuppression darstellen könnte wurde das akute Modell mit DEREG Mäusen erneut durchgeführt.

Depletiert man Tregs während der Behandlung mit VacA in diesem transgenen Mausstamm durch die Gabe von Diphterie Toxin ist der protektive Mechanismus nicht mehr nachweisbar.

Die gewonnenen Daten zeigten, dass VacA neben der prophylaktischen Gabe kurz nach Geburt auch zur Behandlung von adulten Tieren eingesetzt werden kann, um die Ausbildung einer allergischen Atemwegserkrankung zu unterdrücken. VacA kann zur Therapie über unterschiedliche Applikationsrouten eingesetzt werden und wirkt wahrscheinlich hauptsächlich über eine Modifikation der DC und Induktion von Tregs.

Um zu überprüfen, ob VacA auch wirksam ist, wenn sich die allergische Atemwegserkrankung bereits vollständig etabliert hat, wurde ein therapeutisches Modell der Lungenerkrankung durchgeführt. Bei diesem Modell werden Tiere wie auch im akuten Modell mit HDM sensibilisiert und provoziert, um eine allergische Atemwegserkrankung zu induzieren. An diese schließt sich eine 6-wöchige Erholungsphase an, nach der die Tiere erneut mit dem Allergen provoziert werden und sich so die Erkrankung, vergleichbar zum Asthmatiker, rezidivierend neu ausbildet.

Auch in diesem therapeutischen Ansatz, führt die Behandlung mit VacA während der sogenannten sekundären Provokation zu einem abgemilderten Asthmaphänotyp der wieder von einer Induktion von Tregs begleitet war. Asthma ist eine chronische Erkrankung der Atemwege, die die Patienten oft ein Leben lang begleitet und neben wiederkehrenden Entzündungsreaktionen auch durch Umstrukturierungsprozesse in den Atemwegen gekennzeichnet ist. Um zu analysieren, ob VacA auch im chronischen Verlauf der Lungenerkrankung wirksam ist und sich durch wiederholte Behandlungen keine Nebenwirkungen aufzeigen wurde ein chronisches Asthmamodell inklusive zwei unterschiedlicher VacA Behandlungsstrategien durchgeführt.

Hierzu wurde wie bereits beschrieben eine allergische Atemwegserkrankung in Tieren induziert, diese dann aber anschließend über einen Zeitraum von 6 Wochen zweimal wöchentlich mit dem Allergen intratracheal provoziert.

Während der langzeit-Provokation wurde ein Teil der Tiere ausschließlich am Ende der Provokationsphase mit VacA behandelt ein anderer Teil erhielt zudem eine zusätzliche Behandlung während der Hälfe der Provokationsphase.

Vergleichbar zum akuten und therapeutischen Modell konnte nach beiden Behandlungsstrategien ein abgemilderter Asthmaphänotyp beobachtet werden.

Neben einer allgemein schwächeren Entzündungsreaktion in Lunge und Atemwegen konnte auch eine verminderte Atemwegsumstrukturierung in VacA behandelten Tieren beobachtet werden. Interessanterweise war die Kurzzeit-VacA-Behandlung, am Ende der Provokationsphase, mit einer Induktion von Tregs und reduzierten DC Aktivierung assoziiert. Wohingegen die Langzeittherapie mit VacA (während und am Ende der Provokationsphase) zu einer verminderten Ausbildung von $CD4^+$ und $CD8^+$ T Gedächtniszellen in der Lunge führte.

Um den Wirkmechanismus von VacA weiter aufzuklären und zu analysieren, ob das Molekül auch im Menschen wirksam sein könnte wurde sein Einfluss auf murine und humane DC *in vitro* untersucht.

Sowohl bei murinen als auch bei humanen DC führte die VacA Behandlung zu einer Aktivierung der Zellen die sich durch eine stärkere Expression der proinflammatorischen Oberflächenmarker CD80 CD40 und CD86 zeigte. Im humanen Modell konnte allerdings gezeigt werden, dass neben den proinflammatorischen Proteine auch die Expression von antiinflammatorischen Rezeptoren wie PD-L1 PD-1 und ILT-3 erhöht war. Auf Zytokinebene konnte in beiden Modellen gezeigt werden, dass auch das antiinflammatorische Zytokin Interleukin (IL)-10 verstärkt im Überstand der mit VacA behandelten Zellen nachweißbar war.

Spannenderweise führte die Kultur von VacA behandelten DC mit autologen T Zellen zu einer Induktion von Tregs.

Die Ergebnisse zeigten, dass VacA vergleichbar auf murine und humane DC wirkt und bestätigen, dass das Molekül auch im Menschen, über eine Modulation von DC und Induktion von Tregs immunologisch wirksam sein könnte.

Um zu untersuchen, ob VacA auch *in vivo* einen antiinflmamatorischen DC Phänotyp induziert, wurde Mäusen VacA i.p. appliziert und 24 bzw. 48 Stunden später die Expression der antiinflammatorischen Rezeptoren betrachtet. Hierbei konnte ein Anstieg von PD-L1 auf DC in der Lunge und der Milz nach 24h nachgewiesen werden. Dieser Effekt war auch noch nach 48 Stunden in der Milz nachweisbar.

Zusammengefasst zeigen die Daten der präklinischen Analysen von beiden Publikationen, dass sich VacA nicht nur prophylaktisch, sondern auch therapeutisch zur Behandlung von allergischen Atemwegserkrankungen eignet. VacA hat hier nicht nur eine positive Wirkung auf die Suppression der akuten Entzündungsreaktion, sondern kann auch die Ausbildung von

Umstrukturierungsprozessen, wie der Entstehung von Mukus-produzierenden Becherzellen oder subepithelialer Kollagenablagerungen entgegenwirken. Die antiinflammatorische Wirkung wird über eine Modulation der DC Antwort und Induktion von Tregs vermittelt.

Da sich diese antiinflammatorische Wirkung auch *in vitro* bei humanen Zellen beobachten lässt, ist davon auszugehen, dass das Molekül nicht nur in Mäusen, sondern auch im Menschen wirksam sein könnte.

Die Analyse zu den unterschiedlichen Applikationsrouten verdeutlicht, das VacA neben einer Injektion auch oral z.B. als Tablette oder inhalativ per Inhalator wirksam eingenommen werden könnte. In allen experimentellen Abläufen konnte in den durchgeführten Analysen keine negativen Nebenwirkungen beobachtet werden.

Die Ergebnisse unterstreichen die Eignung des bakteriellen Proteins als potentielles neues Therapeutikum. Dieses könnte zukünftig als Ergänzung zu bestehenden Therapieoptionen für Asthma eingesetzt werden oder diese vollständig ersetzen. Es ist auch vorstellbar, dass der immunregulative Mechanismus von VacA nicht nur für Asthma, sondern auch für andere Krankheitsbilder mit überschießenden Immunreaktionen von Vorteil ist.

Weitere Optimierung der Formulierungen und präklinische Studien zur Wirksamkeit und Zytotoxizität werden die nächsten Schritte darstellen, hin zu klinischen Studien, die die Eignung des Therapeutikum VacA im Menschen aufzeigen werden.

# Summary

This work focuses on the preclinical testing of the therapeutic effectiveness of the *Helicobacter pylori* protein vacuolating cytotoxin A (VacA) in the treatment of allergic respiratory diseases. The results have been published in the following publications:

- **Treatment with *Helicobacter pylori*-derived VacA attenuates allergic airway disease.**

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Previous studies have shown that neonatal infections with *Helicobacter pylori* (*H. pylori*) can suppress the development of asthma later in life. Specific depletion mutants demonstrated that the VacA protein plays a central role in the bacterium's beneficial effects. In additional prophylactic models, it was shown that, similar to *H. pylori* infection, treatment shortly after birth with bacterial extract and VacA can mitigate the development of allergic respiratory diseases later in life.

The goal of this doctoral work was to demonstrate that VacA could be used not only prophylactically but also therapeutically for the treatment of asthma. Various preclinical experimental approaches were employed to assess the suitability of VacA as a potential new therapeutic agent that could complement or even replace existing asthma treatments.

In these studies, the therapeutic efficacy of VacA was examined using three different murine models of allergic respiratory disease and murine/human *in vitro* cell cultures. The underlying mechanism of action was analyzed, and initial conclusions regarding its effectiveness in humans were drawn.

The studies began with an acute model in which animals were sensitized to house dust mite (HDM) allergens through the respiratory tract, followed by the induction of allergic airway disease through repeated inhalative allergen provocations.

Based on previous experience, animals were initially treated intraperitoneally (i.p.) with VacA during the provocation phase, and then the asthma and immune phenotype were examined. Treatment with VacA resulted in a significant reduction in classic characteristics of asthmatic airway disease, including airway hyperresponsiveness after methacholine provocation, total cell count of inflammatory cells (lymphocytes, neutrophils, and eosinophils) in bronchoalveolar lavage (BAL), lung inflammation, and the number of mucus-secreting goblet cells in the airways. Additionally, a shift from HDM-specific IgG1 to IgG2b immunoglobulins (Ig) was observed in VacA-treated animals compared to untreated animals.

The attenuated asthma phenotype was accompanied by a generally reduced immune response. VacA-treated animals exhibited a lower number of immune cells (dendritic cells (DC), B cells, and T cells) in the draining lymph nodes, decreased DC activation, and the induction of regulatory T cells (Tregs) at the site of VacA application, including the mesenteric lymph nodes, and the spleen.

To investigate whether VacA is effective through routes of administration that may be more acceptable to potential patients, treatment was also administered intratracheally (i.t.) and orally (p.o.) in the acute model. Similar to intraperitoneal treatment, alternative administration methods successfully mitigated the asthmatic response. Interestingly, the induction of Tregs was observed at the site of VacA application in both the case of p.o. administration in the mesenteric lymph nodes and after i.t. application in the lungs.

To examine whether the induction of Tregs could represent a mechanism of VacA-mediated immunosuppression, the acute model was repeated using DEREG mice. Depleting Tregs during VacA treatment in this transgenic mouse strain with diphtheria toxin eliminated the protective mechanism.

The obtained data showed, that in addition to prophylactic administration shortly after birth, VacA could also be used to treat adult animals to suppress the development of allergic respiratory diseases. VacA can be applied through different routes of administration and likely exerts its effects primarily through the modification of DCs and the induction of Tregs.

To determine whether VacA is effective when allergic respiratory disease has already been fully established, a therapeutic model of lung disease was conducted. In this model, animals were sensitized and provoked with HDM to induce allergic respiratory disease, similar to the acute

model. This was followed by a 6-week recovery phase, during which the animals were again provoked with the allergen, leading to recurrent disease development, similar to asthma in humans.

In this therapeutic approach, treatment with VacA during the so-called secondary provocation resulted in a mitigated asthma phenotype, accompanied by the induction of Tregs. Asthma is a chronic respiratory disease that often persists throughout patients' lives, characterized by recurrent inflammatory reactions and structural changes in the airways. To analyze whether VacA is effective in the chronic course of lung disease and whether repeated treatments have any side effects, a chronic asthma model was conducted, involving two different VacA treatment strategies.

In this model, as previously described, allergic respiratory disease was induced in animals, but they were then provoked with the allergen intratracheally twice a week over a 6-week period. During the long-term provocation, one group of animals was treated with VacA only at the end of the provocation phase, while another group received additional treatment during the first half of the provocation phase. Similar to the acute and therapeutic models, both treatment strategies resulted in a mitigated asthma phenotype.

In addition to a generally weaker inflammatory response in the lungs and airways, a reduced airway restructuring was observed in VacA-treated animals. Interestingly, short-term VacA treatment at the end of the provocation phase was associated with the induction of Tregs and reduced DC activation, while long-term VacA therapy (during and at the end of the provocation phase) led to a reduced formation of $CD4^+$ and $CD8^+$ memory T cells in the lungs.

To delve deeper into the mechanism of VacA and assess its potential effectiveness in humans, its impact on murine and DC was examined *in vitro*. In both murine and human DC, VacA treatment activated the cells, characterized by increased expression of proinflammatory surface markers CD80, CD40, and CD86. In the human model, it was demonstrated that, in addition to proinflammatory proteins, the expression of anti-inflammatory receptors such as PD-L1, PD-1, and ILT-3 was also increased. At the cytokine level, it was shown in both models that the anti-inflammatory cytokine interleukin (IL)-10 was more pronounced in the supernatant of VacA-treated cells. Interestingly, cultivating VacA-treated DC with autologous T cells induced the generation of Tregs.

The results indicated that VacA had a comparable effect on murine and human DC and confirmed that the molecule could potentially be immunologically effective in humans through the modulation of DC and induction of Tregs.

To investigate whether VacA also induced an anti-inflammatory DC phenotype *in vivo*, mice were administered VacA i.p., and the expression of anti-inflammatory receptors was examined 24 and 48 hours later. An increase in PD-L1 on dendritic cells in the lungs and spleen was observed after 24 hours, and this effect was still detectable in the spleen after 48 hours.

The data from the preclinical analyses in both publications show that VacA is suitable not only for prophylactic but also for therapeutic treatment of allergic respiratory diseases. VacA not only has a positive effect on suppressing acute inflammatory responses but can also counteract the development of structural changes, such as the formation of mucus-producing goblet cells or subepithelial collagen deposits. The anti-inflammatory effect is mediated through modulation of the DC response and induction of Tregs. Since this anti-inflammatory effect was also observed in human cells *in vitro*, it is likely that the molecule could be effective not only in mice but also in humans.

The analysis of different routes of administration demonstrates that VacA could be effectively administered orally, for example, as a tablet, or via inhalation with an inhaler, in addition to injection. In all experimental procedures, no negative side effects were observed.

The results highlight the suitability of the bacterial protein as a potential new therapeutic agent. In the future, it could be used as an addition to existing asthma treatment options or even as a replacement. It is also conceivable that the immunoregulatory mechanism of VacA may be beneficial not only for asthma but also for other conditions characterized by excessive immune responses.

Further optimization of formulations and preclinical studies on efficacy and cytotoxicity will be the next steps toward clinical trials that will demonstrate the suitability of the therapeutic VacA in humans.

# Introduction

Bronchial asthma is a persistent respiratory ailment affecting over 300 million individuals worldwide (1). The asthmatic disease is described by an array of symptoms. In adults, these symptoms include shortness of breath, often in sudden attacks, breathlessness when exercising sounds when breathing commonly described as wheezing, whistling, or rattling, chest tightness and coughing or the urge to cough (2). The symptoms can be episodic and vary in intensity. Allergen exposure, viral respiratory infections, irritants, exercise, and stress can trigger exacerbations. These episodes involve acutely worsening symptoms, which can have life-threatening consequences and often lead to emergency rooms visits.

Pathophysiologically, the disease is characterized by recurrent inflammatory reactions, which are often induced by allergens or other inhaled irritants and lead to airway remodeling in the chronic course. This causes obstruction and hypersensitivity of the airways.

For a long time, the disease was regarded as an allergy of the lower respiratory tract. Today we know that it is rather a heterogeneous syndrome that can present with different clinical phenotypes, which are based on varying immunological endotypes.

In addition to atopic patients whose inflammatory reactions are induced by allergens and mast cells and are dominated by $T_H2$ cells, their cytokines and eosinophil granulocytes, there are also patients with non-atopic forms of asthma. To enhance the characterization of distinct patient groups, the current consensus is to categorize them as either type 2-high or type 2-low asthma. The type 2-high asthma group includes endotypes whose immunological profile is dominated by type 2 cytokines such as IL-4, IL-5 and IL-13. These can be secreted by $T_H2$ cells, as in the case of the already described allergic asthma, but can also be released by innate lymphoid cells (ILC), in the case of non-atopic forms. The release of these mediators is responsible for numerous pathophysiological effects, such as the proliferation and attraction of eosinophils, the hypertrophy of smooth muscles cells and the development of airway hypersensitivity and mucus-producing goblet cells. On the other hand, patients with type 2-low asthma show inflammatory reactions that are determined by $T_H1$ or $T_H17$ cells and their key cytokines IL-17 and IFNy. These cytokines trigger neutrophilia or a combined neutrophilic/eosinophilic immune response, leading to subsequent pathophysiological alterations in the lungs during the chronic phase of the disease.

Due to the variety of pathomechanisms, which can also differ in severity between patients, it is not surprising that asthmatics respond differently to therapies.

While the group of patients with allergic asthma can still be relatively well controlled with standard therapeutic agents such as corticosteroids and ß2 sympathomimetic drugs, it is mainly non-atopic type 2-high and type 2-low asthmatics who are more difficult to control.

A better understanding of the mechanisms that contribute to the development, progression and exacerbation of the disease could help to develop new therapeutic intervention strategies.

## Pathogenesis of asthma

The pathogenesis of asthma is best described for allergic asthma, which is the most common asthma phenotype (3). It is assumed that a combination of environmental and genetic factors is responsible for the development of the disease, based on a hypersensitivity reaction of the immune system to normally harmless inhaled antigens from pollen, animal dander, house dust and molds(4).

The development of atopic asthma can be divided into two main phases: The allergic sensitization phase, leading to the development of allergen-specific immunological memory against the inhalant allergens, and the chronic inflammation phase that ultimately transitions into asthmatic disease. In the sensitization phase, the immunological memory is polarized into a $T_H2$ response. This priming against certain allergens can already occur *in utero*(5). It is the result of allergen contact via the mother and the downregulation of the $T_H1$ response, as this response can damage the placenta (6).

The downregulation of the $T_H1$ response occurs via the production of various cytokines such as prostaglandin E2, progesterone, IL-4, and IL-10, which promote the $T_H2$ response and inhibit the $T_H1$ response (7). After birth, these weakly primed $T_H2$ cells then encounter large amounts of allergen, and an allergy can develop. After birth, the immune response should actually be redirected towards the $T_H1$ response, which has a protective effect against the allergen (8) however, genetic predispositions to atopy can slow down or prevent this change (9). Another factor for the postnatal development of the $T_H1$ response is contact with microorganisms (10). Atopic allergy does not necessarily result in the development of asthmatic disease. For the development of asthma, repeated allergen-driven $T_H2$ inflammation of the airways and the associated wound healing processes that set the remodeling process of the airways in motion are needed. The inflammation becomes permanent and chronic, resulting in asthma. This inflammation process is usually intensified and supported by co-factors that cause tissue damage in the lung, such as repeated respiratory viral infections (11), high antigen levels in the environment, but also environmental factors such as tobacco smoke or diesel exhaust fumes (12).

The $T_H2$ immune response results from activation of innate and adaptive immunological pathways. Dendrtitic cells (DC), natural killer cells, and type 2 innate lymphoid cells (ILC2) are responsible for the innate immune response and the activation of the adaptive pathways mediated by $CD4^+$ T cells (13). Alarmins released by epithelial cells such as IL-25, IL-33, and thymic stromal lymphopoietin (TSLP) activate T cells and ILC2s. IL-25 and IL-33 lead to the development of the $T_H2$ $CD4^+$ T cells and TSLP activates DC to induce a $T_H2$ environment (14). Common cytokines produced by $T_H2$ cells are IL-4, IL-13, IL-5 and IL-9 (15). Il-4 affects B cells and promotes, together with IL-13, class-switching and allergen specific IgE production. When IgE binds to mast cells and basophiles and cross-links with an allergen, mediators like histamine, serotonin and tryptase are released, causing the bronchoconstriction and mucus hypersecretion (16). Mast cells also release prostaglandin $D_2$ that interacts with its receptor CRTH2 on $T_H2$ lymphocytes but also on other involved cells and helps to sustain the $T_H2$ inflammation by triggering the release of $T_H2$ cytokines (17). IL-5 and IL-13 are involved in the recruitment of eosinophils in the lung and their survivability and contribute to the airway remodeling. The pathogenesis and the immunological cascade are well described for allergic asthma. In recent years, the role of ILC is more explored especially in asthma subtypes, which are non-allergic but $T_H2$ driven. ILC2 seem to be capable of releasing the same $T_H2$ Cytokines like $T_H2$ T cells in response to alarmins but without antigen presentation (18). ILC2 are also less responsive to common treatment methods of asthma like steroids, leading to a more complicated disease management (19).

Other forms of asthma are often described as non-$T_H2$ asthma or $T_H2$-low asthma because of the absence of the underlying $T_H2$ inflammation. The inflammation often consist mainly of neutrophils. There is still a limited understanding of the biology behind these asthma phenotypes, but it is proposed that the neutrophilic inflammation originates from a dysregulation of the innate immune response. The cytokines IL-8 and IL-1β are increased in non-$T_H2$ asthma phenotypes and recruit neutrophils into the lung tissue (20). The airway remodeling in these types of asthma seems to be driven by an IL-17-mediated inflammation (21). Patients with $T_H2$-low asthma endotypes frequently develop more severe cases of asthma that often don not respond to steroids and other forms of treatment(22,23).

Due to the various phenotypes and endotypes of bronchial asthma and the intricate and multifaceted mechanisms underlying the pathogenesis of this condition, its treatment presents significant challenges. As there is currently no curative therapy available, the primary objective of the treatment is to manage symptoms by reducing the underlying inflammatory response, minimizing acute exacerbations and the prevention or reopening of contracted airways.

Achieving this goal necessitates an ongoing cycle of assessing the disease and adjusting the treatment plan.

## Asthma treatment

For the treatment of asthma, two different goals need to be achieved. The disease has to be managed on a long-term basis with so-called controller medication and on an emergency basis with relievers to reduce symptoms of acute asthma exacerbations. Varieties of different treatment agents are available for this purpose. A distinction is mainly made between beta2-adrenergic agonists, which can be inhaled, and corticosteroids, which can either be inhaled (ICS) or be administered orally (OCS). $B_2$-agonists are strong bronchodilators. They bind to $\beta_2$ receptors on smooth muscle cells and increase the intracellular cAMP concentration, resulting in the relaxation of the muscle cells. They also stimulate the epithelial cilia, leading to better transport of mucus out of the airways (24). For rapid and effective reduction of bronchoconstriction, "short-acting beta-agonists" (SABAs) are used, relaxing the smooth muscles in the airways. While SABAs with a duration of 3 to 6 hours are effective for acute symptoms, their impact on the underlying inflammatory response is limited (25).

Corticosteroids bind to the intracellular glucocorticoid receptor thereby modulating different kind of genes involved in the inflammatory process (26). They inhibit the migration and activation of inflammatory cells into the lung tissue, reduce vascular permeability, suppress the secretion of mucus as well as airway hyperresponsiveness, and inhibit cytokine production (27). Because of their broad effects on gene transcription, corticosteroids have a variety of side effects like cutaneous manifestations of hypercortisolism, hypokalemia, myopathy, glucose intolerance, pancreatitis, hypertension and psychologic and neurologic side effects (28).

ICS are the main kind of steroids used to control asthmatic disease. They are highly effective and have a lower risk of side effects compared to OCS, which are only used in exceptional cases of uncontrolled asthma over short periods. To manage and treat the underlying inflammation, low-dose inhaled corticosteroids and combinations of ICS and "long-acting beta-agonists" (LABAs), such as Formoterol, are employed. The combination of those two therapeutics complements each other, ICS treatment leads to an increased production of $\beta_2$-receptors, amplifying the effect of the LABAs, while the LABAs ensure that more corticoid receptors are translocated into the nucleus (29), making it possibly to reduce the amount of corticosteroids and further decrease the risk of side effects.

For effective treatment of asthma, it is essential to use ICS therapies early and consistently to control the inflammatory response. However, patients often discontinue treatment when symptoms are absent and turn to SABAs when symptoms worsen (30).

In addition to ICS, the treatment of allergic asthma can be complemented by allergen immunotherapy (AIT). The prevalence of allergen sensitization among asthma patients ranges from 30% to 70% in children (31) and 30% to 60% in adults (32). AIT's mechanism of action is based on the induction of immunological tolerance to the specific allergen, characterized by an increased number of Tregs, elevated production of IgG4, and the presence of anti-inflammatory cytokines like IL-10 and TGF-β. The objective of AIT is to regulate the allergic mechanisms underlying asthma, thereby achieving a sustained treatment effect. However, AIT is only effective in patients with allergen sensitization, has no impact on intrinsic asthma, and offers just a partial effectiveness in mixed forms of the disease. Therefore, it is crucial to conduct a comprehensive characterization of the patient's asthma form before initiating therapy, to assess the effectiveness and appropriateness of treatment, and to tailor the approach precisely to the patient.

In recent years, biologics have been developed for the treatment of corticosteroid-resistant forms of severe non-allergic and allergic asthma. These humanized recombinant antibodies target specific messenger molecules in the inflammatory pathways of the disease. The first biologic that was approved in 2005 was Omalizumab, which targets IgE(33). Other biologics include antibodies directed against Type 2 cytokines, like IL-5 or its receptor, such as Mepolizumab and Reslizumab, or Benralizumab, or Dupilumab, which blocks IL-4 and IL-13, by interacting with a shared receptor chain(34). Most recently, the anti-TSLP antibody Tezepelumab, which targets the alarmin TSLP released by epithelial cells, has been approved. Due to its position in the signaling cascade, blocking TSLP demonstrated beneficial effects for both, type 2-high as well as type 2-low asthmatics. Antibody therapies are currently only used in patients with severe forms of the disease. Here, they clearly counteract the annual exacerbation rates and have a positive effect on the quality of life of patients. Unfortunately, these therapies are expensive and may lose their effectiveness over time, necessitating a change in prescription. It is important to emphasize that, except for AIT, all the mentioned therapies only treat the symptoms and not the root cause of asthma.

The search is still on for forms of therapy that can modulate the disease to a large extent and thus prevent its development or suppress its progression and exacerbation. A look at bacteria and, in particular, their anti-inflammatory interaction with the host offers a promising source of inspiration for the identification of new therapeutic strategies.

## The Microbiome – a new path to the therapy of asthma

The human microbiome has come into focus as a key area for discovering new treatment options for asthma. The relationships between microbes and humans are intricate and span a spectrum from harmful pathogenic associations to more neutral coexistence and even mutually beneficial symbiotic partnerships. Various hypotheses have emerged to explain how beneficial microbial interactions can help prevent diseases, while the absence of certain microbial species, often due to lifestyle changes like increased hygiene or antibiotic usage, can contribute to disease development.

The "hygiene hypothesis," originally proposed by Strachan, posited that early childhood infections and improved hygiene standards in developed countries might increase the risk of developing allergies and other atopic diseases (35). Subsequent research, including gnotobiotic animal studies, provided further evidence of the protective role of environmental and commensal bacteria in preventing allergies.

Recent advances in microbiology, such as the ability to culture anaerobic bacteria and the use of high-throughput techniques like matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF), 16s RNA sequencing, phylogenetic microarrays, and taxon-targeted qPCR, have revealed the remarkable diversity of bacterial species residing in various exposed organs, including the gut, skin, and even the lung. Additional methodologies, such as metabolomics, proteomics, transcriptomics, metagenomics, and single-cell sequencing, have provided valuable insights into the functions of these microbial communities.

In the gastrointestinal tract, the microbiome's functions are well-documented and encompass impacts on immunity(36), host defense (37), metabolic processes (38), fat storage (39) vitamin synthesis (40), and even behavior (41). Microbial products serve a range of functions, from protecting against pathogens to facilitating communication within microbial communities and between microbes and their human hosts. These signals can act over short distances but can also influence distant organs (42). Pathogen-associated molecular patterns (PAMPs) and metabolic products, such as indole-3-aldehyde (a ligand for the aryl hydrocarbon receptor) and short-chain fatty acids, have been shown to modulate host immune responses, playing roles in both pro- and anti-inflammatory processes that contribute to maintaining immune homeostasis.

Importantly, alterations in the relationship between the microbiome and the host have been associated with various diseases affecting different organs, including asthma, autism, stress, stroke (brain), atopic dermatitis (skin), inflammation, and obesity (adipose tissue), as well as conditions like type 3 diabetes, systemic lupus erythematosus, and atherosclerosis.

For example, our interaction with the bacterium *H. pylori* serves as a compelling demonstration of the dynamic between microbiota and our immune system. *H. pylori* is considered one of the long-standing microbial companions of humans. For over 58,000 years, this bacterium dominated a specific organ, the stomach. It was once widespread among all human populations, colonizing individuals during their youth and persisting throughout their lifetime if not eradicated (43). Presently, approximately 50% of the global population carries this bacterium, with colonization rates being lowest in industrialized nations and highest in developing countries (44).

*H. pylori* has evolved various strategies to evade the immune system and protect itself from the harsh gastric environment (45). Utilizing its flagella (46), it navigates and establishes itself within the stomach's mucus layer, responding to chemotactic signals (47). Remarkably, the bacterium appears to shield itself from detection by pathogen recognition receptors, resulting in subdued activation of the adaptive immune response upon infection. These interactions between *H. pylori* and adaptive immunity play a crucial role in fostering immunological tolerance toward the bacterium (48). Epidemiological studies have shown that the risk of developing an asthmatic disease decreases with the colonization of *H. pylori* in early childhood (49). Based on these studies the role of *H. pylori* infections in the development of asthma was examined. Using murine models it was shown that a neonatal infection with *H. pylori* lead to an attenuated asthma phenotype later in life. Other experiments showed the role of Tregs and modulated DC in the alteration of the Asthma phenotype by *H. pylori* (50). Through the use of *H. pylori* mutants, lacking different virulence factors, it could be demonstrated that VacA or gamma-glutamyl transpeptidase (GGT) play an important role for the beneficial effects of the bacterium (51). In addition, studies with *H.pylori* extract and the isolated protein VacA were conducted to mitigate the downside of a live *H.pylori* infection. Mice that received VacA prophylactically showed a decreased asthma phenotype later in life.(52).

The aim of this doctoral thesis was the preclinical testing of the therapeutic efficacy of VacA for the treatment of allergic respiratory diseases. The first object was to evaluate the therapeutic efficacy of VacA treatment in animal models. This involved testing different doses and therapeutic routes of VacA administration in the acute model of allergic respiratory diseases. The treatment was then tested as a therapy in both a secondary provocation model and a chronic model of allergic airway inflammation. In addition to the analysis of characteristics of the airway disease (lung function, inflammation in BAL and lung tissue and the development of goblet cell metaplasia), further cell and molecular biological investigations like flowcytometric analysis of the immune phenotype were carried out, in order to draw conclusions about the

mechanism of action. The effect of VacA on the allergen-specific and -nonspecific interaction of DC and T cells was investigated. These analyses were performed with both murine and human cell *in vitro* cultures. The results of the experiments were published in two publications this year.

- **Treatment with *Helicobacter pylori*-derived VacA attenuates allergic airway disease.**

  Reuter S, **Raspe J**, Uebner H, Contoyannis A, Pastille E, Westendorf AM, Caso GC, Cover TL, Müller A and Taube C

  Front. Immunol. 14:1092801. doi: 10.3389/fimmu.2023.1092801 (2023)

- **Therapeutic properties of *Helicobacter pylori*-derived vacuolating cytotoxin A in an animal model of chronic allergic airway disease.**

  **Raspe J**, Schmitz MS, Barbet K, Caso GC, Cover TL, Müller A, Taube C and Reuter S

  Respir Res 24, 178. doi: 10.1186/s12931-023-02484-5 (2023)

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# Treatment with *Helicobacter pylori*-derived VacA attenuates allergic airway disease

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**Background:** Asthma is an incurable heterogeneous disease with variations in clinical and underlying immunological phenotype. New approaches could help to support existing therapy concepts. Neonatal infection of mice with *Helicobacter pylori* or administration of *H. pylori*-derived extracts or molecules after birth have been shown to prevent the development of allergic airway disease later in life. This study evaluated the potential therapeutic efficacy of *H. pylori* vacuolating cytotoxin A (VacA) in allergic airway inflammation and investigated the underlying immunological mechanisms for its actions.

**Methods:** Murine models of allergic airway diseases, and murine and human *in vitro* models were used.

**Results:** In both an acute model and a therapeutic house dust mite model of allergic airway disease, treatment with *H. pylori*-derived VacA reduced several asthma hallmarks, including airway hyperresponsiveness, inflammation and goblet cell metaplasia. Flow cytometry and ELISA analyses revealed induction of tolerogenic dendritic cells (DC) and FoxP3 positive regulatory T cells (Tregs), and a shift in the composition of allergen-specific immunoglobulins. Depletion of Tregs during treatment with VacA reversed treatment-mediated suppression of allergic airway disease. Human monocyte derived DCs (moDC) that were exposed to VacA induced Tregs in co-cultured naïve autologous T cells, replicating key observations made *in vivo*.

**Conclusion:** *H. pylori*-derived VacA suppressed allergic airway inflammation *via* induction of Tregs in both allergic airway disease models. These data suggest that the immunomodulatory activity of VacA could potentially be exploited for the prevention and treatment of allergic airway disease.

KEYWORDS

**asthma, helicobacter pylori, vacuolating cytotoxin A (VacA), therapy, regulatory T cells**

# 1 Introduction

Asthma is one of the most common noncommunicable diseases, affecting an estimated 358.2 million people worldwide (1). There is now in-depth knowledge about the pathophysiology of asthma, and important underlying mechanisms have been identified (2). However, all currently available treatments (including biologics) must be given over the long term, and no cure for asthma is currently available.

Bacteria are known to interact with the host through symbiotic, commensal or pathogenic host-bacteria relationships and can exert immune-modulatory effects. There is mounting evidence that interactions between microbes and the immune system are an important factor influencing susceptibility to allergic disease and asthma (3). The gastric bacterium *Helicobacter pylori* has the ability to induce immune suppression that, on one hand prevents clearance of the bacteria and on the other hand diminishes the risk of allergic diseases (4).

Data from epidemiological studies (5–8) that has been corroborated by studies in murine models (3, 9) suggest a protective effect of *H. pylori* to reduce the incidence of allergic asthma, hay fever, and other allergic disease manifestations. In addition, animal data suggest that exposure of mothers to *H. pylori* during pregnancy can induce a tolerogenic immunophenotype in their pups that attenuates the development of allergic diseases later in life (10). In this setting, the effects of *H. pylori* on allergic airway disease were found to be mediated by the modulation of dendritic cell (DC) and T cell responses (3, 9). However, all currently available data come from postnatal prophylactic models in which infection or administration of bacterial lysates were performed long before allergen sensitization and/or challenge.

In these models, using *H. pylori* strains lacking specific bacterial proteins showed that vacuolating cytotoxin A (VacA) plays a central role in mediating the immunosuppressive effects of the *H. pylori* bacterial infection (3). *H. pylori*-derived VacA is a secreted bacterial immunomodulator that differs in sequence and structure from other known bacterial virulence and persistence factors (11). Best known for its pore-forming function, it plays an important role in *H. pylori* colonization of the stomach (11). VacA can interact with different structural and immune cells, thereby influencing cellular mechanisms in several ways (12).

This study investigated the potential effectiveness of VacA as a therapeutic intervention for the treatment of allergic airway disease. To achieve this, purified *H. pylori*-derived VacA and recombinant VacA (rVacA) were assessed in acute and therapeutic models of allergic airway disease. In addition, a murine Treg depletion model, and murine and human DC/T cell co-cultures were used to determine the mechanistic basis by which VacA modulates the severity of allergic asthma.

# 2 Material and methods

## 2.1 Animals

C57BL/6J mice [Janvier Labs] were housed in the Laboratory Animal Facility of the University Hospital Essen. All mice were females and used at the age of 8–12 weeks. C.B6-Tg(Foxp3-DTR/EGFP)23.2Spar/Mmjax (DEREG) mice were kindly provided by Astrid Westendorf (Institute of Medical Microbiology, University Hospital Essen, University of Duisburg-Essen, Essen). These mice express the diphtheria toxin (DT) receptor and green fluorescent protein (GFP) under control of the FOXP3 promoter. Application of DT results in depletion of Tregs (13). Male and female DEREG mice aged 8–15 weeks were evenly distributed across all experimental groups.

All animal procedures were conducted in accordance with current federal, state, and institutional guidelines, and all experiments were approved by local regulatory authorities (Landesamt für Natur, Umwelt und Verbraucherschutz North Rhine Westphalia, Germany; reference number: AZ 81-02.04.2018.A084).

## 2.2 HDM

To mimic a sensitization with a human relevant allergen, extract derived from whole bodies of Dermatophagoides pteronyssinus was used in the experiments. D. pteronyssinus is one of the most frequently found mite species in german households represents the major allergen source of atopic people with a sensitization towards house dust mite in Europe (14).

HDM was obtained from Greer Laboratories [# XPB82D3A2.5]. The delivered lyophilized cake was reconstituted with PBS to a concentration of 1mg/mL total protein containing 13µg/mL Der p 1.

## 2.3 Experimental protocol

### 2.3.1 Acute HDM-specific murine allergic airway disease model

Induction of house dust mite (HDM)-dependent allergic airway disease was performed using a previously described model (15–17). Briefly, on day 0, isoflurane-anesthetized mice were sensitized using intranasal application of 1 µg HDM protein [Greer Laboratories, USA] dissolved in 50 µL phosphate-buffered saline (PBS). Animals were then challenged with 10 µg HDM protein in 50 µL PBS given intranasally to isoflurane-anesthetized mice from day 7–11. Based on experience from previous studies, the following VacA treatment regimen was implemented: animals received 20 µg of VacA dissolved in 100 µL PBS *via* intraperitoneal (i.p.) injection on days 6, 7, 9 and 11 (Figure 1A). In these experiments, either an active strep-tagged oligomeric s1m1 type VacA (VacA) or an inactive strep-tagged VacA mutant protein (VacAΔ6-27; mutVacA) purified from modified forms of *H. pylori* strain 60190, or a C-terminal 6xHistidine-tagged s1m1 recombinant VacA protein derived from the same *H. pylori* strain (rVacA; provided by GBC-HpVac [Geneva, Switzerland]) was used. VacA and mutVacA were purified as described previously (18–20), and rVacA was purified using similar standard purification steps as follows: the rVacA was captured in a NiNTA affinity chromatography step, followed by size-exclusion chromatography using a S-200 resin as a polishing step. The resulting protein was >98% pure, as judged by analytical gel filtration and SDS-PAGE (not shown).

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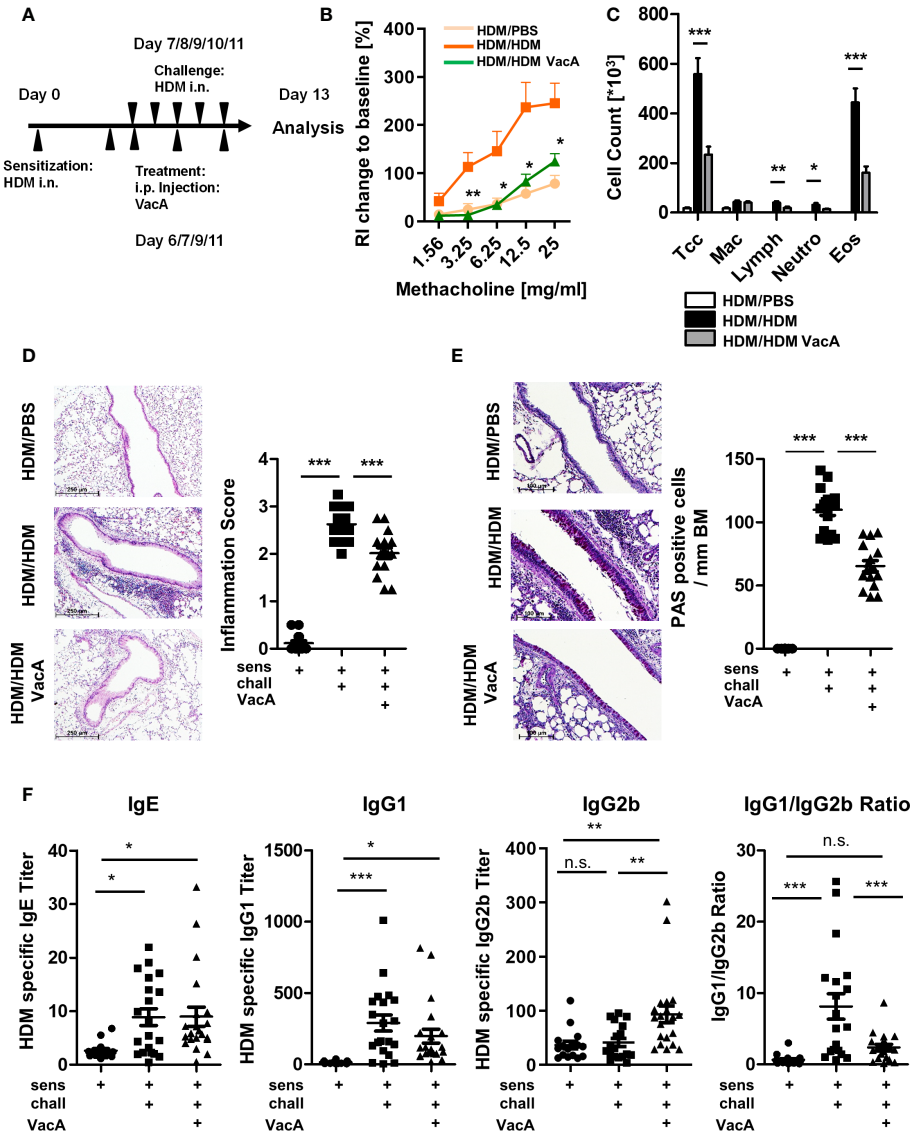


FIGURE 1
VacA treatment attenuates the asthma phenotype. **(A)** Animals were sensitized (day 0) and challenged intranasally (day 7–11) with house dust mite (positive control, HDM/HDM). VacA (HDM/HDM VacA) was given intraperitoneally (i.p.) on days 6, 7, 9 and 11. Phosphate-buffered saline (PBS)-challenged animals served as negative controls (HDM/PBS). **(B)** Change of airway resistance (RI): percentage change of airway resistance in response to increasing doses of methacholine vs. PBS; HDM/HDM (dark orange), HDM/HDM VacA (green) and HDM/PBS mice (light orange). Asterisks indicate difference between HDM/HDM and HDM/HDM VacA. **(C)** Cellular composition of bronchoalveolar lavage (BAL): total cell count (Tcc), macrophages (Mac), lymphocytes (Lymph), neutrophils (Neutros) and eosinophils (Eos); HDM/HDM (black), HDM/HDM VacA (dark gray) and HDM/PBS mice (white). **(D)** Inflammation in lung tissue: pictures show representative sections of each indicated group (x100). Scatter plot: inflammation score in HDM/PBS, HDM/HDM and HDM/HDM VacA animals. **(E)** Mucus-producing cells: pictures show representative sections from each group (x200). Scatter plot: number of mucus-producing cells/mm basement membrane in HDM/PBS, HDM/HDM and HDM/HDM VacA animals. **(F)** VacA treatment affects immunoglobulin subtypes. Graphs: house dust mite (HDM)-specific immunoglobulin (Ig)E, -IgG1, -IgG2b titers and ratio of HDM IgG1 to IgG2b in HDM/phosphate-buffered saline (PBS), HDM/HDM and HDM/HDM VacA animals. **(B, C)** results from five independent experiments, n=15–20 per group. **(D–F)** each symbol represents one animal. **(D, E)** results from four independent experiments, n=12–16 per group. **(F)** Results from five independent experiments, n=16–21 per group. Analysis of variance: *p<0.05, **p<0.01, ***p<0.001; ns, not significant.

Asthma phenotype and immunological readouts were assessed on day 13 (Figures 1, 2). To determine the functional role of Tregs in VacA-mediated immune suppression, DEREG mice and a modified version of the previously described allergic airway disease/VacA treatment model were used. In addition to the HDM sensitization/challenge and treatment with VacA, wild type (WT) and DEREG mice received 30 ng/g DT dissolved in 100 µL PBS i.p. on days 4, 6, 8 and 10 (Figure 3A) to deplete Tregs and control side effects.

### 2.3.2 Therapeutic HDM-specific murine allergic airway disease model

The therapeutic effectiveness of VacA was determined in a modified model of allergic airway disease. To induce allergic airway disease, animals were sensitized and challenged with HDM as already described. After a resting phase of 6 weeks, animals received a secondary HDM challenge on days 54, 55 and 56 (10 µg HDM protein/50 µL PBS). Therapeutic VacA treatment (20 µg/100 µL PBS i.p.) was administered on days 53, 54 and 56.

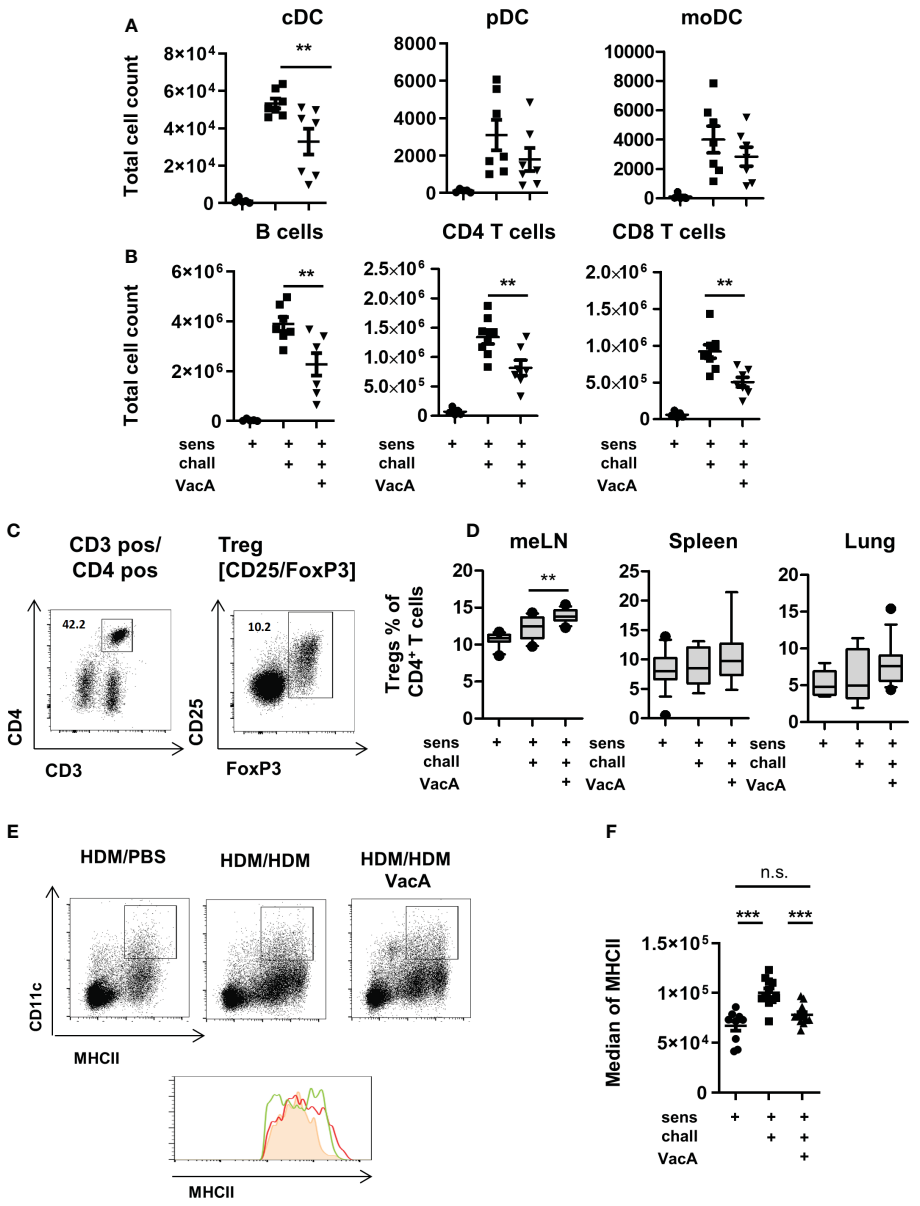


FIGURE 2
VacA treatment affects immune phenotype. Total cell counts of **(A)** conventional dendritic cells (cDC), plasmacytoid DC (pDC) or monocyte-derived inflammatory DC (moDC) and **(B)** B cells. CD4+ and CD8+ T cells in lung draining lymph nodes (tLN) of house dust mite (HDM)/phosphate-buffered saline (PBS), HDM/HDM and HDM/HDM VacA animals. (C, D) VacA treatment increases proportion of Tregs. **(C)** Tregs were characterized by the expression of FoxP3 within the population of CD3+/CD4+ T helper cells. **(D)** Boxplots (Whiskers 10-90 percentile) show percentage of Tregs within the population of CD4 + T cells in mesenteric lymph nodes (meLN), Spleen and tLN of HDM/PBS, HDM/HDM and HDM/HDM VacA animals. **(E, F)** DC in VacA-treated animals showed reduced major histocompatibility complex II (MHCII) expression in draining lymph nodes. **(E)** Representative dot plots show MHCII/CD11c expressing DC in draining lymph nodes of HDM/PBS, HDM/HDM and VacA-treated HDM/HDM mice. Histogram shows MHCII expression in HDM/PBS (shaded orange), HDM/HDM (red line) and HDM/HDM VacA animals (green line). **(F)** Graph shows median MHCII expression on DC in the draining lymph nodes of HDM/PBS, HDM/HDM and HDM/HDM VacA-treated mice. Each point represents one animal. **(A, B)** results from two independent experiments, n=5–7 per group. **(C, D)** results from 3–5 independent experiments, n=10–21 per group. **(E, F)** results from three independent experiments, n=9–12 per group. Analysis of variance: *p<0.05, **p<0.01, ***p<0.001.

Asthma phenotype and immunological readouts were assessed on day 57 (Figure 4A). Three different experimental groups were compared: 1. negative control (sensitized and 1st challenged to HDM, 2nd challenge with PBS – HDM/HDM/PBS), 2. positive control (sensitized and 1st challenged and 2nd challenge with HDM – HDM/HDM/HDM) 3. VacA treatment group (sensitized and 1st challenged and 2nd challenge with HDM + treatment with VacA – HDM/HDM/HDM VacA) (Details in Figure 4A).

Each of these experiments was performed at least twice (actual animal numbers are indicated in the figure legends).

## 2.4 Assessment of asthma hallmarks

Lung function, differential bronchoalveolar lavage (BAL) cell counts, and histological analysis of lung slides were performed to analyze the asthma phenotype.

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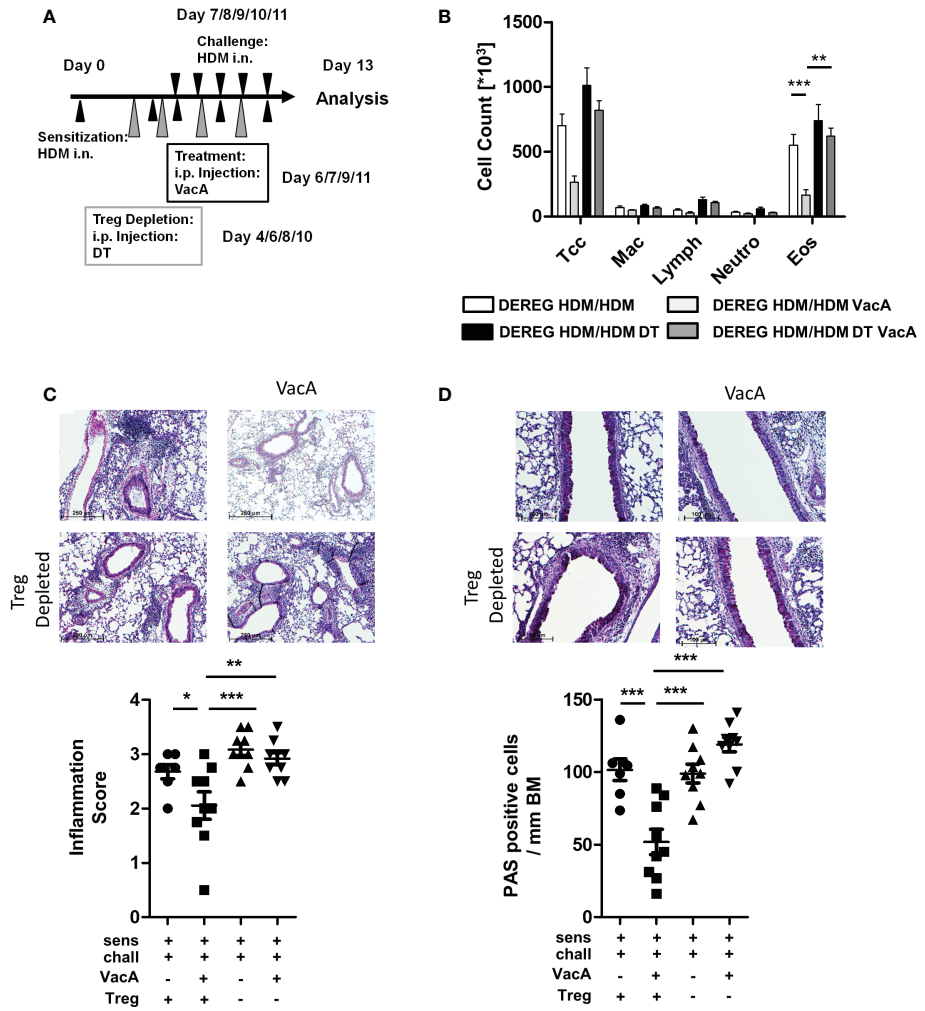


FIGURE 3
Depletion of Tregs in VacA-treated DEREG mice abolished therapeutic effects in the house dust mite (HDM)-specific allergic lung disease model. **(A)** Tregs were depleted by repeated intraperitoneal administrations of diphtheria toxin (DT) on days 4, 6, 8 and 10. **(B)** Cellular composition of bronchoalveolar lavage (BAL): total cell count (Tcc), macrophages (Mac), lymphocytes (Lymph), neutrophils (Neutro) and eosinophils (Eos); DEREG HDM/ HDM (white), DEREG HDM/HDM VacA (light gray), DEREG HDM/HDM DT (black) and DEREG HDM/HDM DT VacA (dark gray) mice. **(C)** Inflammation in lung tissue: pictures show representative sections from each indicated group (x100). Scatter plot: inflammation score in DEREG HDM/HDM, DEREG HDM/HDM VacA and DEREG HDM/HDM DT and DEREG HDM/HDM DT VacA mice. **(D)** Mucus-producing cells: pictures show representative sections from each group (x200). Scatter plot: number of mucus-producing cells/mm basement membrane in DEREG HDM/HDM, DEREG HDM/HDM VacA and DEREG HDM/HDM DT and DEREG HDM/HDM DT VacA mice. Results from three independent experiments, n=5-9; Analysis of variance: *p<0.05, **p<0.01, ***p<0.001

### 2.4.1 Analysis of lung function

Invasive measurement of lung function in response to increasing doses of methacholine (MCh, Sigma-Aldrich; mg/mL PBS) was performed on anesthetized, intubated mechanically ventilated mice using the Fine Point system (FinePointe RC units; Data Sciences International, New Brighton, MN). The percentage change in maximum resistance after each dose of MCh compared with PBS nebulization was calculated.

### 2.4.2 Analysis of BAL

Lungs were lavaged with 1 mL ice-cold PBS *via* an intratracheal tube. BAL cell counts were determined. Differential cell counts of at least 200 counted cells for macrophages, lymphocytes, neutrophils, and eosinophils were performed on cytocentrifuged preparations stained with Hemacolor-Set (Merck). From these, percentages and absolute cell counts were calculated.

### 2.4.3 Analysis of lung histology

After preparation of the left lung lobe for flow cytometric analysis, right lobes were fixed by inflation and immersion in Histofix (Roth) and embedded in paraffin. Tissue sections were prepared and stained with hematoxylin/eosin (HE) to analyze tissue inflammation, or with combined Periodic Acid Schiff (PAS)/HE staining to identify mucus-producing goblet cells. Analysis was performed as described previously (21).

Airway inflammation was scored semi-quantitatively on HE slides. For this, five randomly selected areas were scored by two experienced observers blinded to experimental groups. Inflammation was scored on a scale from 0 to 4 (detailed description in [21]). The

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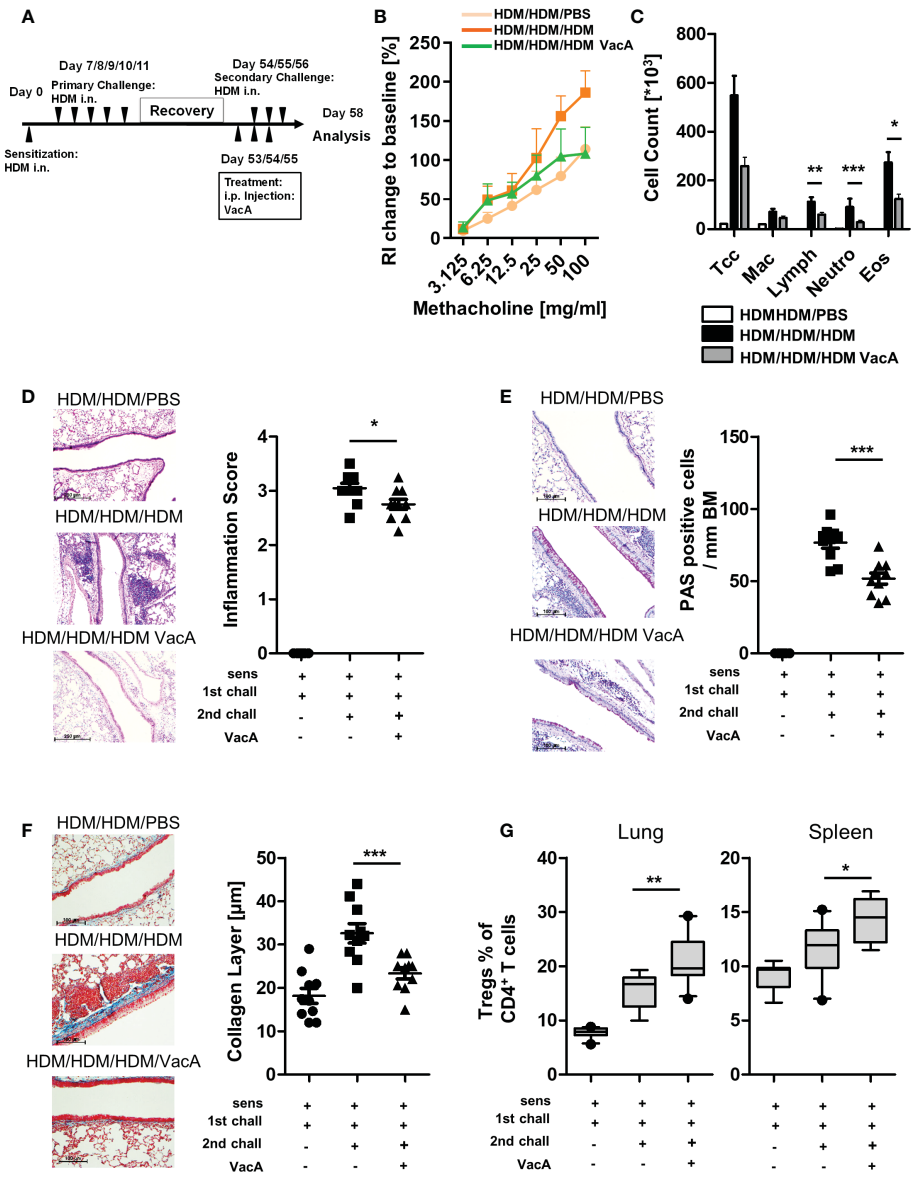


FIGURE 4
VacA attenuates asthmatic reactions in mice with already established lung disease. **(A)** secondary allergen challenge was performed after house dust mite(HDM) sensitization and challenge, and 6 weeks' rest. VacA treatment was given during the secondary challenge. **(B)** Change of airway resistance (RI):percentage change in airway resistance in response to increasing doses of methacholine vs. PBS; HDM/HDM/HDM (dark orange), HDM/HDM/HDM VacA(green) and HDM/HDM/PBS mice (light orange) **(C)** Cellular composition of BAL: number of total cells (Tcc), macrophages (Mac), lymphocytes (Lymph),neutrophils (Neutro) and eosinophils (Eos); negative control (HDM/HDM/PBS [white]), positive control (HDM/HDM/HDM [black]) and HDM/HDM/HDMVacA (dark gray). **(D)** Inflammation in lung tissue: pictures show representative sections from each indicated group (x100). Scatter plot: inflammationscore in HDM/HDM/PBS, HDM/HDM/HDM and HDM/HDM/HDM VacA mice. **(E)** Mucus-producing cells in lung airways: pictures show representativesections from each group (x200). Scatter plot: number of mucus-producing cells/mm basal membrane of HDM/HDM/PBS, HDM/HDM/HDM and HDM/HDM/HDM VacA mice. **(F)** Subepithelial collagen deposition: pictures show representative sections from each group (x200). Scatter plot: averagedsubepithelial collagen layer thickness in µM in HDM/PBS, HDM/HDM and HDM/HDM VacA animals. **(G)** VacA treatment increases Treg frequencies.Boxplots (Whiskers 10-90 percentile) show proportion of Tregs within the population of CD4-positive T cells in the lung and spleen of HDM/HDM/PBS,HDM/HDM/HDM and HDM/HDM/HDM VacA animals. Each symbol represents one animal. Data are results from two independent experiments, n=10 per group. Analysis of variance: *p<0.05, **p<0.01, ***p<0.001.

score is based on the following inflammatory conditions: 0, all airways and vessels are free of inflammatory infiltrates; 1, some infiltrates are detectable around airways and vessels, and are up to two layers in thickness; 2, the majority of airways and vessels show infiltrates with thickness of up to two layers, with more layers found only occasionally; 3, the majority of airways and vessels show inflammatory infiltrates, and many layers are thicker than two layers, ranging from 3–8 layers; 4, all airways and vessels are surrounded by thick layers of inflammatory cells.

PAS-positive goblet cells were quantified per millimeter of basal membrane on at least three different representative airways on PAS-stained slides. To analyze subepithelial collagen deposition, Masson Trichrome staining was performed on lung slides from the secondary challenge model. At least three different airways were randomly selected, and thickness of the collagen layer was measured at five different areas of each airway. Mean collagen layer thickness of each airway was calculated and then averaged between all airways.

## 2.5 Preparation of single cell suspensions from different tissues

### 2.5.1 Lungs

After perfusion was performed, *via* the right ventricle, ligated left lung lobes were eviscerated, minced and transferred into 50-mL tubes. Collagenase type I (0.5 mg/mL; catalog no. C9891, Sigma) was added and after incubation in a shaking water bath for 45 minutes at 37°C, cells were passed through a cannula (20G 0.9 mm x 40 mm) in a 10-mL syringe and then through a 70-µm cell strainer at least three times to obtain a single cell suspension. Erythrocytes were lysed, and cell counts were determined.

### 2.5.2 Lymph nodes (mesenteric lymph node; tracheal lymph node)

Lymph nodes were carefully grinded between the slides, cells were washed into Hanks' balanced salt solution (HBSS) and transferred through a 70 µm cell strainer into a tube. Cell counts were determined.

### 2.5.3 Spleen

Organs were minced and transferred through a 70 µm cell strainer into a 50 mL tube. Red blood cell (RBC) lysis was performed as already described, cells were washed, and cell counts determined.

All single cell preparations were adjusted to $1x10^7$ cells/mL IMDM containing 10% fetal calf serum (FCS; PAA Laboratories), 1% Pen/Strep (Gibco; 10,000 U/mL penicillin; 10,000 µg/mL streptomycin) and stored on ice until further processing.

## 2.6 Flow cytometric analysis

Prepared single cell suspensions ($5x10^5$ cells) were plated in 96 well plates (TC Plate 96 Well Suspension, R [Sarsted]). Unspecific antibody binding was blocked with 0.5 µL of Fc receptor–blocking antibodies [TruStain FcX™ (anti mouse CD16/32) (Isotyp: Rat IgG2a, λ clone: 93 [BioLegend])]. Antibody panels defined in Supplementary Table 1 were used to analyze DC and B cells (murine DC/B cell analysis) and T cells (murine Treg analysis panel). To analyze Tregs, intracellular staining against the transcription factor FoxP3 was performed on surface-stained T cells using the FoxP3/Transcription Factor Staining Buffer Set [eBioscience]. All cells were finally fixed with Fixation Buffer [PBS + 2% paraformaldehyde [Sigma-Aldrich] and analyzed.

To determine T cell populations following analysis strategy was used. First, debris and doublets were excluded based on size and shifting properties seen by analysis of FSC-Height vs. FSC-Area. autofluorescent cells, positive cells in an empty channel were excluded. Based on the expression of CD8/CD3 cytotoxic T cells were differentiated from CD3/CD4 positive T Helper cells. Tregs were then defined as FoxP3 positive cells within the population of CD3/CD4 positive cells (22). Treg subpopulations were further subdivided in $CD25^+$ cell and $CD25^-$ cells (Supplementary Figure 1B) (23).

To determine DC subpopulations and B cells, first debris and doublets were excluded, and then B cells were identified as CD19-positive cells. Within the remaining cells, DC were characterized as CD11c/MHCII-positive cells. Subpopulations like pDC ($CD11c^+/MHCII^+/B220^{positive}$), cDC ($CD11c^+/MHCII^+/Ly6c^{negativ}B220^{negative}$), inflammatory DC ($CD11c^+/MHCII^+/Ly6c^{positive}B220^{negative}$), were identified using the indicated marker combination (Supplementary Figure 1A).

The absolute cell count of the individual cell populations was calculated using the relative proportions of the individual cell populations and the absolute cell counts of the organ determined during the experiments.

Measurements were performed on a CYTOFLEX flow cytometer (Beckman Coulter), FCS files were analyzed and graphics were generated using FlowJo Software (version 10.6.1).

## 2.7 HDM-specific ELISA

To analyze HDM-specific immunoglobulins (Ig - IgG1; IgG2b and IgE) serum was collected at day 13 in the HDM-specific murine allergic airway disease model. Enzyme-linked immunosorbent assay (ELISA) plates (Microplate, 96 Well, PS, F-Bottom, clear, Microlon®, High Binding [Greiner bio-one]) were coated with HDM coating solution (3,125 µg/mL HDM in 50 µL coating buffer (100 mM carbonate-bicarbonate, pH 9.5) for 24 hours. Plates were blocked with 300 µL PBS containing 2% bovine serum albumin (BSA; SIGMA) and incubated for 1 hour. After three washing steps, 50 µL of adequately diluted serum duplicates were applied to the plate and serial diluted over four steps and incubated for 2 hours (IgE dilutions 1:10/20/40/80, IgG1 dilutions 1:200/400/800/1600, IgG2b dilutions 1:100/200/400/800). After removal of the serum and three washing steps, biotin conjugated primary antibodies for IgG1 (Biotin Rat Anti-Mouse IgG1, clone: A35-1, BD Pharmingen™), IgG2b (Biotin Rat Anti-Mouse IgG2b, clone: R12-3, BD Pharmingen™) or IgE (Biotin Rat Anti-Mouse IgE, clone: R35-118, BD Pharmingen™) were applied in 1% BSA in PBS and incubated for 1 hour. After three washing steps, streptavidin conjugated horseradish peroxidase (BD Pharmingen™) was applied and incubated for 1 hour and removed with three washing steps. For the color detection reaction, the tetramethylbenzidine (TMB) Substrate Reagent Set from BD OptEIA™ was added; colorimetric reaction was stopped by addition of 1M sulfuric acid. Optical density (OD) was measured with plate reader Microplate Reader (BIO-RAD iMark™). The antibody titer was defined as the reciprocal serum dilution yielding an absorbance reading of OD=0.2 after linear regression analysis.

## 2.8 Analysis of impact of VacA on murine bone marrow-derived DCs

DCs were generated from bone marrow of C57BL/6 mice as described previously by Beckert et al. (24).

On day 8, $1x10^5$ cells were seeded into a 96-well round bottom plates and three different conditions were performed: a) naïve (cultured without any further stimulus); b) activated, cultured in presence of lipopolysaccharide (LPS [1 µg/mL; Sigma, *E.coli* O111:B4]); c) activated and allergen-treated, cultured following a standardized protocol in presence of the allergen ovalbumin (OVA; [EndoGrade® (purity >98%,

endotoxin c: <1 EU/mg)]) loading (5 µg/mL) on day 7 and activated with LPS (1 µg/mL added at day 8). In each condition, VacA was applied without any further modifications at a concentration of 10 µg/mL, one hour before activation was performed (Figure 5A).

The activation state of naïve, activated and activated cells additionally treated with allergen was analyzed by flow cytometry 24 hours later. Using the antibodies listed in Supplementary Table 2 (murine DC activation panel), cells were stained as already described and the following strategy was used to characterize DC. Following exclusion of doublets and dead cells, DCs were identified by the expression of CD11c and MHCII. Expression of the activation markers CD80, CD86, CD40 was analyzed. Mean expression intensity was determined using FlowJo. To better compare different experiments with each other, the percentage change in expression between VacA treatment and the corresponding comparison group was calculated. Supernatants of DCs were stored at –20°C.

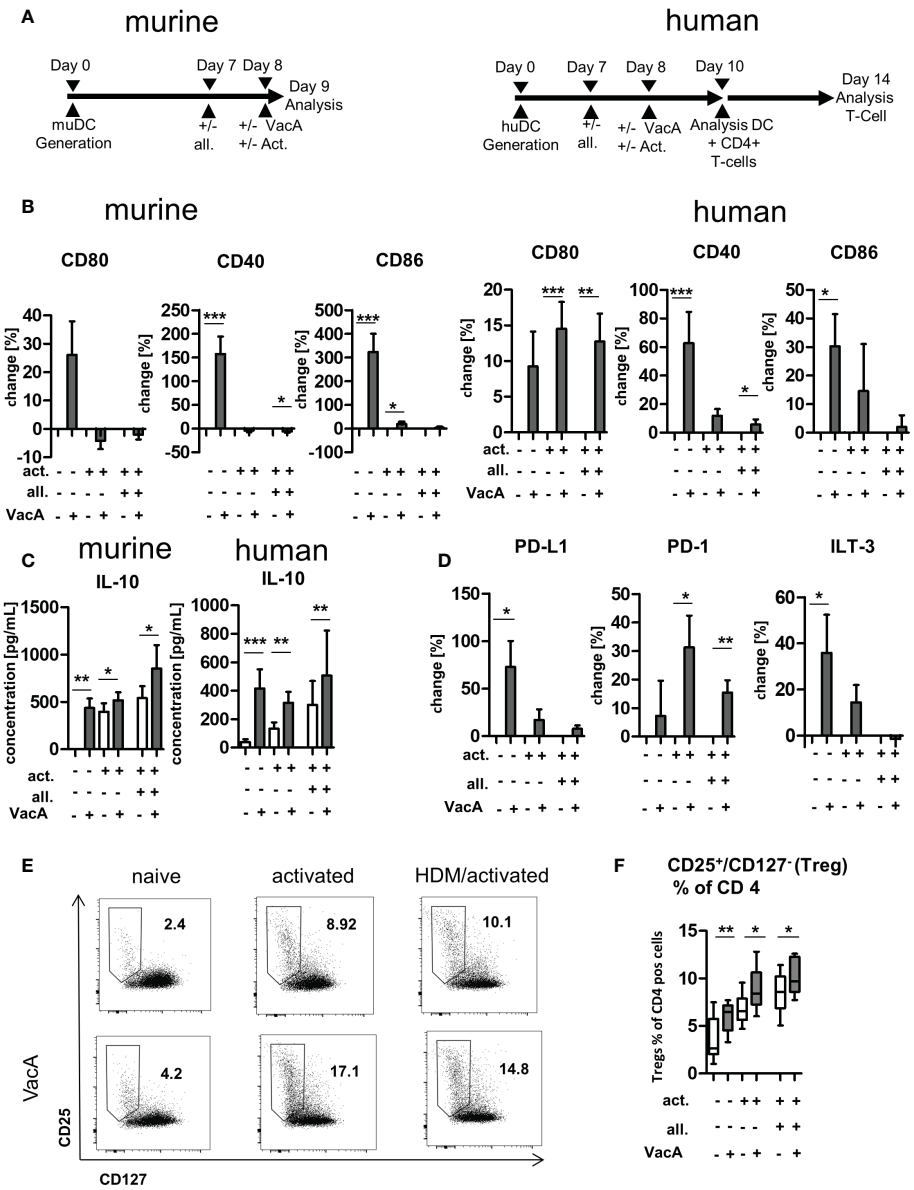


FIGURE 5
VacA modulates expression of stimulatory and inhibitory co-receptors and cytokines in murine and human dendritic cells (DC) and induces humanregulatory T cells (Tregs). **(A)** Treatment scheme: murine bone marrow DC (BMDC)/human DC: after 7 days of differentiation DC were treated +/-allergen (all), then DC activation (act.) was performed and treatment with VacA was given. Cultivation of human DC with autologous T cells wasperformed from day 10–14. **(B)** Change in expression of the indicated surface marker after treatment with VacA versus naïve, activated, or activated andallergen-supplemented murine and human DC. **(C)** Supernatant concentrations of interleukin (IL)-10 in naïve, activated, and activated and allergensupplementedmurine and human DC cultures with or without VacA. **(D)** Change in expression of the indicated surface marker with inhibitory capacityversus VacA treatment on the surface of naïve, activated, or activated and allergen-supplemented human DC (murine, n=14; human, n=10). AutologousT cells were cultured with the differentially activated and VacA-treated DC and the proportion of regulatory T cells was then examined. **(E)** Dot plots:regulatory T cells were identified by gating CD25+/CD127- cells within the population of CD4+/CD3+ T helper cells. Dot plots show representativeexamples for CD25/CD127 staining of naïve, activated, and activated and allergen-supplemented human DC/T cell cultures with or without VacAtreatment. Gate and proportion of Tregs is highlighted. **(F)** Percentage of Tregs within the CD4+ T cell population. VacA treatment significantly increasedTregs in all analyzed culture conditions (n=10). Wilcoxon signed rank test: *p<0.05, **p<0.01, ***p<0.001.

## 2.9 Analysis of impact of VacA on PBMC-derived human DC and their interaction with autologous T cells

Human DCs were differentiated from monocytes isolated from PBMC using a modified version of a previously published protocol (25). In brief, after isolation of PBMC using BioColl® (Bio&Sell), monocytes were isolated from PBMC with a magnetic bead sorting system from Miltenyi (Pan Monocyte Isolation Kit, human), as recommended by the manufacturer. In a Nunc EasyFlask 75 $cm^2$ Nunclon™ Delta Surface cell flask, $10^7$ cells/10mL were seeded and incubated (5% $CO_2$ at 37°C) for seven days in DC medium (X-Vivo-15 medium [Lonza]) supplemented with granulocyte-macrophage colony-stimulating factor (800 U/mL; MiltenyiBiotech), interleukin (IL)-4 (1000 U/mL; PeproTech), 2% autologous serum, and 1% penicillin streptomycin (Gibco). After 4 days, the same amount of medium was added to the cells. After 7 days, cells were collected with PBS/EDTA buffer (PBS, 2 mM EDTA pH 7.4–7.9) and $10^6$ cells/mL were plated into 96-well round bottom plates in DC medium. The impact of VacA was analyzed in three different conditions: a) naïve cells, cultured without any further stimulus; b) activated cells, cultured in presence of an activation cocktail containing 30 ng/mL IL-1β (Miltenyi), 30 ng/mL tumor necrosis factor (TNF)-α (Miltenyi Biotech) and 3 µg/mL prostaglandin-D2 (Cayman Chemical) for 48 hours from day 8–10; and c) allergen-treated/-activated cells, incubated with HDM (6.5 µg/mL Greer) at day 7 and treated with the activation cocktail from day 8–10. In all groups, VacA (10 µg/mL) was added at day 8 without any further modifications (Figure 5A).

Impact on activation state was analyzed *via* flow cytometry at day 10 using antibodies listed in Supplementary Table 2 (human DC activation panel) and the following gating strategy. Staining of dead cells, blocking of FcγR (Human TruStain FcX™; BioLegend) and staining with specific antibodies was performed as described above. Following exclusion of doublets and dead cells, moDC were identified by expression of HLA-DR and CD11c. Expression of surface molecules with immune-activatory (ITAMs) and inhibitory (ITIMs) properties were analyzed. Comparable to the mouse model, mean expression of the ITAMs CD80, CD40 and CD86 were measured. Additionally, surface expression of the ITIMs programmed death ligand (PD-L) 1, programmed death (PD)-1 and ILT3 was measured (Supplementary Figure 1B). To better compare different experiments with each other, the percentage change in expression of the MFI of the corresponding molecule was compared between VacA treatment and the corresponding comparison group. moDC supernatants were stored at –20°C.

To analyze the impact of VacA on Treg modulation, differentially treated moDC were cultivated with PBMC-derived autologous T cells. PBMC were isolated using BioColl®. CD4-positive T cells were isolated as per the manufacturer's instructions (CD4+ T Cell Isolation Kit, human, Miltenyi). T cells were cultured with moDCs (5:1 ratio) for 4 days in Xvivo supplemented with 1% PBS and 2% autologous medium (Figure 5A). To identify Tregs, doublets and dead cells were excluded, $CD4^+$ T cells were chosen, and Tregs were characterized as $CD127^-/CD25^+$ cells as described previously (26, 27). For detailed antibody information, see Supplementary Table 2 (human Treg analysis panel). The ratio of Tregs analyzed using FlowJo and expression were compared between all treatment groups.

All experiments with human samples were performed in accordance with the ethics vote 18-8069-BO approved by the medical faculty of the University Duisburg-Essen, Germany.

## 2.10 Cytokine detection in DC supernatants

To detect production of IL-10 and other cytokines, the LEGENDplex™ HU Th Cytokine Panel (12-plex) or the LEGENDplex™ MU Th Cytokine Panel (12-plex) assays were performed with supernatants from human moDC or murine *in vitro* DC assays, respectively. Assays were performed and cytokine concentrations were calculated according to the manufacturer's instructions.

## 2.11 Statistical analysis

Values for all measurements are expressed as mean ± standard error of the mean (SEM). Analysis of variance (ANOVA) was used to determine all between-group differences. To compare differences between two groups, an Anderson–Darling test was first performed to control gaussian approximation, and then an appropriate test (unpaired Student t-test or Mann–Whitney U test) was used for analyses. The Wilcoxon signed ranked test was used for comparisons in *in vitro* experiments. The p-value cutoff for statistical significance was set at 0.05.

# 3 Results

## 3.1 Treatment with *H. pylori*-derived VacA ameliorates allergic airway disease

HDM sensitization and challenge (Figure 1A) resulted in development of airway hyperreactivity, airway inflammation and goblet cell metaplasia, all of which were significantly attenuated by treatment with VacA (Figures 1B–E). No suppression of allergic airway disease was detectable when animals were treated with a mutant VacA protein (VacA Δ6-27) (Supplementary Figure 2).

Treatment of animals with rVacA also suppressed airway inflammation and goblet cell metaplasia (Supplementary Figure 3), supporting the therapeutic effect of VacA in the allergic airway disease model.

In sensitized and challenged animals there were no differences between VacA-treated and placebo-treated animals with respect to changes in bodyweight over time, signs of peritoneal inflammation and animal distress scores (data not shown).

## 3.2 Treatment with VacA modulates the composition of allergen-specific immunoglobulin subtypes

Sensitization and challenge with allergen increased production of allergen-specific IgE and IgG1 compared to mice treated with PBS during the challenge phase (Figure 1F). Treatment with VacA did not affect

serum IgE, and was associated with a small, statistically insignificant decrease in IgG1 levels compared with sensitized and challenged animals. However, animals treated with VacA showed an increase in serum levels of allergen-specific IgG2b, resulting in a significant reduction in the IgG1/IgG2b ratio compared with the positive control.

## 3.3 Treatment with VacA modulates the immune phenotype

Animals treated with VacA showed significantly reduced numbers of conventional (c)DCs, B cells, and CD4- and CD8-positive T cells in the lung draining lymph node (Figures 2A, B). Compared with cDCs, there were no significant detectable differences in the number of plasmacytoid and inflammatory monocyte-derived DCs between animals in the positive control group and those in the VacA-treated group (Figure 2A).

Treatment with VacA slightly but significantly increased the frequencies of $CD4^+$/$FoxP3^+$ Tregs in mesenteric lymph nodes (Figures 2C, D). In spleen, lung and lung draining lymph node was no statistically significant between untreated and VacA treated animals detectable. (Figure 2D).

To evaluate if VacA affects Treg subset, CD25 positive and negative FoxP3 positive Tregs were distinguished. None of the analyzed organs demonstrated a significant difference in the composition of these two subpopulations between VacA treated and untreated groups (Supplementary Figure 4).

Animals treated with rVacA showed comparable induction of Tregs in mesenteric lymph nodes (meLNs) and demonstrated a significantly increase of Tregs in the spleen as well (Supplementary Figure 3D). In comparison to untreated sensitized and challenged animals demonstrated DCs in the lung draining lymph nodes of animals treated with VacA a slightly reduced expression of MHCII molecules on their surface (Figures 2E, F and Supplementary Figure 1C).

## 3.4 Tregs play a pivotal role during VacA-mediated immune suppression

To assess the role of Tregs in mediating the protective effects of VacA on allergen-induced airway disease, the same treatments as shown in Figure 1 were performed in DEREG mice (13), which allows the depletion of Tregs by injecting DT (Figure 3A).

Similar to the previous experiments, sensitized and challenged DEREG mice developed eosinophilia in BAL (Figure 3B), inflammatory infiltrates in lung tissue (Figure 3C), and goblet cell metaplasia (Figure 3D). Treatment with VacA reduced BAL eosinophils and improved lung histology. Depletion of Tregs *via* administration of DT in sensitized and challenged DEREG mice did not affect the inflammatory phenotype. However, depletion of Tregs in VacA-treated mice abolished VacA-mediated immune suppressive effects, and animals developed eosinophilia in BAL, lung inflammation and goblet cell metaplasia that was comparable to the untreated positive control (Figures 3B–D).

DEREG animals receiving DT showed a lower proportion of GFP-positive Tregs in meLN, spleen and tLN (Supplementary Figure 5). There was no difference in the number of inflammatory cells in sensitized and challenged WT animals, and sensitized and challenged WT animals treated with DT (data not shown).

## 3.5 Effectiveness of VacA in a therapeutic model

To further analyze the effectiveness of VacA as a therapeutic molecule, a secondary challenge model was utilized. In this setting, treatment of VacA was applied to animals with already developed allergic airway disease during a secondary, repeated exposure to the allergen (Figure 4A). In this model, treatment with VacA ameliorated the lung function decline (Figure 4B) and attenuated the inflammatory phenotype of allergic airway disease, including reducing the numbers of lymphocytes, neutrophils and eosinophils in BAL (Figure 4C), and attenuating both inflammation (Figure 4D) and goblet cell metaplasia in the lung (Figure 4E). Induction of subepithelial collagen deposition, a clear sign of airway remodeling, was significantly reduced in mice treated with VacA (Figure 4F). Furthermore, there was a significant increase of Tregs in the spleen and lung after treatment with VacA (Figure 4G).

## 3.6 VacA exposure drives tolerogenic reprogramming of murine and human DC

To characterize the influence of VacA on DC and T cells in more detail and to draw first conclusions about its effectiveness in humans, murine and human cell culture experiments were carried out. Murine and human DCs were generated from bone marrow and PBMCs, respectively, and the effects of VacA were analyzed alone and in combination with allergen exposure (Figure 5A). Application of VacA to naïve murine and human DC resulted in upregulation of the co-stimulatory molecules CD40, CD80 and CD86 (Figure 5B). Adding VacA to activated cells or activated cells treated with allergen did not further increase the expression of these markers (Figure 5B). The application of VacA strongly induced the production of the immune suppressing cytokine IL-10 in both murine and human DCs. Increased concentrations of IL-10 after VacA exposure were detectable in all analyzed conditions (Figure 5C). Naïve human DCs treated with VacA showed increased surface expression of PD-L1 and ILT3; these markers only tended to be increased in VacA-treated activated and allergen treated activated cells. However, these cells showed increased expression of PD-1 after VacA treatment compared with the respective control (Figure 5D).

Exposure of human T cells to autologous DCs treated with VacA resulted in the induction of $CD127^-$/$CD25^+$ $CD4^+$-positive T cells in all investigated conditions (Figures 5E, F). In addition, flow cytometric analyses of dead cells did not reveal any VacA-mediated cytotoxic effects on the *in vitro* cultures (Supplementary Figure 6).

# 4 Discussion

In the present study, we report that the systemic therapeutic administration of purified VacA (either the form secreted by *H. pylori* or a recombinant version) reduced allergic airway disease, as shown by decreased airway inflammation, tissue inflammation, PAS-positive cells and airway reactivity. Unlike previous studies that only showed a prophylactic efficacy of VacA after long-term treatment started shortly after birth, the current data demonstrate the potential therapeutic efficacy of this approach in asthma for the first time (9, 19). In both acute and therapeutic HDM-specific models of allergic airway disease, treatment of adult mice during the primary and secondary allergen challenge phase was associated with a reduction of the asthma phenotype. Beneficial effects on lung function, the inflammatory reaction in the lungs and BAL, and on restructuring processes in the lungs could be observed. The results of this study also suggest that VacA can modulate the function of both murine and human DCs in a similar manner. Furthermore, the VacA-mediated induction of Tregs in human *in vitro* cultures supports observations from the murine *in vivo* experiments and could thus represent a first indication of the effectiveness of VacA in humans.

The current results are consistent with previous studies investigating the activity of crude *H. pylori*-derived bacterial lysates containing all kinds of bacterial compounds in allergic airway disease (28, 29). Interestingly, treatment with pure VacA appeared to be more effective than the *H. pylori* lysate in our therapeutic model: subepithelial collagen deposition was reduced in VacA-treated mice but not in mice treated with *H. pylori* lysate. Collagen formation around the airways is one hallmark of airway remodeling that plays a central role in airway obstruction for individuals with chronic asthma. This observation is therefore important for the potential therapeutic efficacy of VacA but needs to be investigated in more detail in follow-up projects.

To further assess the functionality of VacA, we repeated the experiments using a mutant form of VacA (VacA Δ6–27), which lacks vacuolating activity (30). Treatment with this mutant showed no suppressive effect on airway inflammation and goblet cell metaplasia, similar to previous findings in preventive models (18, 19). This suggests that a full-length functional form of the protein is required for the observed activity. To further evaluate the therapeutic potential of VacA, we compared purified VacA derived from *H. pylori* with a recombinantly generated form of VacA (rVacA). Both were found to be similarly effective in suppressing airway inflammation and goblet cell metaplasia.

Allergen-specific immunoglobulins were analyzed to further assess the immunomodulatory impact of VacA. While there did not appear to be any modulation of allergen-specific IgE and IgG1, a significant induction of HDM-specific IgG2b was detectable after administration of VacA, resulting in a decreased IgG1/IgG2b ratio. Interestingly, while allergen-specific IgG1 is associated with development of high affinity IgE responses (31, 32), induction of allergen-specific IgG2 responses are described as having the ability to antagonize IgE-mediated allergic reactions (33).

Attenuation of the disease phenotype accompanied by a IgG2b-biased shift of immunoglobulins has also been reported in models of allergic conjunctivitis testing the therapeutic effectiveness of either superoxide dismutase 3 (34) or rapamycin (35). Induction of IgG2b is mediated by Th1 cells (36), which have also been shown to counterbalance Th2-mediated pathology in asthma (37).

DCs in particular, but also macrophages, are mediators of *H. pylori*-driven immune suppression (18). The therapeutic administration of VacA resulted in DC with a slightly reduced MHCII expression in the draining LN; such "semi-mature" DCs are associated with T cell tolerance (38) and could therefore contribute to the reduced inflammatory phenotype seen with the acute therapeutic approaches. Murine *in vitro* and DC transfer approaches have previously highlighted the central role of DCs in *H. pylori*-mediated asthma suppression and showed that VacA contributed to the induction of Tregs (3). Altobelli et al. confirmed that VacA targets myeloid cells (DCs and macrophages) of the gastric lamina propria (18). In macrophages, VacA promotes the secretion of the anti-inflammatory molecules IL-10 and transforming growth factor-β, and thus probably promotes the development of Tregs locally and also in the periphery.

Also in our models, administration of VacA modulated Treg responses. In both the acute and secondary challenge models we found induction of Tregs locally at the application site (mesenteric LN). In the therapeutic model of allergic airway disease, increased proportions of Tregs were also detectable in the spleen and lung. These differences might occur to different treatment protocols and time points of analysis.

Initial analyzes of Treg subpopulations did not show any differences in their composition at the time of analysis. Proportion of $CD3^+/CD4^+/FoxP3^+/CD25^{high}$ cells which are considered as the dominant natural Treg population consisting of thymic derived and induced Tregs and $CD3^+/CD4^+/FoxP3^+/CD25^{low}$ cells which are described as ancillary regulatory arm with an higher activation threshold (23) were comparable. Moreover, also a detailed analyses of Neuropilin 1 and Helios expression in (Data not shown) in the population of FoxP3 positive Tregs in the therapeutic model did not reveal a VacA mediated induction of a specific Treg subtype like induced or naturally occurring Tregs (39).

Nevertheless, a VacA mediated induction of a particular Treg subpopulation is imaginable. It could be that at the time of the analysis differences between subpopulations are no longer visible due to migration movements of the cells within the body. Kinetic experiments, which are focus of ongoing work might help to reveal the impact of VacA on Treg subtypes.

In prophylactic neonatal studies, *H. pylori* infection has been reported to reduce susceptibility to allergic airway disease, an effect mediated by Tregs (40, 41). The inability of VacA-deficient mutants to induce Tregs *in vitro* and mediate asthma protection *in vivo*, and the observation that prophylactic application of purified VacA attenuated the development of allergic airway disease, underlines the capability of this protein to induce Treg-mediated immune suppression (9, 42). To analyze the role of Tregs in the present therapeutic model, depletion assays were performed using DEREG mice. In these animals, application of DT can temporally deplete Tregs, allowing direct assessment of the functional role of these cells. Interestingly, Treg depletion during VacA treatment completely abolished the beneficial effects of the molecule.

Although only modest effects on the number of Tregs and the activation status of DC were seen, the findings of the the depletion

assay suggest that the suppressive effect of VacA is mediated *via* Tregs.

Using two different murine therapeutic models for allergic airway disease, the present study showed that *H. pylori*-derived VacA mediated immune suppression in adult animals and could therefore be useful as a therapeutic approach to asthma that might complement, or even replace, existing therapies.

To obtain initial data on whether VacA could be effective in humans, we compared its influence on the activation of DCs derived from humans (moDC) and mice (bone marrow derived DC). Application of VacA was associated with the upregulation of costimulatory molecules associated with the induction of adaptive immune responses, and was accompanied by the production of a variety of cytokines. In both mice and in human DC cultures, the immunosuppressive molecule IL-10 was detectable in increased concentrations. Induction of IL-10 is key for the protective effects mediated by various bacterial strains and compounds with well-documented therapeutic activity. In addition to extracts of *H. pylori* (9), other microbiota constituents such as *Bifidobacteria breve* (43) and *Faecalibacterium prausnitzii* (44) are also capable of inducing IL-10 secretion in DCs, and therefore the induction of beneficial Tregs. Interestingly, treatment with *F. prausnitzii* also induced tolerogenic factors in DCs (44). Moreover, VacA- and *F. prausnitzii*-treated naïve human moDCs showed induced expression of PD-L1, a surface molecule that is strongly associated with attenuation of T cell responses (45, 46). In addition to PD-L1, VacA is also capable of inducing production of ILT3, an inhibitory receptor that is expressed on tolerogenic DCs (47) and appears to be necessary for the induction of Tregs (48). Interestingly, VacA induces the expression of PD1 specifically in activated moDCs. Transfer models have shown that expression of PD1 on DCs attenuates the proliferation of antigen-specific $CD8^+$ T cells (49). To assess Treg-inducing capacities in the current study, human moDCs were cultured with autologous T cells. VacA increased the proportion of Tregs under all conditions, underlining its regulatory properties in both murine and human systems.

Several epidemiological studies demonstrate that *H. pylori* strains containing particularly immunogenic forms of VacA are associated with increased gastric cancer risk. However, there is little direct evidence in animal models demonstrating that VacA contributes to the pathogenesis of gastric cancer (50). In the current study, no negative effects of treatment with VacA were seen in either the prophylactic or the therapeutic models nor in the murine and human *in vitro* models, suggesting that VacA may be a safe therapeutic intervention for the treatment of asthma.

However, the administration of such a therapeutic must be balanced to avoid potential unwanted side effects that could be mediated by the induction Tregs.

In summary, these data show suppression of allergic airway disease by administration of VacA in a therapeutic setting. VacA induced the generation of Tregs *via* modulation of DCs, and this induction of Tregs is a key event in VacA-mediated immune suppression in therapeutic asthma models.

Modulation of Treg responses might represent an interesting therapeutic approach not only for asthma but also for other diseases with an underlying exaggerated immune pathology. The prophylactic application of VacA demonstrated already beneficial effects in model of food allergy (19). Here but also in other allergic diseases is the therapeutic treatment approach of great interest and will be focus of future work.

VacA exerts similar tolerogenic effects on human moDCs, and can readily be produced in recombinant form, making it an attractive candidate for therapeutic intervention in patients with asthma.

## Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## Ethics statement

The studies involving human participants were reviewed and approved by Ethics committee of the medical faculty of the University Duisburg-Essen, Germany (ethics vote 18-8069-BO). The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Landesamt für Natur, Umwelt und Verbraucherschutz North Rhine Westphalia, Germany; reference number: AZ 81-02.04.2018.A084.

## Author contributions

SR designed the experiments. SR, JR, HU, AC performed the experiments. SR and JR, analyzed the data. SR and CT conceived and supervised the project. EP, AW, GC, TC, AM supported the writing process and supplied VacA and animals. SR, JR and CT wrote the manuscript. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1092801/full#supplementary-material

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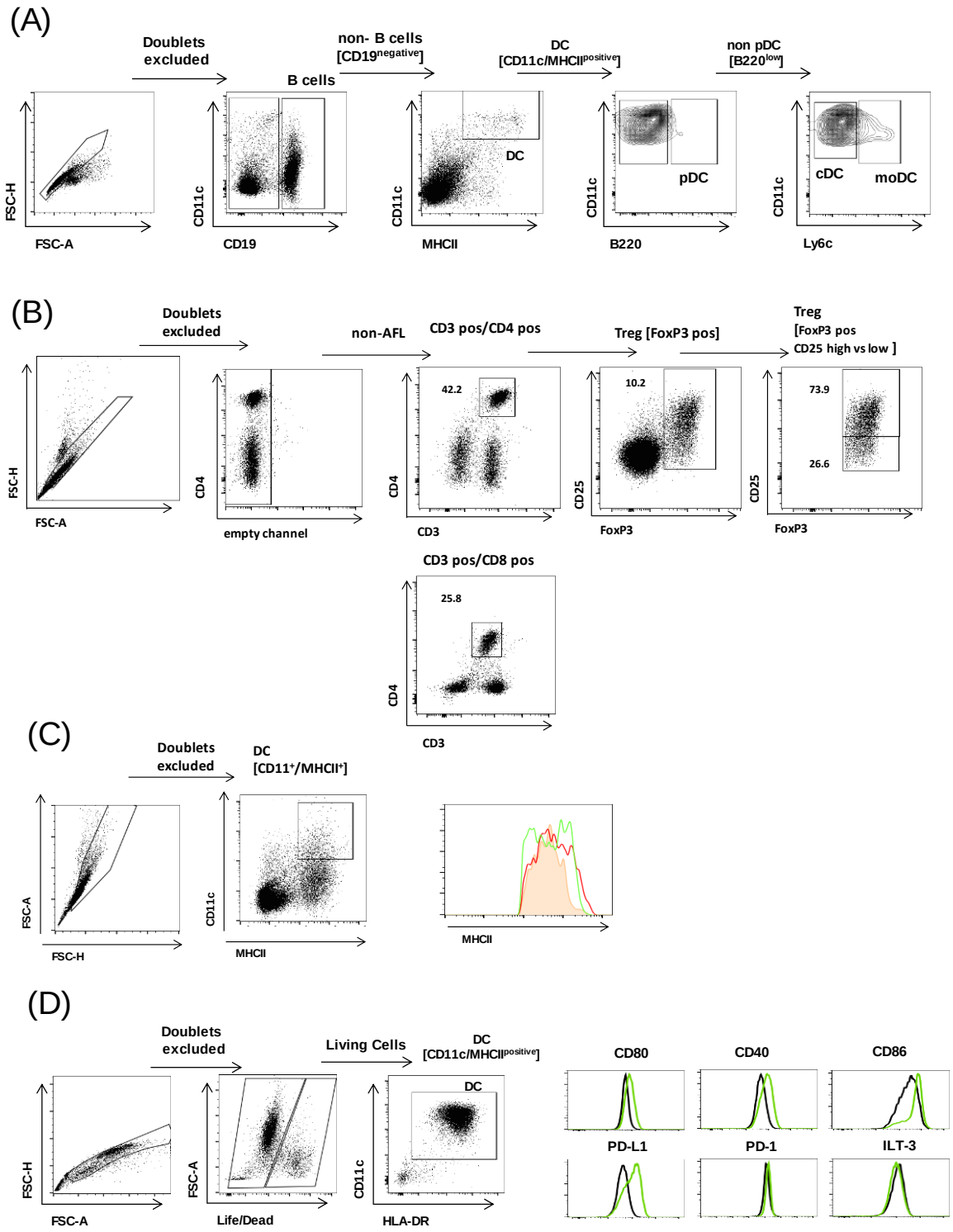
# *Supplementary Material*

| Panel | murine Treg Analysis | | | | |
|---|---|---|---|---|---|
| **Fluochrome** | **Specificity** | **Clone** | **Reactivity** | **Antibody Host Species** | **Manufacturer** |
| PerCP-Cy™5.5 | CD3 | 145-2C11 | mouse | rat | Biolegend |
| PE/Cyanine7 | CD4 | 53-6.7 | mouse | rat | Biolegend |
| Pe | CD25 | PC61 | mouse | rat | BD Pharmingens |
| Brilliant Violet 650™ | CD25 | PC61 | mouse | rat | Biolegend |
| APC | FoxP3 | FJK-16s | mouse | rat | Biolegend |
| | | | | | |
| **Panel** | **murine DC/B cell Analysis** | | | | |
| **Fluochrome** | **Specificity** | **Clone** | **Reactivity** | **Antibody Host Species** | **Manufacturer** |
| FITC | MHCII | M5/114.15.2 | mouse | rat | Biolegend |
| PE/Dazzle™ 594 | CD19 | 6D5 | mouse | rat | Biolegend |
| PE/Cyanine7 | CD11c | N418 | mouse | hamster | Biolegend |
| APC/Cyanine7 | CD3 | 145-2C11 | mouse | hamster | Biolegend |
| Alexa Fluor® 700 | CD45 | 30F11 | mouse | rat | Biolegend |
| Brilliant Violet 421™ | Ly6c | HK1.4. | mouse | rat | Biolegend |
| Brilliant Violet 785™ | B220 | RA3-6B2 | mouse/human | rat | Biolegend |

**Supplementary Table 1. Antibody List murine ex vivo Analysis**

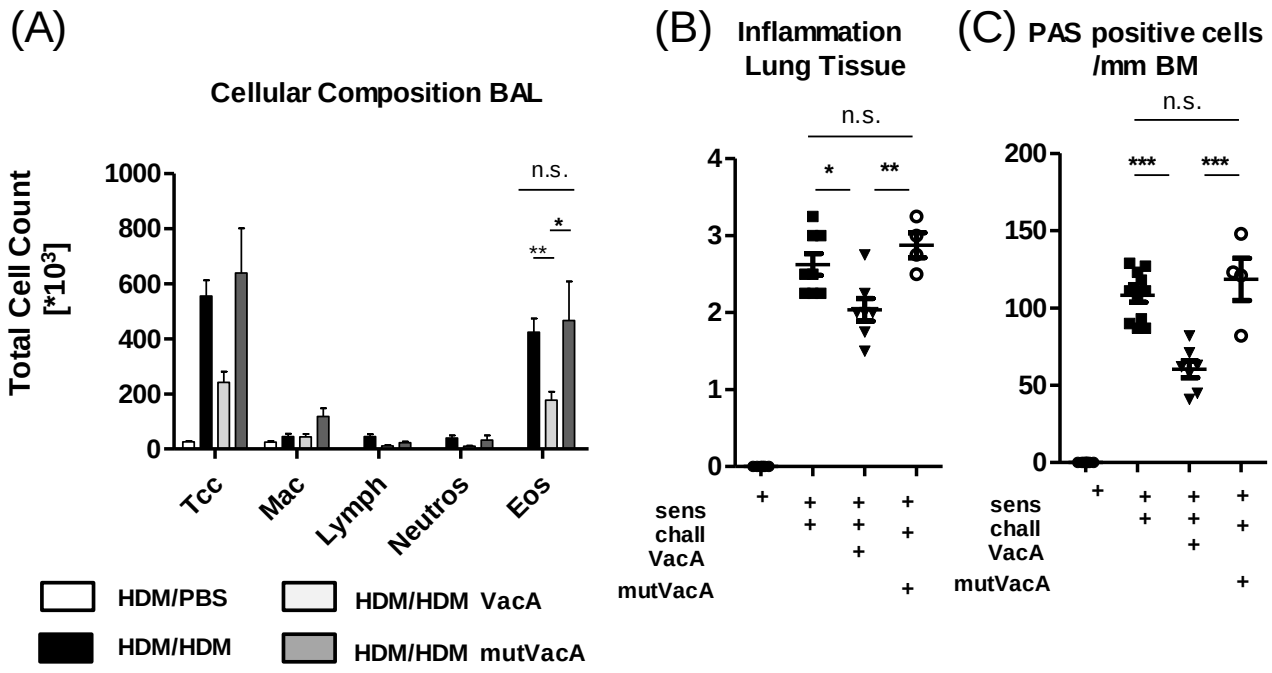
| Panel | murine DC activation | | | | |
|---|---|---|---|---|---|
| **Fluochrome** | **Specificity** | **Clone** | **Reactivity** | **Antibody Host Species** | **Manufacturer** |
| FITC | MHCII | M5/114.15.2 | mouse | rat | Biolegend |
| PE | CD86 | GL1 | mouse | rat | Biolegend |
| PerCP-Cy™5.5 | CD80 | 16-10A1 | mouse | hamster | Biolegend |
| PE/Cyanine7 | CD11c | N418 | mouse | hamster | Biolegend |
| Pacific Blue | CD40 | 3/23 | mouse | rat | Biolegend |
| NUV450 | Zombie UV | | | | Biolegend |
| | | | | | |
| **Panel** | **human DC activation** | | | | |
| **Fluochrome** | **Specificity** | **Clone** | **Reactivity** | **Antibody Host Species** | **Manufacturer** |
| FITC | PD-L1 | MIH3 | human | mouse | Biolegend |
| PE/Dazzle™ 594 | PD1 | EH12.2.H1 | human | mouse | Biolegend |
| PE/Cyanine7 | ILT3 | ZM4.1 | human | mouse | Biolegend |
| Brilliant Violet 421™ | HLA-DR | L243 | human | mouse | Biolegend |
| Brilliant Violet 510™ | CD80 | 2D10 | human | mouse | Biolegend |
| Brilliant Violet 605™ | CD40 | 5c3 | human | mouse | Biolegend |
| Brilliant Violet 650™ | CD11c | 3.9 | human | mouse | Biolegend |
| Brilliant Violet 785™ | CD86 | IT2.2 | human | mouse | Biolegend |
| NUV450 | Zombie UV | | | | Biolegend |
| | | | | | |
| **Panel** | **human Treg Analysis** | | | | |
| **Fluochrome** | **Specificity** | **Clone** | **Reactivity** | **Antibody Host Species** | **Manufacturer** |
| PercP-Cy5.5 | CD4 | RPAT4 | human | mouse | Biolegend |
| APC | CD127 | AO19D5 | human | mouse | Biolegend |
| AF700 | CD3 | UCHT1 | human | mouse | Biolegend |
| Brilliant Violet 421™ | CD25 | HIB 19 | human | mouse | Biolegend |
| NUV450 | Zombie UV | | | | Biolegend |

**Supplementary Table 2. Antibody List murine/human in vitro Analysis**

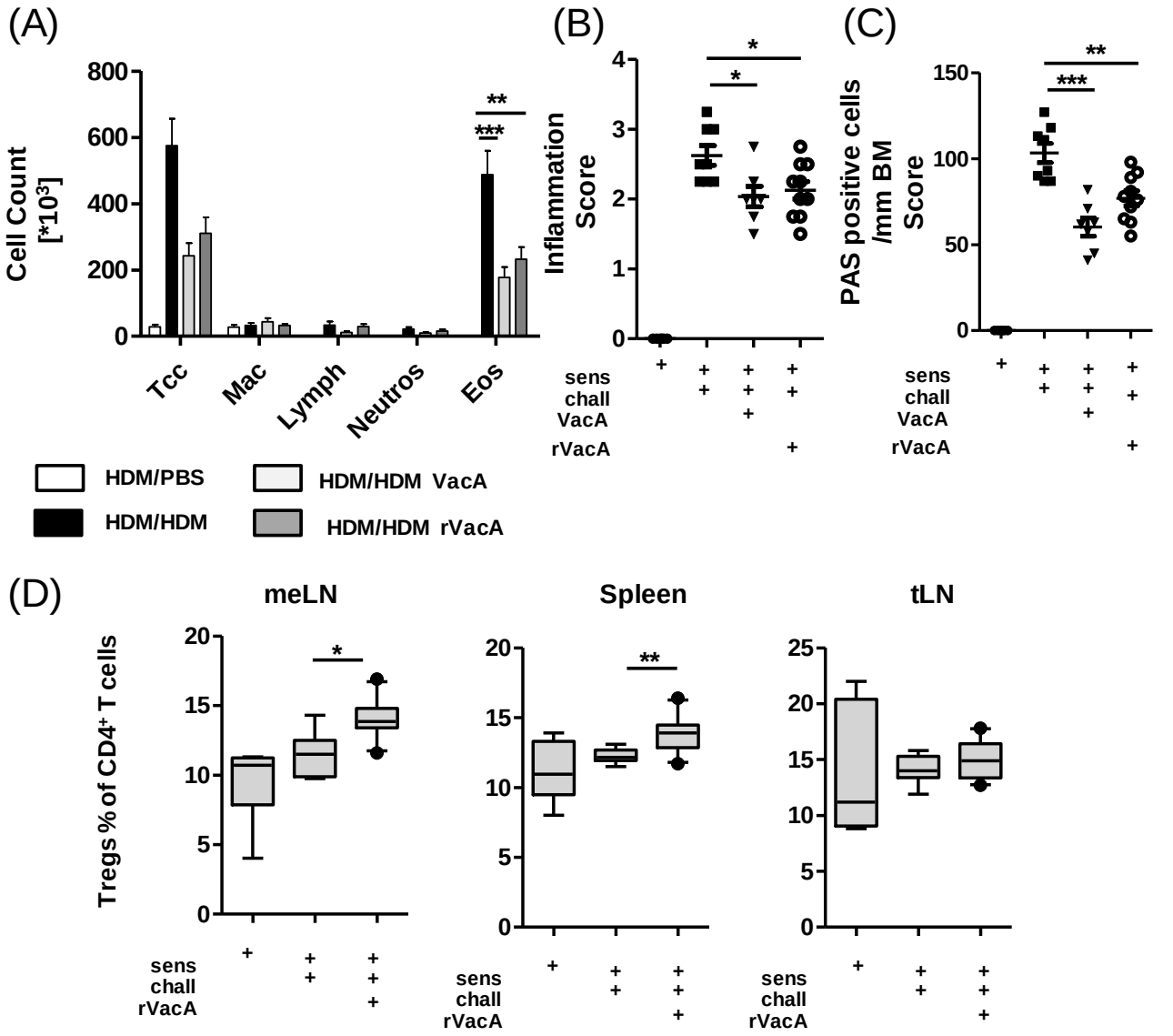



**Supplementary Figure 1: Flow cytometry gating strategies B cells/DC Subtypes, Tregs and DC MHCII expression *ex vivo*/DC activation *in vitro*.** A) The dot plots presented show the gating strategy used to analyze B cells and DC ex vivo. Following exclusion of doublets via FSC-A vs. FSC-H B cells were characterized as CD19 positive cells. Within the group of CD19 negative cells DC were analyzed as CD11c/MHCII positive cells. To further distinguish subpopulation expression

of B220 was analyzed and positive cells were characterized as pDC. Further, Ly6c expression was analyzed on B220 negative cells. Ly6c negative cells were identified as cDC and positive cells as moDC. B) The gating strategy shows the in vitro analysis of regulatory T cells ex vivo. First, debris and doublets were excluded based on size and shifting properties seen by analysis of FSC-Height vs. FSC-Area. Autofluorescent cells, positive cells in an empty channel were excluded. Based on the expression of CD8/CD3 cytotoxic T cells were differentiated from CD3/CD4 positive T Helper cells. Tregs were then defined as FoxP3-positive cells within the population of CD3/CD4 positive cells. Treg subpopulations were further subdivided in $CD25^+$ cell and $CD25^-$ cells. C) The gating demonstrates identification of DC and analysis of their MHCII expression in tLN: First, debris and doublets were excluded based on size and shifting properties seen by analysis of FSC-Height vs. FSC-Area. DC were identified by the expression of CD11c and MHCII. Within the population mean fluorescence intensity of MHCII was analyzed. Histogram shows MHCII expression in HDM/PBS (shaded orange), HDM/HDM (red line) and HDM/HDM VacA animals (green line). D) The gating strategy shows the in vitro analysis of DC activation using human DC as an example. Following exclusion of doublets living cells were identified based on the reduced expression of a life/dead marker. Within the living cells DC were characterized as CD11c/HLA-DR positive cells. Within the DC population surface expression of the markers shown in the histograms was analyzed. Histograms show the expression of the indicated markers on naïve DC (black line) and naïve DC treated with VacA (green line).

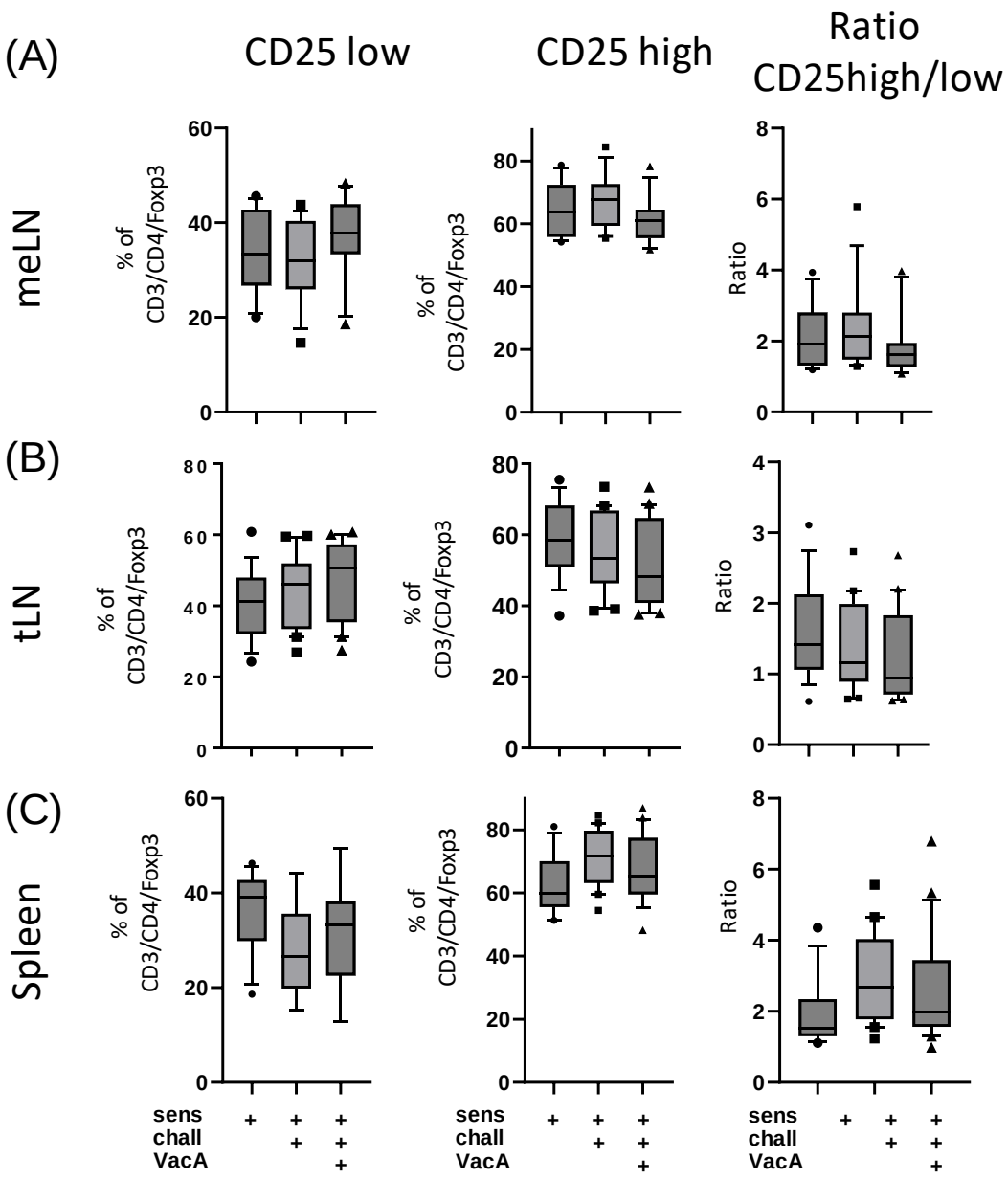



**Supplementary Figure 2: Effect of a VacA mutant on asthma hallmarks in an acute murine asthma model. A**) **Cellular composition of bronchoalveolar lavage (BAL) fluid**: The positive control (HDM/HDM, black bars) showed increased total cell count (Tcc), macrophages (Mac), lymphocytes (Lymph), neutrophils (Neutros) and eosinophils (Eos) compared with the negative Control (HDM/PBS, white bars). Eosinophil numbers were reduced in animals treated with VacA (HDM/HDM VacA, light gray) and comparable to the positive control in animals treated with the VacA mutant (HDM/HDM mutVacA, dark gray). **B**: **Inflammation in lung tissue:** Scatter plot shows inflammation score for HDM/PBS, HDM/HDM, HDM/HDM VacA or HDM/HDM mutVacA animals. **C**: **Mucus-producing cells in lung airways:** Scatter plot shows averaged number of mucus-producing cells/mm basal membrane in HDM/PBS, HDM/HDM and HDM/HDM VacA or HDM/HDM mutVacA animals.



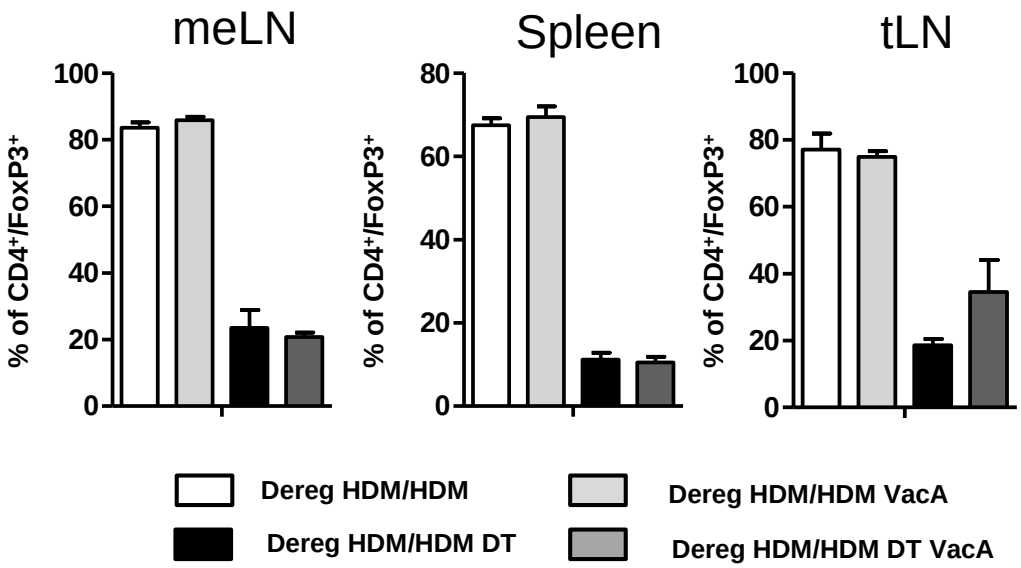

**Supplementary Figure 3: Recombinant produced VacA attenuates allergic airway disease in adult mice. A**) **Cellular composition of bronchoalveolar lavage (BAL) fluid**: The positive control (HDM/HDM, black bars) showed increased total cell count (Tcc), macrophages (Mac), lymphocytes (Lymph), neutrophils (Neutros) and eosinophils (Eos) compared with the negative Control (HDM/PBS, white bars). Numbers eosinophils were reduced detectable in animals treated with VacA (HDM/HDM VacA, light gray) or rVacA (HDM/HDM VacA, dark gray). **B**) **Inflammation in lung tissue:** Scatter plot shows inflammation scores for HDM/PBS, HDM/HDM, HDM/HDM VacA or HDM/HDM rVacA animals. **C**) **Mucus-producing cells in lung airways:** Scatter plot shows averaged number of mucus producing cells/mm basal membrane in HDM/PBS, HDM/HDM and HDM/HDM VacA or HDM/HDM rVacA animals. **D**) Box plots (Whiskers 10-90 percentile) show proportion of CD25/FoxP3 positive cells within the CD4/CD3 positive T helper cell population in

mesenteric lymph nodes (meLN), spleen and lung draining lymph nodes (tLN) of negative and positive controls, and rVacA-treated animals. **B-D**: Each point represents one animal, Data are results from two independent experiments, n=6–10 per group; Analysis of variance, *$p<0.05$, **$p<0.01$, ***$p<0.001$.



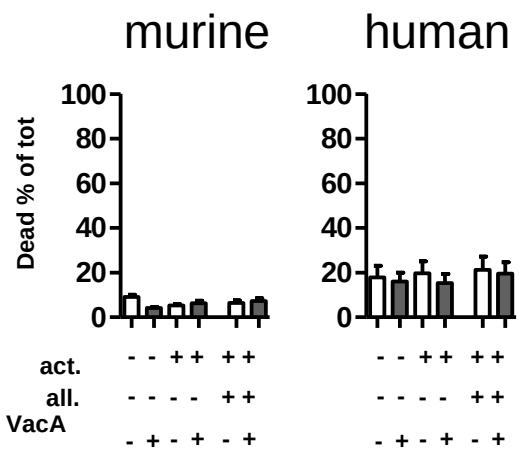

**Supplementary Figure 4: Treatment with VacA did not affect Treg Subtypes** Within the population of $CD3^+CD4^+/FoxP3^+$ cells $CD25^{low}$ and $CD25^{high}$ cells were analyzed. Boxplots (Whiskers 10-90 percentile) show percentage of $CD25^{low}$, $CD25^{high}$ and Ratio of $CD25^{high}/CD25^{low}$ Tregs in A) meLN B) tLN and C) spleen of HDM/PBS, HDM/HDM and HDM/HDM VacA-treated mice. Results are from 4-5 independent experiments, n=13-21 mice per group.

Analysis of variance, *p<0.05, **p<0.01, ***p<0.001.




**Supplementary Figure 5:**

Bar graphs show the proportion (%) of green fluorescent protein (GFP)-positive regulatory T cells in DEREG mice sens/chall (white bars), DEREG mice sens/chall treated with VacA (light gray bars), DEREG mice sens/chall with DT depletion (black bars) and DEREG mice sens/chall treated with VacA and DT depletion (dark gray bars).




**Supplementary Figure 6: VacA did not demonstrate cytotoxic effects.** Bar graphs demonstrate % of dead cells in the in vitro cultures of murine and human naïve, activated and activated VacA supplemented dendritic cells.

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Respiratory Research

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# Therapeutic properties of *Helicobacter pylori*-derived vacuolating cytotoxin A in an animal model of chronic allergic airway disease

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## Abstract

**Background** It has previously been shown that the *Helicobacter pylori* (*H. pylori*)-derived molecule vacuolating cytotoxin A (VacA) could be suitable for the treatment of allergic airway disease. The therapeutic activity of the protein, which acts through modulation of dendritic cells (DC) and regulatory T cells (Tregs), was demonstrated in murine short-term acute models. The aim of this study is to further evaluate the therapeutic potential of VacA by determining the effectiveness of different application routes and the suitability of the protein for treating the chronic phase of allergic airway disease.

**Methods** VacA was administered by the intraperitoneal (i.p.), oral (p.o.) or intratracheal (i.t.) routes, and long-term therapeutic effectiveness, allergic airway disease hallmarks, and immune phenotype were analyzed in murine models of acute and chronic allergic airway disease.

**Results** Administration of VacA via the i.p., p.o or i.t. routes was associated with a reduction in airway inflammation. The i.p. route showed the most consistent effect in reducing airway inflammation and i.p. treatment with VacA was the only treatment that significantly reduced mucus cell hyperplasia. In a murine model of chronic allergic airway disease, both short- and long-term treatment with VacA showed a therapeutic effect, with a reduction in a variety of asthma hallmarks, including bronchoalveolar lavage eosinophilia, lung inflammation and goblet cell metaplasia. Short-term treatment was associated with induction of Tregs, while repetitive long-term administration of VacA influenced immunological memory in the lung.

**Conclusions** In addition to showing therapeutic efficacy in short-term models, treatment with VacA also appeared to be effective in suppressing inflammation in a chronic airway disease model. The observation that treatment was effective after administration via several different routes highlights the potential of VacA as a therapeutic agent with different routes of administration in humans.

**Keywords** Asthma, *Helicobacter pylori*, Vacuolating cytotoxin A, Chronic allergic airway disease model, Regulatory T cells, Tissue residential memory T cells

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## Background

It is estimated that 300 million people worldwide are affected by asthma [1], making it one of the most common non-communicable chronic diseases. Asthma is characterized by a multitude of different disease phenotypes and endotypes [2]. Although the underlying pathophysiological mechanisms of asthma are now well described, existing treatment options are purely symptomatic rather than curative. The microbiome and its interactions with the immune system are promising starting points in the search for new therapies [3].

Surprisingly, the gastric bacterium *Helicobacter pylori* (which is associated with gastritis and gastric ulcer) has emerged as a promising candidate for treating asthma. Epidemiological studies [4, 5] and investigations in mouse models [6] have shown that neonatal infection with *H. pylori* protects against the development of asthma in later life. Studies with knock-out *H. pylori* mutants showed that the protein vacuolating cytotoxin A (VacA) plays a substantial role in mediating the protective effects of *H. pylori* in asthma [7]. It was subsequently shown that both prophylactic and therapeutic intraperitoneal (i.p.) treatment with purified VacA can suppress the development of an allergic respiratory disease in mice [8, 9].

In both prophylactic and therapeutic models, the suppressive effect of VacA was associated with immunomodulation of dendritic cells (DC) and the induction of regulatory T cells (Tregs). Furthermore, we were able to obtain initial indications that VacA might be translationally effective in humans [9]. Treatment of human DC with VacA induced the secretion of anti-inflammatory cytokine interleukin (IL)-10 and induced Tregs in autologous DC/T cell co-culture.

This study was designed to shed light on two central questions concerning the therapeutic effectiveness of VacA. Firstly, which route of administration is the most effective and would be best suited for the treatment of allergic respiratory disease? Secondly, is VacA effective in an animal model of chronic allergy respiratory disease, and are there differences between therapeutic short-term and long-term treatment approaches with respect to attenuation of asthma hallmarks and immune-modulating effects? Investigating these questions should provide further insights into the therapeutic potential of VacA for the treatment of asthma.

## Methods

### Animals

The animals used in the experiment were SPF C57BL/6JRj mice purchased from Janiver Labs [Le Genest-Saint-Isle, France] and housed under SPF conditions at the Laboratory Animal Facility of the University Hospital Essen. The mice were all female and aged between 8 and 12 weeks at the beginning of the experiments.

### VacA

The VacA used in these experiments was an active strep-tagged oligomeric s1m1 type VacA derived from modified forms of the *H. pylori* strain 60,190. VacA was isolated and purified as described previously [10].

### House dust mite (HDM)

A lyophilized extract derived from whole bodies of *Dermathophagoides pteronyssinus* (*D. pteronyssinus*) was obtained from Greer Laboratories [#XPB82D3A2.5] and used for all experiments. *D. pteronyssinus* represents the most common mite species in Germany and Europe, and is therefore the main source of HDM allergens for European individuals with atopy [11]. The lyophilized cake was reconstituted with phosphate-buffered saline (PBS) to a concentration of 1 mg/mL adjusted to the protein content. The Der p 1 concentration was 13 µg/mL.

### Acute allergic airway disease model

Acute allergic airway disease was induced as previously described [12]. On day 0, 1 µg of HDM protein was dissolved in 50 µL of PBS and administered intranasally (i.n.) to isoflurane-anesthetized C57BL/6JRj mice to trigger sensitization. To induce allergic airway disease, the anesthetized mice were challenged i.n. with 10 µg HDM dissolved in 50 µL PBS daily from day 7–11. 20 µg VacA dissolved in PBS was given via the i.p., per oral (p.o.) or intratracheal (i.t.) route on days 6, 7, 9 and 11; i.t. treatments were administered to animals that had been anesthetized with a mixture of ketamine and xylazine [both Serumwerk Bernburg]. Final analysis was performed on day 13 (Fig. 1A).

### Activation model

C57BL/6JRj mice were treated i.p. with VacA 20 µg dissolved in PBS, and analyzed after 24 and 48 h (Fig. 2A).

### Chronic allergic airway disease model

C57BL/6JRj mice were sensitized and challenged as described in the acute allergic airway disease model. Subsequently, mice received either 50 µL PBS or 1 µg HDM dissolved in 50 µL PBS twice a week for 10 weeks. Allergen and PBS were applied i.t. to avoid oral tolerance towards HDM. Animals were anesthetized with ketamine/xylazine and then the PBS or HDM solution was carefully injected directly into the airways with a pipette.

Two different treatment regimens with VacA were performed. For short-term treatment (T1), VacA 20 µg dissolved in 100 µL PBS was administered i.p. on three consecutive days in the last week of the experiment (week 10, days 66/67/68). For long-term treatment (T2), VacA 20 µg dissolved in 100 µL PBS was administered i.p. on three consecutive days in the middle and the last week of the experiment (week 6, days 39/40/41 and week 10, days

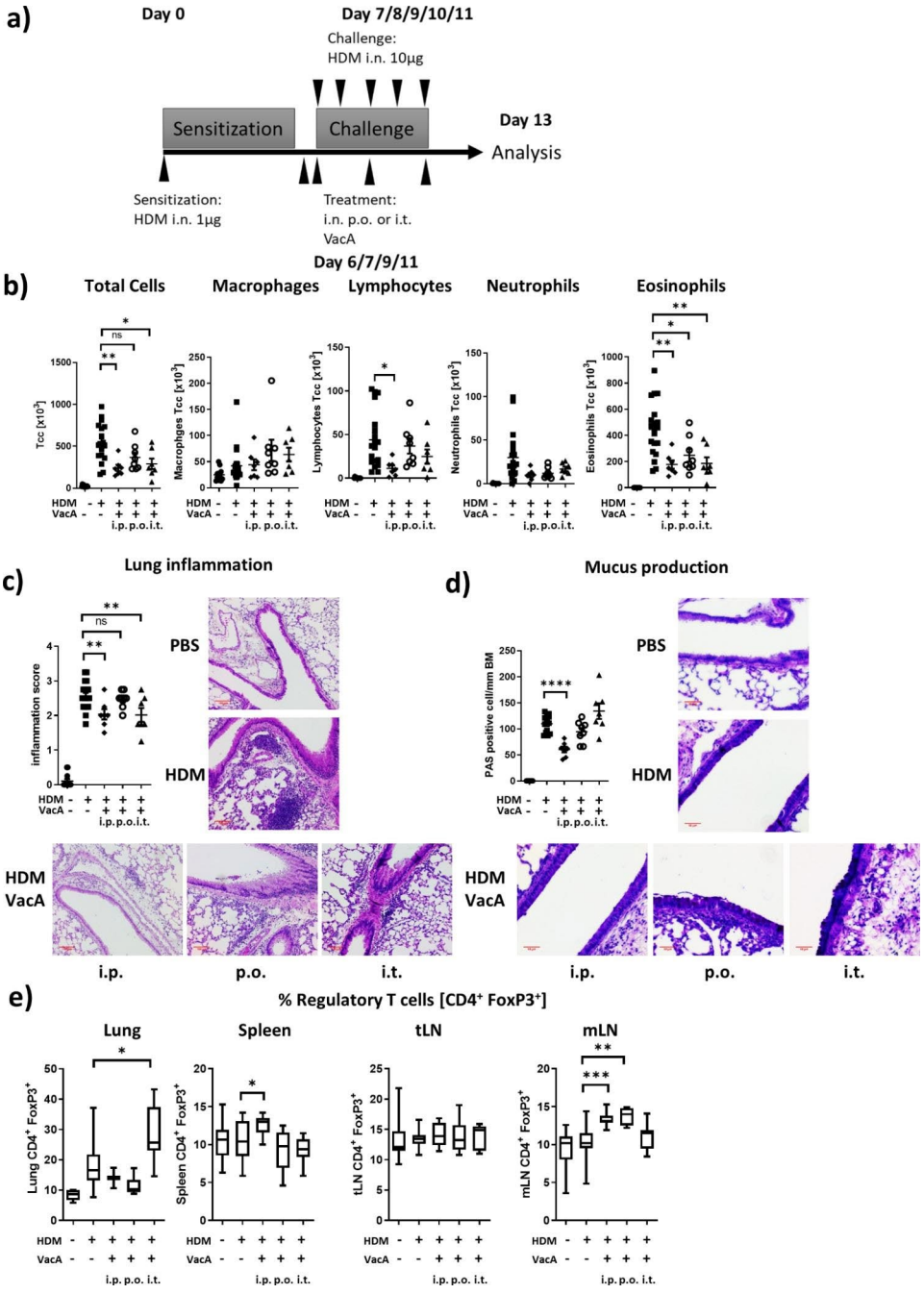


**Fig. 1** Application route dependent effects of VacA on the asthma phenotypes. **a)** On day 0 all animals were sensitized intranasally (i.n.) with 1 µg house dust mite (HDM); on day 7–11, the positive control and the three different VacA-treated groups were challenged i.n. with 10 µg HDM. The negative control group was challenged with phosphate-buffered saline (PBS). On days 6, 7, 9 and 11, animals were treated 20 µg VacA given via the intraperitoneal (i.p.), oral (p.o.) or intratracheal (i.t.) route. **b)** Cellular composition of bronchoalveolar lavage (BAL): total cell count (Tcc), macrophages, lymphocytes, neutrophils and eosinophils. **c)** Lung tissue inflammation: representative sections of each indicated group are shown (x100); scatter plot of inflammation score. **d)** Mucus-producing cells: pictures show representative sections from each group (x200); scatter plot of the number of mucus producing cells per mm of basement membrane. **e)** VacA treatment increases proportions of Tregs. Tregs were characterized by the expression of $FoxP3^+$ $CD25^+$ cells in the $CD3^+$ $CD4^+$ cell population; box plots show the percentage in lung, spleen, tracheal lymph node (tLN) and mesenteric lymph node (mLN); results from six independent experiments n = 6–15 per group. n.s. not significant; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001

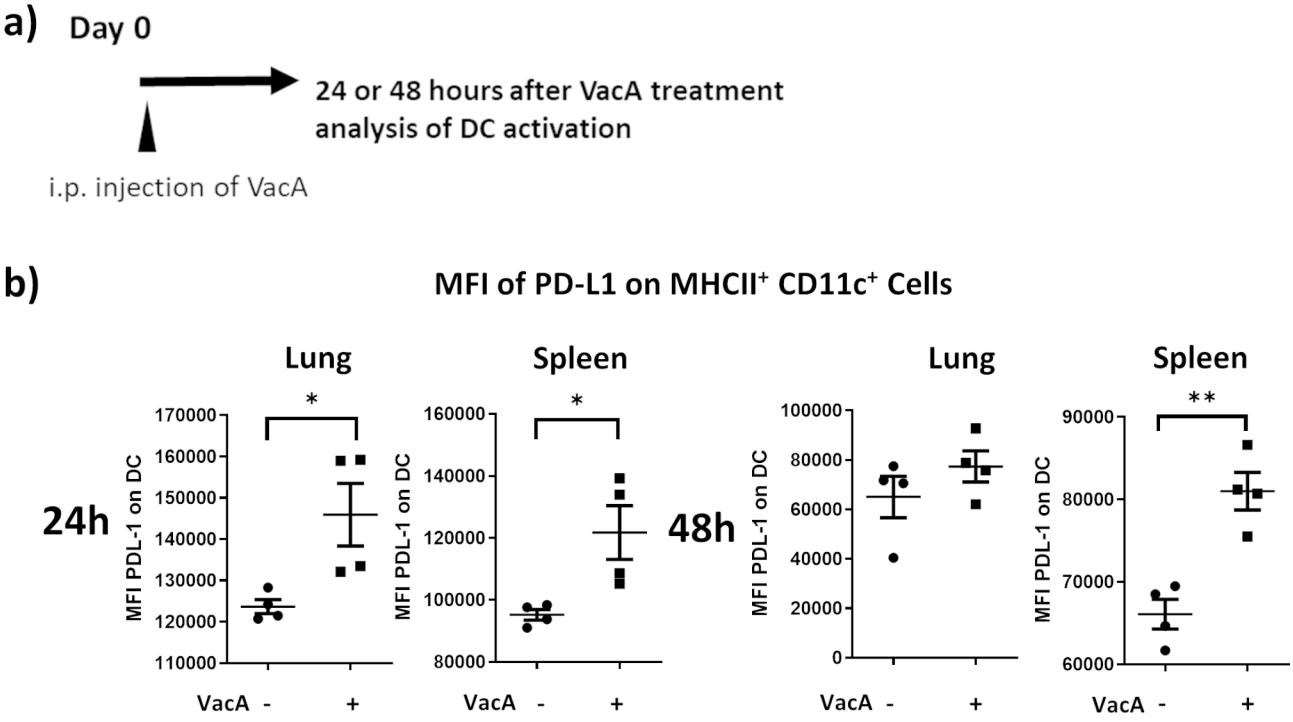


**Fig. 2** VacA treatment affects PD-L1 expression on MHCII$^+$CD11c$^+$cells. **a)** 20 µg intraperitoneal (i.p.) VacA was injected into C57BL/6JRj mice and PD-L1 on dendritic cells (DC) was analyzed after 24 or 48 h. **b)** Scatter plots show geomean fluorescence intensity (MFI) of PD-L1 on MHCII$^+$ CD11c$^+$ cells after 24 or 48 h in lung and spleen; results from n = 4 per group. *p < 0.05; **p < 0.01

66/67/68). Analyses were performed 24 h after the last VacA treatment (Fig. 3A).

#### Assessment of asthma hallmarks

To assess the impact of VacA on the asthma phenotype, the composition of the bronchoalveolar lavage (BAL) fluid, infiltration of immune cells into the lung tissue, the number of mucus-producing cells, and subepithelial collagen deposition were analyzed, and the immune phenotype was assessed.

#### Analysis of BAL

Lungs were flushed with 1 mL ice-cold PBS through an i.t. tube and total cell counts were determined. To determine differential cell counts, cytocentrifuged preparations of BAL were stained with Hemacolor-Set (Merck) and at least 200 cells were counted and differentiated into macrophages, lymphocytes, neutrophils and eosinophils based on histological properties. The total cell count for each cell type was calculated based on relative cell counts and the initially determined total cell number.

#### Lung histology

Tissue sections for histological analyses were prepared as previously described [13]. In short, after the removal of the left lung lobe, the right lung lobe was inflated with Histofix (Roth) and then transferred to the fixative. After embedding in paraffin, lung sections with a thickness of 2.5 µm were cut with a microtome. Sections were stained with hematoxylin/eosin (HE) to assess the degree of inflammation, or with combined Periodic Acid Schiff (PAS)/HE staining to identify mucus-producing goblet cells. Tissue sections were stained with Masson Trichrome staining to determine subepithelial collagen deposition.

The degree of lung inflammation was assessed by observers who were unaware of experimental groups, who scored five randomly selected areas on a scale from 0 (no visible infiltrate around airway vessels and parenchyma) to 4 (several layers thick cellular infiltrates on nearly all visible vessels and airways). A more detailed description of the individual scores has been reported previously [13]. Mucus-producing cells were quantified per millimeter of basal membrane on three different representative airways on PAS-stained slides. Subepithelial collagen deposition was determined by measuring the thickness of collagen deposition on five different positions in at least three airways. The mean thickness was first calculated for each airway and then across the three airways.

#### Assessment of the immune phenotype

The immune phenotype was analyzed in four different organs: lung, spleen, lung draining tracheal lymph node (tLN), and mesenteric lymph nodes (mLN) using flow cytometry. First, single cell suspensions were produced as

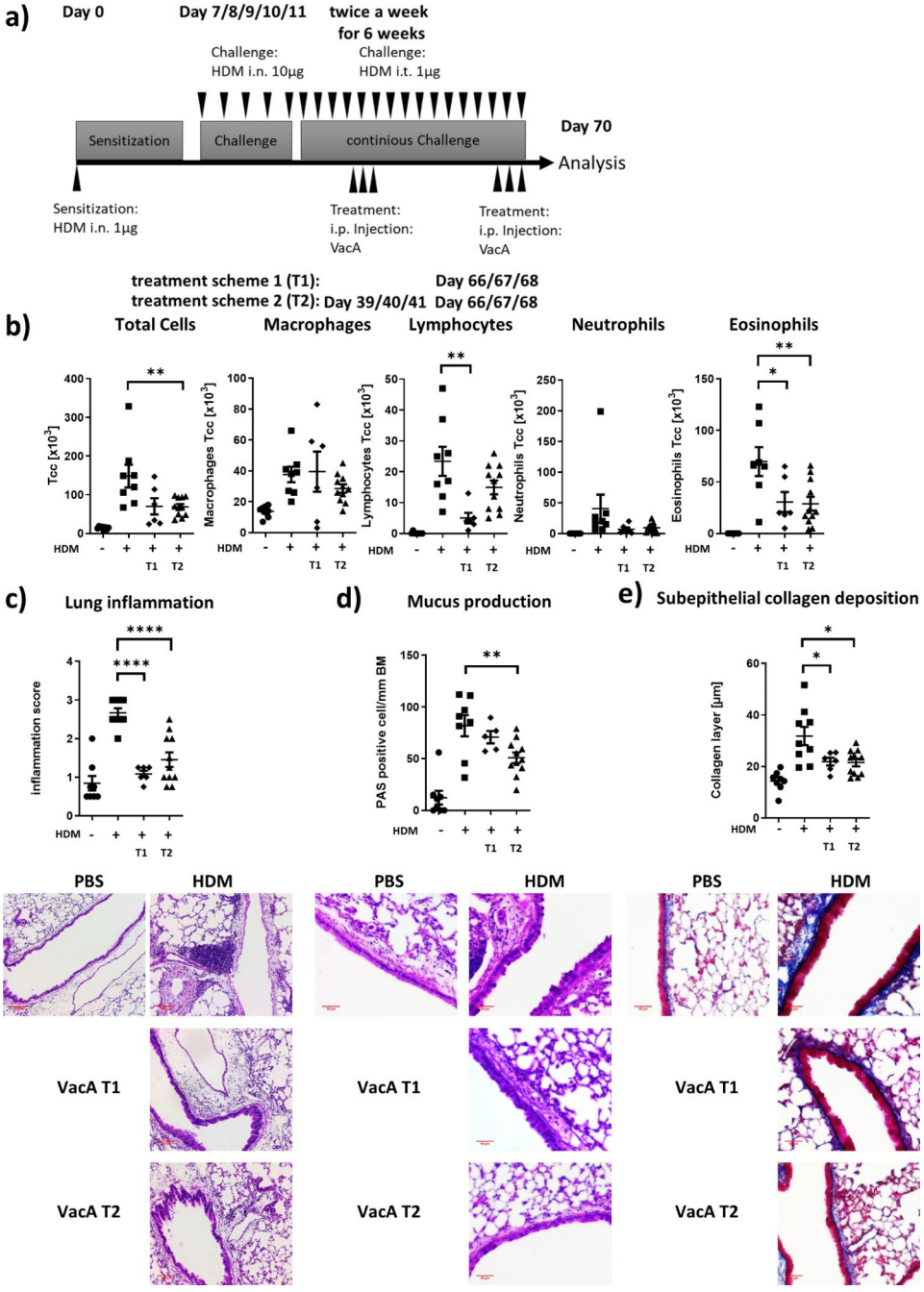


**Fig. 3** Effects of VacA treatment in a model of chronic allergic airway disease. **a)** Mice were sensitized on day 0 with 1 µg of house dust mite (HDM) given intranasally (i.n.). Mice were challenged with 10 µg HDM on days 7, 8, 9, 10 and 11. Subsequently, the positive control and the VacA-treated groups were challenged with 1 µg HDM intratracheally (i.t.) twice a week for 6 weeks. During the chronic challenge phase, two treatment schemes were applied: short-term (T1) = 20 µg intraperitoneal (i.p.) VacA at days 66, 67 and 68, and long-term (T2) = 20 µg VacA i.p. at days 39, 40, and 41 plus days 66, 67 and 68. **b)** Cellular composition of bronchoalveolar lavage (BAL): total cell count (Tcc), macrophages, lymphocytes, neutrophils and eosinophils. **c)** Lung tissue inflammation: representative sections of each indicated group are depicted (x100); scatter plot depicts inflammation score. **d)** Mucus-producing cells: pictures show representative sections from each group (x200); scatter plot depicts the number of mucus producing cells per mm of basement membrane. **e)** Subepithelial collagen deposition: pictures show representative sections from each group (x200); scatter plot shows averaged subepithelial collagen layer thickness in µm for each group; results from two independent experiments n = 5–9 per group. *p < 0.05; **p < 0.01; ****p < 0.0001

briefly described below. The lung was flushed with PBS via the right ventricle to eliminate venous blood before preparation. The left lung lobe was eviscerated, minced with a scalpel and transferred into 50 mL reaction tubes. A collagenase digestion was performed by adding collagenase type I (0.5 mg/mL; catalog no. C9891, Sigma) and incubating in a shaking water bath at 37 °C for 45 min. Subsequently, cells were passed through a cannula (20G 0.9 mm x 40 mm) and transferred through a 70 µm cell strainer into a new tube. Cell counts were determined after erythrocyte lysis ($KHCO_3$ 20mM/L [Roth], $NH_4Cl$ 310mM/L [Roth], EDTA 200µM/L [Sigma] in aqua dest.). The spleen was extracted and pushed through a 70 µm cell strainer, erythrocytes were lysed, and cell counts were determined. Lymph nodes were extracted and ground between the roughened ends of two glass microscope slides, washed with Hanks' balanced salt solution, and filtered with a 70 µm cell strainer, then cell counts were determined. All single cell solutions were adjusted to $1\times10^7$ cells/mL with IMDM (PAN Biotech; w L-Glutamine; 25mM HEPES, 3,024 g/L $NaHCO_3$) containing 10% fetal calf serum (FCS; PAA Laboratories), 1% Pen/Strep (Gibco; 10.000 U/mL penicillin; 10.000 µg/mL streptomycin) and stored on ice until further processing.

#### Flow cytometry

Measurements were performed on the CYTOFLEX LX platform of Beckman Coulter. The generated data were analyzed using the FlowJo Software Version 10.6.1 which also was used to generate the cytometry graphics.

Single-cell solutions containing $5\times10^5$ cells were used for each flow cytometric staining. Staining was performed in a 96-well plate (TC Plate 96 Well Suspension, R [Sarsted]). Live- dead staining was performed using Zombie UV in a dilution of 1:1000 (Zombie UV™ Fixable Viability Kit [BioLegend]) to exclude dead cells. Subsequently, binding cells were blocked with 0.5 µL of Fc receptor-blocking antibodies (TruStain FcX™ [anti mouse CD16/32 Isotype Rat igG2a, λ clone: 93 Biolegend]) to reduce non-specific antibody binding.

To identify DC and T cells, samples were incubated for at least 15 min with the appropriate antibody mixtures. After surface staining, a FoxP3 intracellular staining was performed to mark Tregs, as recommended by the manufacturer.

*General analysis strategy* at the beginning of each analysis, doublets and debris were excluded based on their size and shifting properties in FSC-A and FSC-H. After exclusion of dead cells (Zombie UV positive cells), CD45-positive cells were selected and then auto-fluorescent cells were excluded based on their signal in an empty channel not occupied by antibodies.

*Strategy to identify Tregs* within the non-auto-fluorescent cells, T helper cells were identified based on the expression of CD3 and CD4. Within these, Tregs were selected as FoxP3-positive cells. Details of the antibodies used and a gating example are provided in Supplementary Tables 1 and Supplementary Fig. 2.

*Strategy to identify MHCII expression on DC* within non-fluorescent hematopoietic cells, $CD19^+$ B cells were excluded and CD11c/MHCII-positive cells were selected. After exclusion of $CD3^+$ cells in this population, the geo-mean fluorescence intensity (MFI) of MHCII on DC was determined. The antibodies used are listed in Supplementary Tables 2 and an example gating is shown in Supplementary Fig. 3.

*Strategy to identify memory T cells* within the group of hematopoietic cells, T cells were identified as CD3-positive cells; T helper cells were distinguished from CD8-positive cytotoxic T cells. Naïve cells were characterized as $CD62L^+CD44-$ cells, central memory cells as $CD62L^+CD44^+$ cells, effector memory cells as $CD62L^-CD44^+$ cells, and tissue residential memory cells as $CD69^+CD103^+$ cells. The antibodies used are detailed in Supplementary Tables 3 and an example gating strategy is shown in Supplementary Fig. 4.

#### Statistical analysis

An Anderson Darling test was performed to determine whether data followed a Gaussian approximation, and then the appropriate statistical test (unpaired Student t-test or Mann-Whitney U test) was used to compare between groups. Statistical significance was defined as a p-value of $\leq 0.05$.

## Results

**VacA administration by different routes attenuates allergic respiratory disease**.

An acute model of allergic airway disease in mice was used to test whether VacA is therapeutically effective via different routes of administration and to identify the most effective route (Fig. 1A). Administration of VacA via i.p., p.o., and i.t. routes reduced the number of eosinophilic granulocytes compared with the untreated positive control (Fig. 1B). Although none of the treatment regimens reduced the number of neutrophils and macrophages, i.p. application of VacA significantly reduced lymphocytes in the BAL. Both i.p. and i.t. application of VacA also significantly reduced total cells in the BAL.

Administration of VacA via the i.p. route reduced the inflammation in the lung tissue (Fig. 1C) and decreased the number of mucus-producing goblet cells in the airways (Fig. 1D); lung inflammation was also reduced after i.t. (but not p.o.) administration of VacA (Fig. 1C),

but and neither of these routes of administration altered the number of mucus producing cells in the airways (Fig. 1D).

Flow cytometric analysis of FoxP3$^+$/CD25$^+$ Tregs showed an organ-specific increase of Tregs depending on the VacA route of administration. After i.t. treatment there was a significant increase in Tregs in the lungs, after i.p. treatment there was an increase of Tregs in the spleen and the mLN, and after p.o. administration there was an induction of Tregs in the mLN compared with the positive control (Fig. 1E).

#### VacA treatment induces PD-L1 on DC in vivo

In our previous work we could observe an induction of PD-L1 on the surface of human DC after stimulation with VacA in vitro. To investigate whether VacA can also modulate the immunosuppressive surface ligand on DC in vivo, animals were treated i.p. with VacA, and then PD-L1 expression on DC in different organs was examined 24 or 48 h later (Fig. 2A). A significant upregulation of PD-L1 was observed in the lung- and spleen-derived DC as early as 24 h after treatment with VacA. This was still detectable in spleen DC at 48 h after treatment (Fig. 2B).

#### VacA suppresses the inflammatory respiratory phenotype in a chronic model of allergic airway disease

The therapeutic effectiveness of VacA in the chronic phase of allergic airway disease was determined in animals sensitized to HDM and continuously challenged with the allergen over 10 weeks. Both short- and long-term treatment with VacA were associated with improvement in the inflammatory phenotype (Fig. 3A). Short-term treatment significantly reduced lymphocytes and eosinophils in the BAL, and decreased lung inflammation and sub-epithelial collagen deposition compared with the positive control. Long-term treatment with VacA reduced eosinophilia in the BAL, which was accompanied by a lower lung inflammation score, lower numbers of mucus-secreting goblet cells in the airways and less sub epithelial collagen deposition (Fig. 3B-E).

#### Effects of VacA on the immune phenotype vary with short- and long-term treatment

To analyze the effect of the different VacA treatment approaches on the immune phenotype, Tregs, MHCII expression on DC and induction of memory T cells were analyzed in lung, spleen, mLN and tLN in the chronic model (Fig. 4). Short-term treatment was associated with an increased proportion of Tregs in spleen, mLN and tLN. After long-term treatment, an increased proportion of Tregs could only be detected in the spleen compared to the positive control (Fig. 4A). Previously, we could demonstrate that DC in the draining lymph nodes of VacA treated animals exhibited reduced expression of MHCII on the surface in an acute model of allergic airway disease.

Similarly, also in the chronic model short-term (but not long-term) treatment with VacA decreased expression of MHCII on DC (CD11c$^+$/MHCII$^+$) in the lung draining lymph node and the spleen (Fig. 4B).

Development of an immunological memory represents a central step in adaptive immunity, and is important for the induction of recall responses against pathogens, but is a disadvantage if these responses are directed against a harmless allergen [14]. Treatment with VacA, especially long-term treatment, attenuated the development of an immunological memory in the lung. CD4$^+$ and CD8$^+$ tissue residential memory (TRM- CD103$^+$/CD69$^+$) were detectable in reduced numbers in the lung. In addition, numbers of CD4$^+$ and CD8$^+$ central memory cells (CM - CD62L$^{high}$/CD44$^{high}$) and effector memory cells (EM – CD62L$^{low}$/CD44$^{high}$) were lower after long-term treatment with VacA (Fig. 4C).

To evaluate any possible side effects of VacA treatment, animals were observed throughout the experiments and their body weight was recorded; no visible signs of distress or differences in body weight were seen compared with the positive control group (Supplementary Fig. 1A). In addition, no cytotoxic effects were detected in VacA-treated animals based on flow cytometry analyses (Supplementary Fig. 1B).

## Discussion

The results of this study confirm and substantiate the suitability of VacA as therapeutic treatment option for asthma. These findings extend previous data showing that the i.p. administration of VacA can suppress the development and progression of an allergic respiratory disease [9]. New insights from the current study include the documentation of the therapeutic effects of VacA after administration via several different routes, and demonstration that VacA can downregulate inflammatory responses in a chronic model of allergic airway disease, without any signs of adverse effects after repeated applications.

The inhaled, oral, subcutaneous or intravenous routes are the typical ways patients with asthma take their medications. Of these, oral intake of tablets or inhalation using an inhaler have the best acceptability and compliance. In the present study, we compared the effectiveness of equivalent concentrations of VacA administered via i.p. injection, p.o. delivery via gavage or i.t. delivery of droplets into the lung in a murine model of acute allergic airway disease. All three of these were effective, although there were differences in the magnitude of the effects of VacA given via the different routes. All were associated with a significant reduction in the eosinophil count in

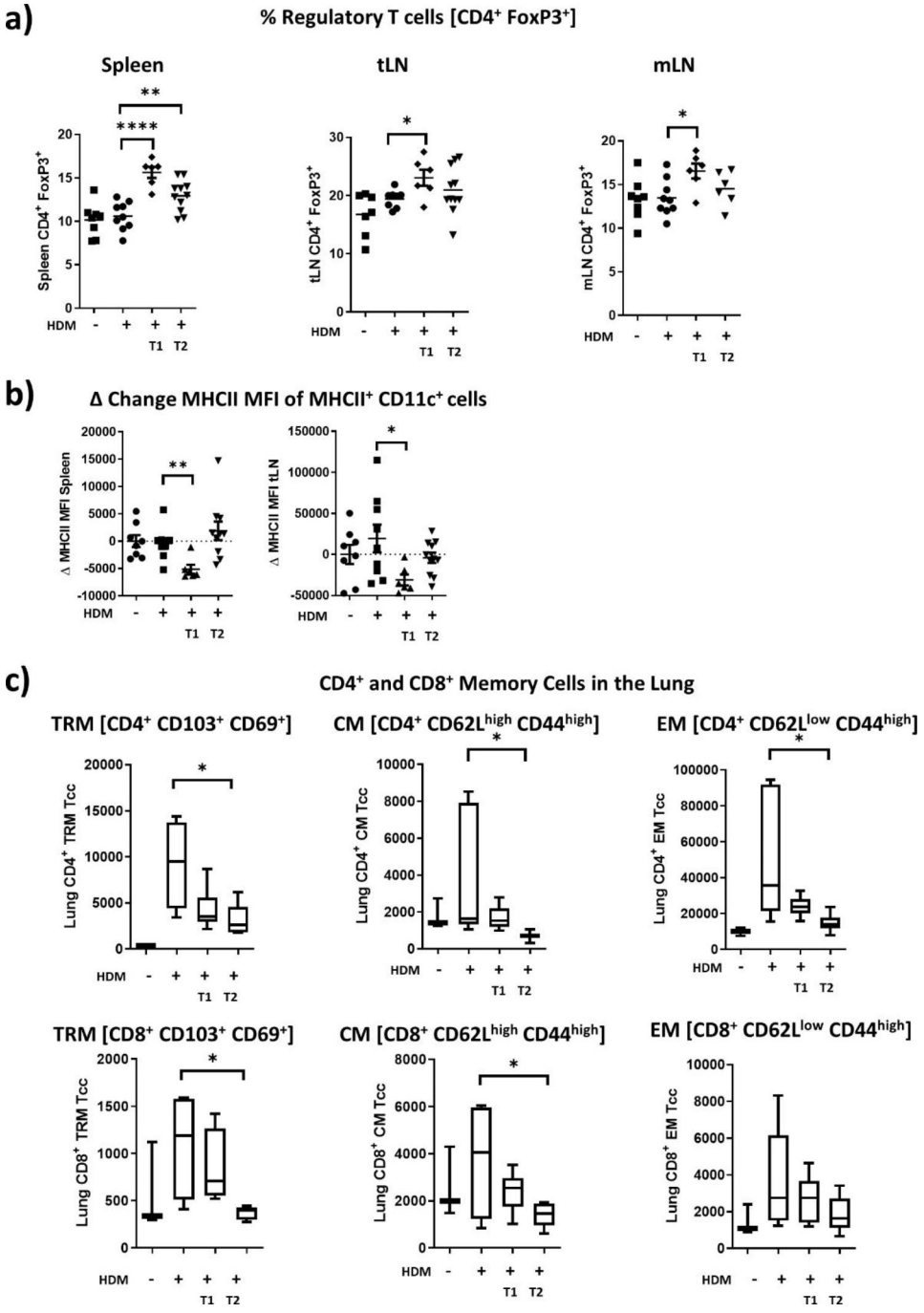


**Fig. 4** Effects of VacA treatment on the immune phenotype in a chronic allergic airway disease model. **a)** VacA treatment increases proportions of Tregs. Tregs were characterized by the expression of FoxP3$^+$ CD25$^+$ cells in the CD3$^+$ CD4$^+$ cell population; scatter plots show the percentage in lung, spleen, tracheal lymph node (tLN) and mesenteric lymph node (mLN). **b)** Scatter plot showing change in geomean fluorescence intensity (MFI) of MHCII on MHCII$^+$ CD11c$^+$ cells versus negative control group. **c)** Box plot of tissue residential memory T cells (CD103$^+$ CD69$^+$), central memory T cells (CD62L$^{high}$ CD44$^{high}$) and effector memory T cells (CD62L$^{low}$ CD44$^{high}$) in the CD3$^+$ CD4$^+$ and CD3$^+$ CD8$^+$ cell populations; results from two independent experiments n = 5–9 per group. *p < 0.05; **p < 0.01; ****p < 0.0001

the BAL, while i.p. and i.t. administration also significantly reduced the total cell count, and i.p. also reduced lymphocytes. In terms of the histological analysis of the inflammatory phenotype, i.p. and i.t. administration of VacA significantly reduced the infiltration of cells into lung tissue, while i.p. treatment also reduced mucus-producing cells in the airways.

Similar to previous studies, all routes of VacA administration in the current study were able to induce Tregs. Tregs, and modulation of DC towards a tolerogenic

phenotype, are key mechanisms of action underlying the therapeutic effectiveness of VacA [15]. In this study, we showed that the induction of Tregs occurs primarily at the site of VacA administration, with significant induction in the lung after i.t. administration, in the spleen and mesenteric lymph nodes after i.p. administration, and in mesenteric lymph nodes after p.o. administration.

Overall, the findings of this study show that VacA can have a therapeutic effect via different application routes. Further, our results indicate that i.p. application represents the most effective and consistent therapy approach with the current formulation of the molecule. However, a different formulation and dosage of VacA could potentially increase its effectiveness via treatment routes like inhalation or oral administration, which are the preferred treatment options for humans with higher patient compliance; this remains to be determined in future studies.

We propose that modulation of DCs represents a central mechanism in VacA-mediated immune suppression. Previous studies have shown that *H. pylori*, and VacA, can induce mouse and human DC with a tolerogenic phenotype [9, 16]. These DC are capable of secreting anti-inflammatory mediators such as IL-10, and also show increased expression of immune receptors with inhibitory motifs on their surface.

Recently, we showed that VacA induced the expression of PD-L1 on the surface of human DCs in vitro [9]. In general, upregulation of the PD-L1/PD-1 pathway is important for the development, maintenance and function of induced Tregs [17]. PD-L1 is strongly associated with the modification of the T-cell immune response [18] and appears to be important for the generation of regulatory T-cells [19]. The role of the PD-L1/PD-1 pathway in allergic diseases and asthma is controversial. PD-L1 expression is associated with reduced proliferation of allergen-specific human $CD4^+$ T cells [20]. A human PD-1 agonist was shown to alleviate neutrophilic asthma [21] whereas new-onset asthma has been reported as a side effect of cancer treatment with the PD-1 inhibitor nivolumab [22]. It is assumed that the PD-1/PD-L1 axis is involved in regulating the severity of allergic asthma by targeting Th17 cell activity [23]. However, PD-L2 (but not PD-L1) has been discussed to have a beneficial effect in asthma [24]. Interestingly, the induction of Treg-inducing tolerogenic PD-L1-expressing human DC is also described as the immune suppressing mechanism of allergoid-mannan, a newly described vaccine for allergen-specific immunotherapy [25]. In the current study, i.p. administration of VacA increased expression of PD-L1 on DCs in the lungs and spleen. By increasing the expression of PD-L1 on DC, VacA can directly modulate the communication between DC and T cells and promote the generation of anti-inflammatory T cells.

To date, all experiments investigating the therapeutic efficacy of VacA have been carried out in acute models, in which the effect of the molecule during the onset of the disease or shortly thereafter has been analyzed. Asthma is a chronic lung disease in which patients experience recurrent inflammatory reactions in the lungs, resulting in remodeling of the airways, which in turn contributes to disease progression. This study used a HDM-specific mouse model of chronic allergic airway disease to determine whether VacA was also effective in the chronic phase of the disease and evaluate the safety of repeated doses of VacA. After an initial sensitization and a one-week challenge period, the allergen was administered i.t. at low doses to mice over 6 weeks. Two different VacA treatment regimens were examined: short-term treatment at the end of the experiment and long-term treatment given in the middle and the end of the experiment. Both treatment regimens significantly reduced eosinophilia in the BAL, lung tissue infiltration and subepithelial fibrosis. However, there were also some differences between effects of the two regimens. Short-term treatment significantly reduced the number of lymphocytes in the BAL, while long-term treatment significantly reduced the total number of cells in the BAL and the mucus-producing cells. These differences in the regulation of the inflammatory phenotype of the allergic airway disease were accompanied with variances in the immune phenotype of the animals.

Similar to observations in the acute and therapeutic model, short-term treatment with VacA in the chronic model was associated with visible induction of Tregs in the lung draining lymph node, spleen and mLN; this induction was less pronounced in animals receiving long-term VacA treatment. In addition to the induction of Tregs, we also observed a modulation of DC after short-term treatment. Similar to what we saw in the acute model of allergic airway disease, we detected reduced expression of MHCII on DC in lung and spleen, but only during short-term (not long-term) treatment. The current findings also showed that VacA can suppress the inflammatory phenotype in the chronic model. Here, the beneficial effects of short-term treatment were accompanied by induction of Tregs and modulation of DC. The anti-inflammatory cytokine IL-10, which has an important role in the induction and maintenance of T cell tolerance, can reduce MHCII expression on DC, and the reduction of MHCII on the surface of DC then leads to T cell unresponsiveness or anergy [26]. These tolerogenic DC can also upregulate the activity of Tregs that subsequently suppress the underlying inflammation [27].

An interesting finding of the current study was that long-term treatment with VacA seemed to have an impact on the development of immunological memory. VacA reduced the number of both $CD4^+$ and $CD8^+$ tissue

resident, central and effector memory T cells in the lung. Studies have shown that CD4 tissue resident memory T cells (TRM) are responsible for the existence and recurrence of asthma [14], and that CD8 TRM also play an important role in the recruitment of immune cells to the lungs, especially in chronic cases [28]. These TRM can persist in the lung for a long time and maintain the local "allergic memory", which is associated with the induction of asthma exacerbations upon repeated exposure to an allergen [14]. This makes them an interesting target for the treatment of allergic asthma.

Overall, the observations in the current study underline the therapeutic capacity of VacA in asthma because both short- and long-term treatment in a chronic disease model were able to alleviate the asthma phenotype. Long-term treatment seems to have an impact on the development of local immunological memory. A reduction of memory against the allergen in the lung could be an interesting new approach to treat asthma. No obvious adverse effects were detected in the current study, but impact on the immune modulation on other immune responses, for example infections, needs to be evaluated.

## Conclusions

The *H. pylori*-derived molecule VacA appears to be a promising candidate for the treatment of allergic diseases such as allergic asthma when given via a variety of administration routes. In addition to demonstrated activity in acute and acute/therapeutic models of allergic respiratory disease, the present data showed that treatment with VacA can also effectively suppress inflammatory reactions in chronic allergic airway disease models. Overall, VacA appears to be an interesting future therapeutic option that can counter-regulate diseases that are characterized by an excessive immune response (such as asthma) by inducing immunosuppressive mechanisms, thus minimizing disease severity and potentially contributing to long-term remission.

### List of abbreviations

| | |
|---|---|
| BAL | bronchoalveolar lavage |
| CM | central memory T cells |
| *D. pteronyssinus* | *Dermathophagoides pteronyssinus* |
| DC | dendritic cell |
| EM | effector memory T cells |
| *H. pylori* | *Helicobacter pylori* |
| HDM | house dust mite |
| i.n. | intranasal |
| i.p. | intraperitoneal |
| i.t. | intratracheal |
| mLN | mesenteric lymph node |
| PBS | phosphate buffered saline |
| PD1 | programmed cell death protein-1 |
| PD-L1 | programmed cell death protein-ligand 1 |
| p.o. | peroral |
| T1 | short-term treatment regimen |
| T2 | long-term treatment regimen |
| tLN | tracheal lymph node |
| Treg | regulatory T cell |
| TRM | tissue residential memory T cells |
| VacA | vacuolating cytotoxin A |

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12931-023-02484-5.

Supplementary Material 1

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### Authors' contributions

SR and JR designed the experiments; JR, SR, MS and KB performed the experiments; JR and SR analyzed the Data; SR and CT conceived and supervised the project; GC, TC, AM and CT supported the writing process; GC and TC supplied VacA; JR, SR wrote the manuscript. All authors approved the final version of the manuscript.

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### Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Declarations

### Competing interests

The authors declare no competing interests.

### Ethics approval and consent to participate

The local regulatory authority approved the animal experiments (Landesamt für Natur, Umwelt und Verbraucherschutz North Rhine Westphalia, Germany, reference number: AZ 81-02.04.2018.A084). The procedures were conducted in accordance with current federal, state and institutional guidelines.

### Consent for publication

Not applicable.

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## Publisher's Note

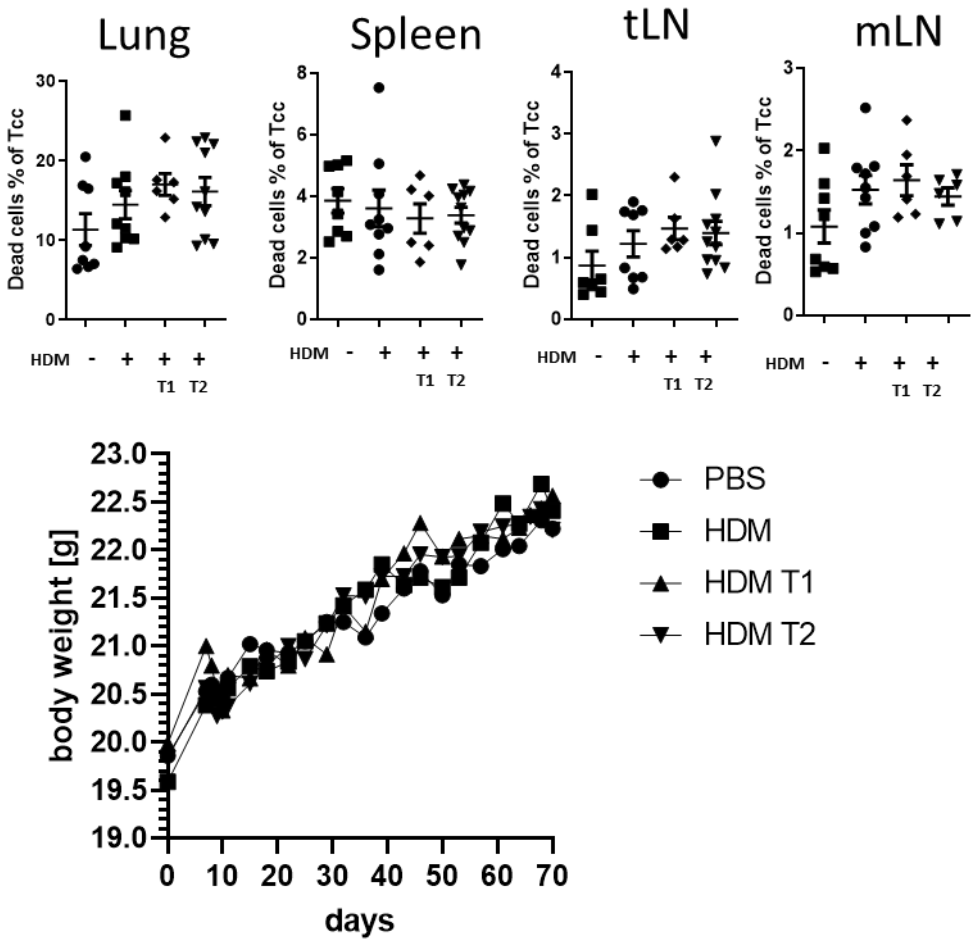
## Supplementary material

**Supplementary Figure 1: No evidence of cytotoxic effects during treatment with VacA.**

a) Scatter plots show the proportion (%) of dead cells in different organs at the end of treatment in the chronic allergic airway disease model; cells were stained with Zombie UV.

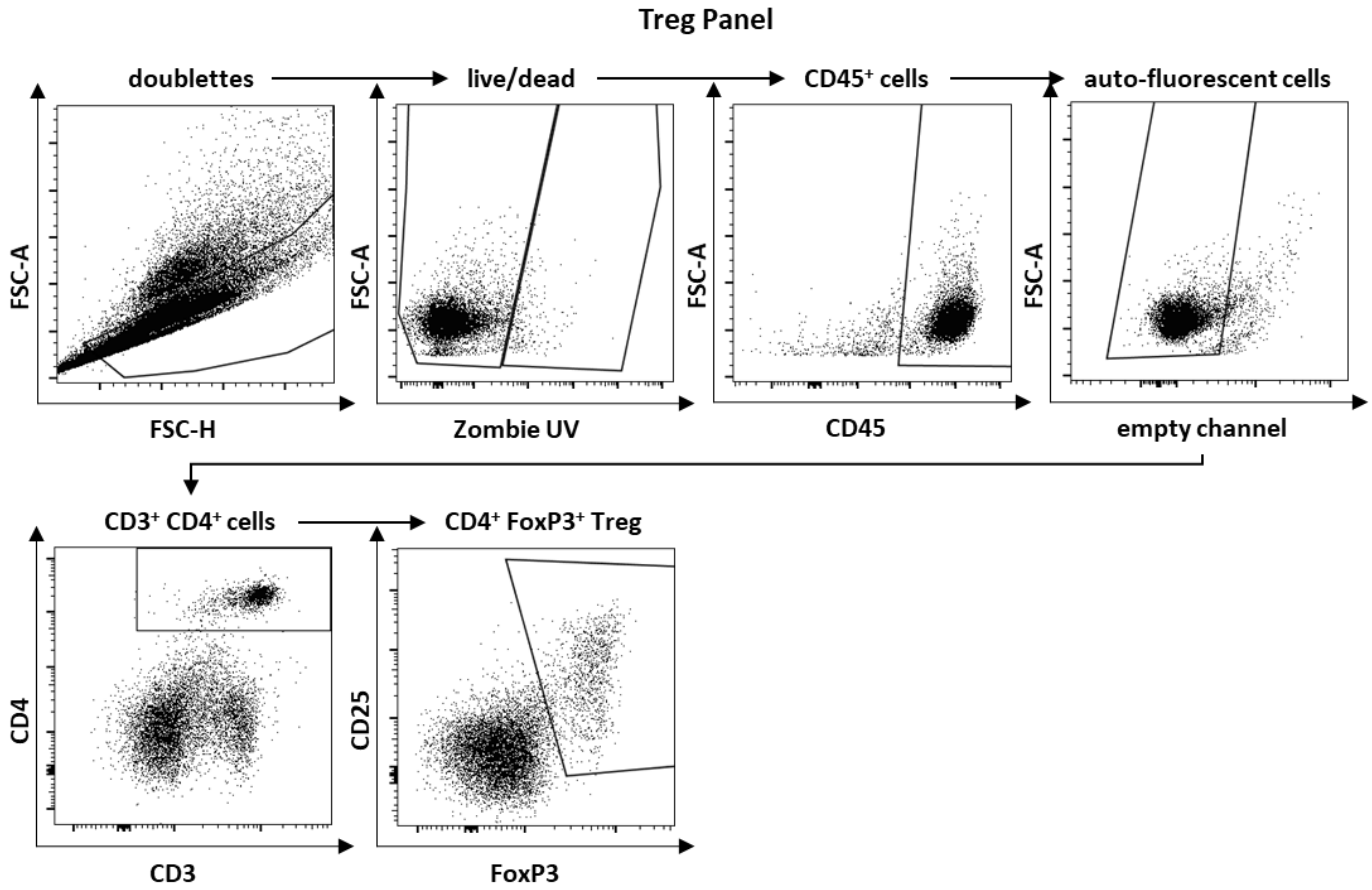
b) Change in body weight [g] of the mice (C57BL/6j) during treatment with VacA in the chronic allergic airway disease model (body weight increases over time).

DM, house dust mite; mLN, mesenteric lymph node; PBS, phosphate-buffered saline; Tcc, total cell count; tLN, tracheal lymph node; T1, short-term VacA treatment; T2, long-term VacA treatment.



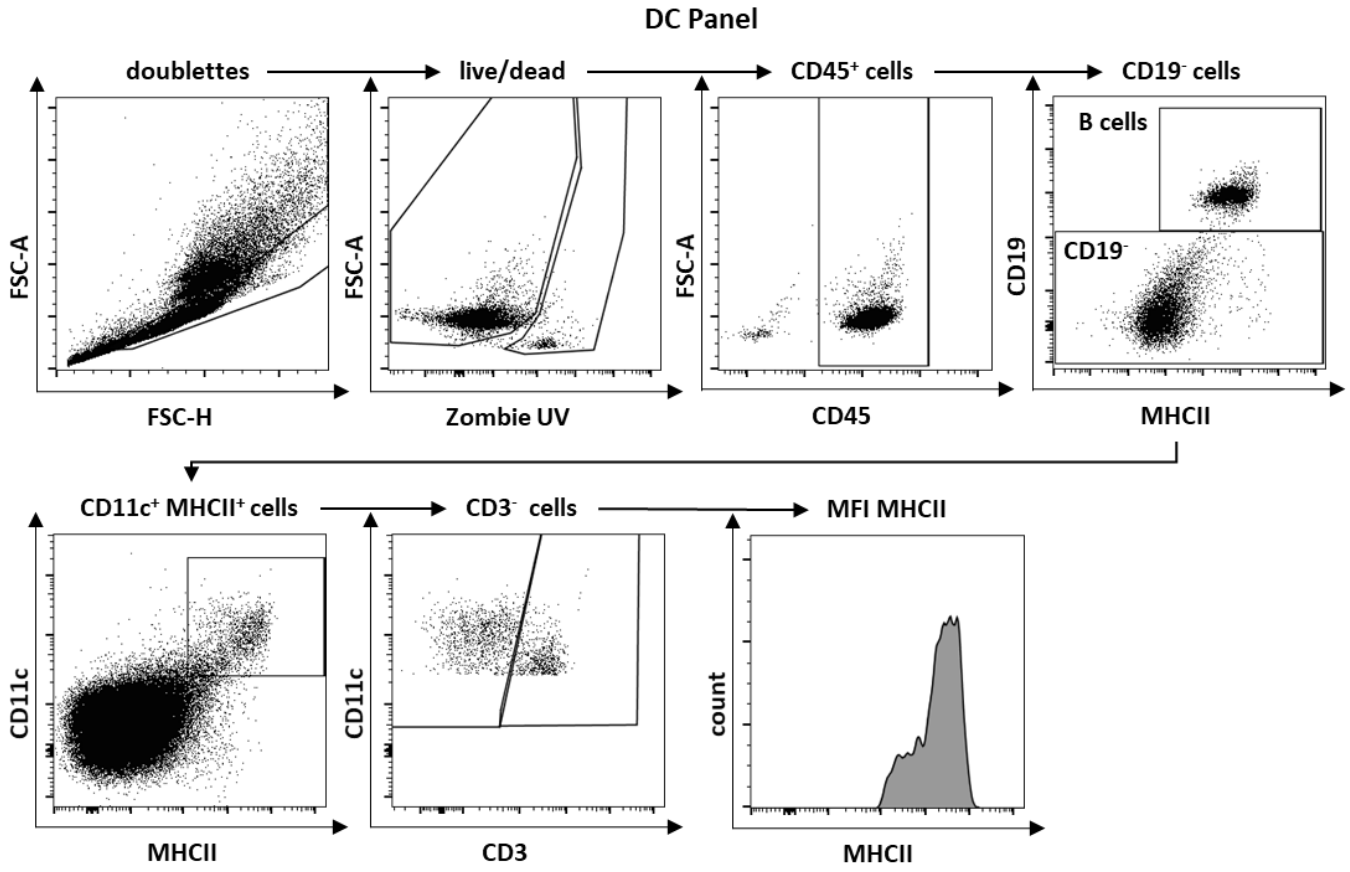
**Supplementary Figure 2: Gating strategy for regulatory T-cells.**

First doublets were excluded then a live/dead staining was performed. $CD45^+$ cells were gated and auto-fluorescent cells were excluded. Regulatory T cells (Treg) were identified as $CD3^+CD4^+FoxP3^+$ cells.



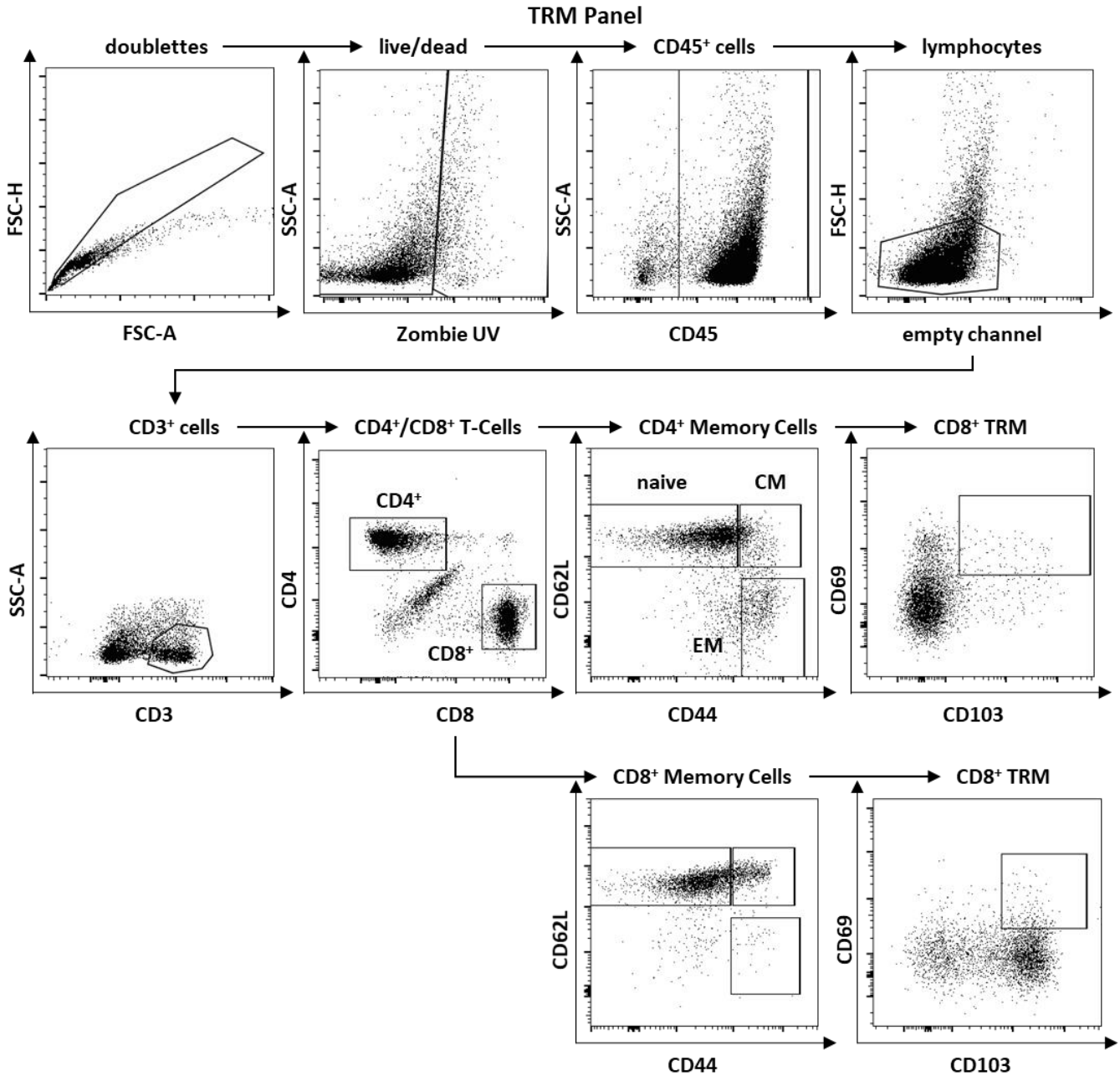
**Supplementary Figure 3: Gating strategy for dendritic cells and mean fluorescence intensity (MFI) of MHCII or PD-L1.**

First doublets were excluded then a live/dead staining was performed. $CD45^+$ cells were gated and $CD19^+$ cells were excluded. $CD11c^+$ $MHCII^+$ cells were identified and after exclusion of $CD3^+$ cells the MFI of MHCII or PD-L1 was determined.



**Supplementary Figure 4: Gating strategy for tissue residential memory T cells (TRM).**

First doublets were excluded then a live/dead staining was performed. $CD45^+$ cells were gated and lymphocytes were identified after gating of $CD3^+$cells, $CD4^+$ and $CD8^+$ cells were separated and on each population the memory gating was performed.



**Supplementary Table 1: Antibody list – regulatory T cells (Treg)**

| Panel | Murine Treg Analysis | | | | |
|---|---|---|---|---|---|
| **Fluochrome** | **Specificity** | **Clone** | **Reactivity** | **Antibody Host Species** | **Manufacturer** |
| Alexa Fluor 700 | CD45 | 30F-11 | Mouse | rat | Biolegend |
| PerCP-Cy™5.5 | CD3 | 145-2C11 | mouse | rat | Biolegend |
| PE/Cyanine7 | CD4 | 53-6.7 | mouse | rat | Biolegend |
| Pe | CD25 | PC61 | mouse | rat | BD Pharmingens |
| Brilliant Violet 650™ | CD25 | PC61 | mouse | rat | Biolegend |
| APC | FoxP3 | FJK-16s | mouse | rat | Biolegend |

**Supplementary Table 2: Antibody list – MHCII expression on dendritic cells (DC)**

| Panel | Murine DC | | | | |
|---|---|---|---|---|---|
| **Fluochrome** | **Specificity** | **Clone** | **Reactivity** | **Antibody Host Species** | **Manufacturer** |
| Alexa Fluor 700 | CD45 | 30F-11 | mouse | rat | Biolegend |
| PE/Dazzle™ 594 | CD19 | 6D5 | mouse | rat | Biolegend |
| PE/Cyanine 7 | CD11c | N418 | mouse | armenian hamster | Biolegend |
| FITC | MHCII | M5/114.15.2 | mouse | rat | Biolegend |
| APC/Cyanine 7 | CD3 | 145-2C11 | mouse | armenian hamster | Biolegend |

**Supplementary Table 3: Antibody list – memory T cells**

| Panel | Murine memory T cells | | | | |
|---|---|---|---|---|---|
| **Fluochrome** | **Specificity** | **Clone** | **Reactivity** | **Antibody Host Species** | **Manufacturer** |
| Alexa Fluor 700 | CD45 | 30F-11 | mouse | rat | Biolegend |
| PerCP-Cy™5.5 | CD3 | 145-2C11 | mouse | rat | Biolegend |
| PE/Cyanine7 | CD4 | 53-6.7 | mouse | rat | Biolegend |
| Brilliant Violet 605™ | CD62L | MEL-14 | mouse | rat | Biolegend |
| Brilliant Violet 785™ | CD44 | IM7 | mouse | rat | Biolegend |
| APC/Cyanine 7 | CD69 | H1.2F3 | mouse | armenian hamster | Biolegend |
| PE | CD103 | 2E7 | mouse | armenian hamster | Biolegend |
| Brilliant Violet 510™ | CD8 | 53-6.7 | mouse | rat | Biolegend |

**Supplementary Table 4: Antibody list – PD-L1 expression on dendritic cell**

| Panel | Murine PD-L1 | | | | |
|---|---|---|---|---|---|
| **Fluochrome** | **Specificity** | **Clone** | **Reactivity** | **Antibody Host Species** | **Manufacturer** |
| Alexa Fluor 700 | CD45 | 30F-11 | mouse | rat | Biolegend |
| PE/Cyanine 7 | CD11c | N418 | mouse | armenian hamster | Biolegend |
| FITC | MHCII | M5/114.15.2 | mouse | rat | Biolegend |
| PE/Dazzle™ 594 | PDL-1 | 10F.9G2 | mouse | rat | Biolegend |

# Participation in publications

## Kumulative Dissertation/Beteiligung an Veröffentlichungen

Kumulative Dissertation von Herrn Jonas Raspe

**Autorenbeiträge**

- **Titel (Journal; IF)**

Therapeutic properties of *Helicobacter pylori*-derived vacuolating cytotoxin A in an animal model of chronic allergic airway disease. *Respir Res* **24**, 178 (2023). https://doi.org/10.1186/s12931-023-02484-5

**(Impact Factor: 7,162 )**

- **Autoren**

**J. Raspe**, M. S. Schmitz, K. Barbet, G. Caso, T. L. Cover, A. Müller, C. Taube, S. Reuter

- **Anteile:**

- Konzept (conception) - %: Beschreibung (specification)

20%

Konzept dieses Manuskripts und die Durchgeführten Experimente sind Teil eines DFG Antrages und wurden von Dr. Sebastian Reuter entwickelt.
Herr Raspe hat bereits existierende Fragestellungen erweitert und selbstständig eigene Ideen einfließen lassen.

- Durchführung der Experimente (experimental work) - %: Beschreibung (specification)

80%

Planung und Organisation der Experimente wurden von Herrn Raspe durchgeführt. Großversuche wurden von Mitgliedern der Arbeitsgruppe unterstützt.

- Datenanalyse (data analysis) - %: Beschreibung (specification)

90%

Herr Raspe hat die Datensätze der Experimente in diesem Manuskript selbstständig analysiert. Daten wurden mit Dr. Reuter besprochen, der einzelne Datensätze ebenfalls untersucht hat.

- Grafiken - %: Beschreibung (specification)

100%

Herr Raspe hat die Grafiken die in das Manuskript eingeflossen sind selbstständig mit GraphPad Prism erstellt. Der finale Aufbau und die Darstellung wurde mit Dr. Reuter besprochen.

- Statistische Analyse (statistical analysis) - %: Beschreibung (specification)

90%

Herr Raspe hat die Datensätze der Experimente in diesem Manuskript selbstständig analysiert, hierbei hat er auch die Statistische Analyse der Datensätze durchgeführt. Daten wurden mit Dr. Reuter besprochen, der einzelne Datensätze ebenfalls untersucht hat.

- Manuskripterstellung (writing the manuscript) - %: Beschreibung (specification)

90%

Der primäre Aufbau des Manuskripts wurde mit Herrn Dr. Reuter geplant. Herr Raspe hat dann selbständig das Manuskript geschrieben und diesen zusammen mit Dr. finalisiert.

- Überarbeitung des Manuskripts (revising the manuscript) - %: Beschreibung (specification)

90%

Unterschrift Doktorand/in

Unterschrift Betreuer/in

**Koautorenbeiträge**

- **Titel (Journal; IF)**

Treatment with Helicobacter pylori-derived VacA attenuates allergic airway disease
Front Immunol. 2023 Jan 24;14:1092801. doi: 10.3389/fimmu.2023.1092801.

**(Impact Factor: 8.786)**

Autoren (authors)

S. Reuter, **J. Raspe**, H. Uebner , A. Contoyannis, E. Pastille, A.M. Westendorf, G.C. Caso, T.L. Cover, A. Müller, C. Taube

Anteile (contributions):

- Konzept (conception) - %: Beschreibung (specification)

10%

Konzept dieses Manuskripts und die Durchgeführten Experimente sind Teil eines DFG Antrages und wurden von Dr. Sebastian Reuter entwickelt.
Herr Raspe hat bereits existierende Fragestellungen erweitert und selbstständig eigene Ideen einfließen lassen.

- Durchführung der Experimente (experimental work) - %: Beschreibung (specification)

30%

Herr Raspe ist im Zuge seiner Doktorarbeit zu dem Projekt gestoßen. Planungen der Experimente wurden mit Dr. Reuter durchgeführt. Während der Durchführung der Experimente wurde er von Herrn Doktor Reuter begleitet.
Ein Teil der Experimente hatte Herr Raspe nach der Einarbeitung bereits selbstständig und alleine durchgeführt.

- Datenanalyse (data analysis) - %: Beschreibung (specification)

30%

Herr Raspe analysierte nach Einarbeitung ein Teil der Daten selbst.
Daten wurden mit Dr. Reuter besprochen.

- Grafiken - %: Beschreibung (specification)

20%

Grafik 5 Daten zur Wirkung von VacA in vitro wurde von Herrn Raspe ausgearbeitet.

- Statistische Analyse (statistical analysis) - %: Beschreibung (specification)

20%

Herr Raspe analysierte einTeil der Daten des Manuskripts selbstständig, hierbei hat er auch die Statistische Analyse der Datensätze durchgeführt.
Daten wurden mit Dr. Reuter besprochen.

- Manuskripterstellung (writing the manuscript) - %: Beschreibung (specification)

10%

Das Manuskript wurde von Doktor Reuter Konzeptioniert und geschrieben. Herr Raspe hat ihn bei diesem Prozess unterstützt.

- Überarbeitung des Manuskripts (revising the manuscript) - %: Beschreibung (specification)

10%

Unterschrift Doktorand/in

Unterschrift Betreuer/in

# Discussion

The two publications provide valuable insights into the potential therapeutic use of VacA in the context of asthma treatment. In both acute and therapeutic murine models of allergic airway disease, which also included a chronic disease model, VacA treatment effectively mitigated airway disease. Similar to findings in prophylactic models, the induction of Tregs was observed. The induction of immune-suppressive DCs and Tregs appears to be a central mechanism underlying the therapeutic effectiveness of VacA. Furthermore, in the chronic model where VacA treatment was administered repeatedly, it appeared to inhibit the formation of local, lung-specific adaptive immunological memory.

In previous studies, the impact of VacA on myeloid cells within the gastric mucosa was shown. It creates an environment conducive to Treg induction and promoting immune tolerance (53). These cells may possess the capability to migrate throughout the body, potentially mediating immune suppression and thereby reducing the likelihood of developing allergic reactions by moderating excessive immune responses (54). In addition, the induction of allergen-specific IgG2b, which can antagonize IgE-mediated allergic reactions, is an intriguing finding (55). This implies that VacA has the potential not only to alleviate asthma symptoms but also to mitigate the immune response to allergens, reducing the risk of allergic reactions.

The fact that VacA demonstrates similar effects in both murine and human cell culture systems bodes well for its potential translation to human therapies. This represents a crucial step in the development of any new treatment, as results in animal models do not always directly translate to humans. The effectiveness of VacA in human DCs and its induction of immune-suppressive factors such as IL-10 and PD-L1 suggest its potential relevance for human asthma treatment.

In addition to i.p. application, VacA has demonstrated therapeutic effectiveness via inhalation and oral treatment routes. Currently, the injection of the molecule appears to be particularly effective in suppressing allergic respiratory disease. Modifying VacA concentrations or making pharmacological adjustments could potentially facilitate its future use in treating conditions like asthma. This might involve administration through inhalers or in the form of tablets, for instance. Due to their simpler and already familiar administration method, patients are likely to find these applications more acceptable, thereby increasing compliance. Our experiments with mutants and recombinant forms of VacA underscore the possibility of pharmacological modifications to the protein.

Before considering clinical trials, it is urgently necessary to conduct further preclinical studies. With the current and new formulations of VacA, cytotoxicity and dose-effect studies on human

*in vitro* cultures would have to be carried out in advance. Although no side effects were observed in the *in vivo* or *in vitro* experiments, it should be clarified in the future whether the modulation of DC/T cell responses has long-term consequences. It would also be interesting to analyze how long the effectiveness of VacA treatment lasts. Does it only provide acute protection after administration and, therefore, must be repeatedly taken, or does the treatment lead to long-lasting protection and an alteration of the disease?

# Conclusion

The data from my work confirm our hypothesis that VacA could be suitable not only prophylactically but also in therapeutic approaches for the treatment of allergic bronchial asthma. It is conceivable that the mechanism of action, a DC-mediated induction of regulatory T cells, could be effective not only in allergic asthma but also in other diseases whose pathophysiology is characterized by an excessive inflammatory response. Following further steps to optimize the pharmacological formulation and safety of the drug, it is conceivable that VacA will be used in clinical trials and could supplement or perhaps even completely replace existing therapies in the future.

# List of abbreviations

| | |
|---|---|
| VacA | vaculating cytotoxin A / vakuolisierendes Zytotoxin A |
| *H. pylori* | *Helicobacter pylori* |
| HDM | house dust mite / Haustaubmilbe |
| BAL | bronchoalveolar lavage / bronchoalveoläre Lavage |
| Ig | immune globuline / Immunglobulin |
| DC | dendritic cell / dendritische Zelle |
| Tregs | regulatory T cells / regulatorische T Zelle |
| i.p. | intraperitoneal |
| p.o. | per oral |
| i.t. | intratracheal |
| IL | interleukin |
| ILC | innate lymphoid cells |
| TSLP | thymic stromal lymphopoietin |
| ICS | inhalative corticosteroids / inhalative Kortikosteroide |
| OCS | orale corticosteroids / orale Kortikosteroide |
| SABA | short acting β agonist |
| LABA | long acting β agonist |
| AIT | allergen immune therapie / Allergenimmuntherapie |
| PAMPS | pathogen associated molecular patterns |
| GGT | gamma-glutamyl transpeptidase |

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# Curiculum Vitae Jonas Raspe

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Hiermit erkläre ich, gem. § 7 Abs. (2) d) + f) der Promotionsordnung der Fakultät für Biologie zur Erlangung des Dr. rer. nat., dass ich die vorliegende Dissertation selbständig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient, bei der Abfassung der Dissertation nur die angegebenen Hilfsmittel benutzt und alle wörtlich oder inhaltlich übernommenen Stellen als solche gekennzeichnet habe.

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Prof. Christian Taube

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