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Pneumococcal pneumonia suppresses allergy development but preserves respiratory tolerance in mice

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ABSTRACT

Colonization with *Streptococcus pneumoniae* (*S. pneumoniae*) is associated with an increased risk for recurrent wheeze and asthma. Killed *S. pneumoniae* showed some potential as an effective immunomodulatory therapy in a murine model of asthma. Murine studies demonstrated protection against allergic asthma by symbiotic bacteria via triggering regulatory T cell response: treatment with killed *S. pneumoniae* resulted in suppressed levels of allergen-specific Th2 cytokines, while early immunization generated a protective Th1 response. We investigated the impact of lung infection with live *S. pneumoniae* on both the development and maintenance of allergic airway inflammation and respiratory tolerance in mice. BALB/c mice were infected intratracheally with *S. pneumoniae* either prior to or after tolerance or allergy were induced, using ovalbumin (OVA) as model allergen. Infection of mice with *S. pneumoniae* prior to sensitization or after manifestation of allergic airway inflammation suppressed the development of an allergic phenotype as judged by reduced eosinophil counts in bronchoalveolar lavage fluid, decreased IgE serum levels and Th2 cytokines, relative to non-infected allergic control mice. In contrast, infection of mice with *S. pneumoniae* after manifestation of allergic airway inflammation combined with late mucosal re-challenge did not affect the allergic response. Moreover, induction and maintenance of respiratory tolerance to OVA challenge were not altered in *S. pneumoniae*-infected mice, demonstrating that mice remained tolerant to the model allergen and were protected from the development of allergic airway inflammation regardless of the time point of infection. Our results suggest that a bacterial infection may decrease the manifestation of an allergic phenotype not only prior to sensitization but also after manifestation of allergic airway inflammation in mice, whereas both, induction and maintenance of respiratory tolerance are not affected by pneumococcal pneumonia. These data may point to a role for undisturbed development and maintenance of mucosal tolerance for the prevention of allergic inflammation also in humans.
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1. Introduction

Within the last decades, the prevalence of allergic airway disease increased several fold particularly within industrialized countries, thus making allergic asthma one of the most common chronic airway diseases [1]. Both genetic and environmental factors have been suggested to drive an immunological imbalance toward the predominance of T-helper 2 (Th2) cells in the absence of counter-regulating immune mechanisms [2,3]. For the last decades, it has been discussed that the rise in allergic diseases was the price to be paid by modern societies for the freedom of infections gained by improved hygiene and public health care. However, the hygiene hypothesis, first introduced by David Strachan in 1989 [4], draws only a simplified picture of the protective role of microbial exposure in childhood. Meanwhile, it is widely accepted that although microbial exposure is stimulating for the developing immune system and promotes immune regulation, severe respiratory infections are risk factors for wheezing and asthma. Several studies revealed an association of viral infections with an increase in asthma exacerbation [5] and recurrent wheeze [6]. In addition, Bisgaard et al. [7] reported on a birth cohort study which found a similar and independent association for bacterial infections, including *S. pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis*, compared to viral

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infections with wheezy episodes. Young adult asthmatics were shown to be more frequently colonized with *S. pneumoniae* than healthy controls [8] and adults suffering from asthma were shown to have a more than six times higher risk of developing invasive pneumococcal disease or pneumonia than those without [9]. Existing asthma therefore defines an independent risk factor for the manifestation of invasive pneumococcal disease in children and adults [10]. On the other hand, high levels of *S. pneumoniae*- and *H. influenzae*-specific IgE were inversely correlated to asthma risk [11]. Nevertheless, for both viral and bacterial infections, the exact timing between infection and allergen exposure seems to play a crucial role in terms of defining either a protective or aggravating effect on the manifestation of asthma [2].

In murine asthma models, it has been demonstrated that symbiotic bacteria provided protection against allergic asthma by triggering regulatory T cell response. For example, Preston et al. applied killed *S. pneumoniae* as an immunomodulatory therapy for the prevention of allergic airway disease in a murine model of asthma. Treatment of mice with killed *S. pneumoniae* during and after allergen sensitization suppressed levels of allergen-specific Th2 cytokines, while early immunization resulted in a protective Th1 response [12].

In the current study, we investigated the effect of a bacterial infection with *S. pneumoniae* on the establishment and maintenance of an allergic phenotype as well as a possible impact on respiratory tolerance using well-established murine models of respiratory tolerance and allergy [13–17]. Systemic sensitization and local challenge with the model allergen ovalbumin (OVA, allergy protocol) result in the development of an allergic phenotype with eosinophilic airway inflammation, mucus hypersecretion and elevated allergen-specific serum IgE levels, which can be prevented by mucosal OVA pre-treatment prior to sensitization (tolerance protocol). In these two models, we examined the effect of lung infection with *S. pneumoniae* before (day −7) and after (day 14) systemic sensitization on either the process of sensitization or respiratory tolerance development to the allergen, or persistence of established allergy or respiratory tolerance. Lastly, possible long-term effects of pneumococcal infection were examined after delayed OVA-challenges at day 38 of a prolonged immunization protocol.

2. Materials and methods

2.1. Mice

Eight to ten week old female BALB/cByJ mice were purchased from Elevage Janvier (Le Genest Saint Isle, France) and maintained under S2 conditions with free access to food and water. We performed two to three independent experiments with groups of four to eight mice. Protocols were approved by the Committee on Animal Welfare of Lower-Saxony.

2.2. Culture and quantification of S. pneumoniae

For infection experiments, we used the capsular group 19 *S. pneumoniae* strain EF3030 as previously described [18,19]. The bacteria were grown in Todd-Hewitt broth (Oxoid, Wesel, Germany) supplemented with 20% FCS to mid-log phase and aliquots were snap frozen in liquid nitrogen and stored at −80 °C until use. Quantification of pneumococci was done by plating thawed aliquots in 10-fold serial dilutions on sheep blood agar plates (BD Biosciences, Heidelberg, Germany), followed by incubation of the plates at 37 °C/5% CO_2 for 18 h and subsequent determination of colony-forming units (CFU). We routinely checked the viability of thawed bacteria and bacterial stocks older than 4 weeks were not used for infection experiments.

2.3. Determination of bacterial loads

In a preceding experiment *S. pneumoniae*-infected mice were killed by an overdose of 100% isoflurane (Baxter, Unterschleissheim, Germany) and the bacterial loads within the lungs were determined at various time points after infection as recently described in detail [18–20]. Lungs were homogenized in Hanks' balanced salt solution without supplements by using a tissue homogenizer (IKA, Staufen, Germany). The resulting homogenates were filtered through a 100-μm-pore-size cell strainer (BD Falcon), and aliquots of each sample were then plated in 10-fold serial dilutions on sheep blood agar plates, followed by incubation at 37 °C in 5% CO_2 for determination of the bacterial loads [18–20].

2.4. Immunization protocols

Mice were immunized with the model allergen OVA (Sigma–Aldrich, Steinheim, Germany). The contaminating lipopolysaccharide (LPS) in Sigma OVA (Grade V) was removed by Detoxi-Gel® endotoxin-removing gel columns (Pierce Biotechnology, Rockford, IL, USA) to a level <10 EU/mg protein (tested with limulus amebocyte lysate (LAL) Assay, Cambrex, Walkersville, MD, USA) [13,14,16,17,20].

For induction of an allergic phenotype ("OVA", allergy protocol), mice were sensitized intraperitoneally (i.p.) twice – on days 0 and 8 – with OVA (20 μg in 200 μl 0.9% saline) adsorbed to 2 mg of an aqueous solution of aluminum hydroxide and magnesium hydroxide (Imject Alum; Perbio Science, Bonn, Germany). Subsequently, intranasal (i.n.) challenges with 20 μg OVA in 40 μl 0.9% saline were performed on three consecutive days: days 8–11 and days 21–23 (Figs. 1A and 2A). Alternatively, i.n. challenges were performed on days 8–11 and days 35–37, (Fig. 3A) under isoflurane anesthesia (100% (v/v), Baxter, Unterschleissheim, Germany). Control mice ("Alum") received 200 μl 0.9% saline/2 mg Imject Alum i.p. and were challenged i.n. with 0.9% saline. Mice were sacrificed on day 24 (Figs. 1 and 2) or 38 (Fig. 3). The same cohort of uninfected mice were used as a comparison for both Spn_{-7} and Spn_{14}, all naïve mice were sacrificed at day 24.

Mucosal application of high-dosed OVA prior to i.p. sensitization prevents the manifestation of an allergic phenotype and results in respiratory tolerance to the allergen ("TOL"). Therefore, mice received OVA i.n. (500 μg OVA, 40 μl 0.9% saline) on days −6 and −3 before they were referred to the allergy protocol described above.

2.5. Infection of mice with S. pneumoniae

Intratracheal infection of mice with serotype 19 *S. pneumoniae* (EF3030) was done using thawed aliquots adjusted to the given infection dose, essentially as recently described [18,19]. After instillation, mice were kept in individually ventilated cages (IVC) with free access to autoclaved food and water and were monitored twice daily for disease symptoms and survival during the entire observation period.

To examine the effect of pneumococcal infection on both induction and persistence of an allergic inflammation and respiratory tolerance, mice were infected with *S. pneumoniae* at two different time points, either on day −7 ("Spn_{-7}") or on day 14 ("Spn_{14}" or "$Spn_{14/35}$", respectively) relative to i.p. sensitization. In detail, mice were anesthetized with tetrazoline hydrochloride (5 mg/kg) and ketamine (75 mg/kg) and then orotracheally intubated with a 29-gauge Abbocath catheter (Abbott, Wiesbaden, Germany), which was inserted into the trachea under visual control with transillumination of the neck region. Infection with *S. pneumoniae* was induced by intratracheal instillation of 5×10^6 CFU of *S. pneumoniae* in a volume of 50 μl Todd-Hewitt broth (THB).