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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<sub>2</sub> 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<sub>2</sub> 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 amoebocyte 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<sub>–7</sub> and Spn<sub>14</sub>, 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<sub>–7</sub>”) or on day 14 (“Spn<sub>14</sub>”) or “Spn<sub>14/35</sub>”, 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<sup>6</sup> CFU of *S. pneumoniae* in a volume of 50 µl Todd-Hewitt broth (THB).

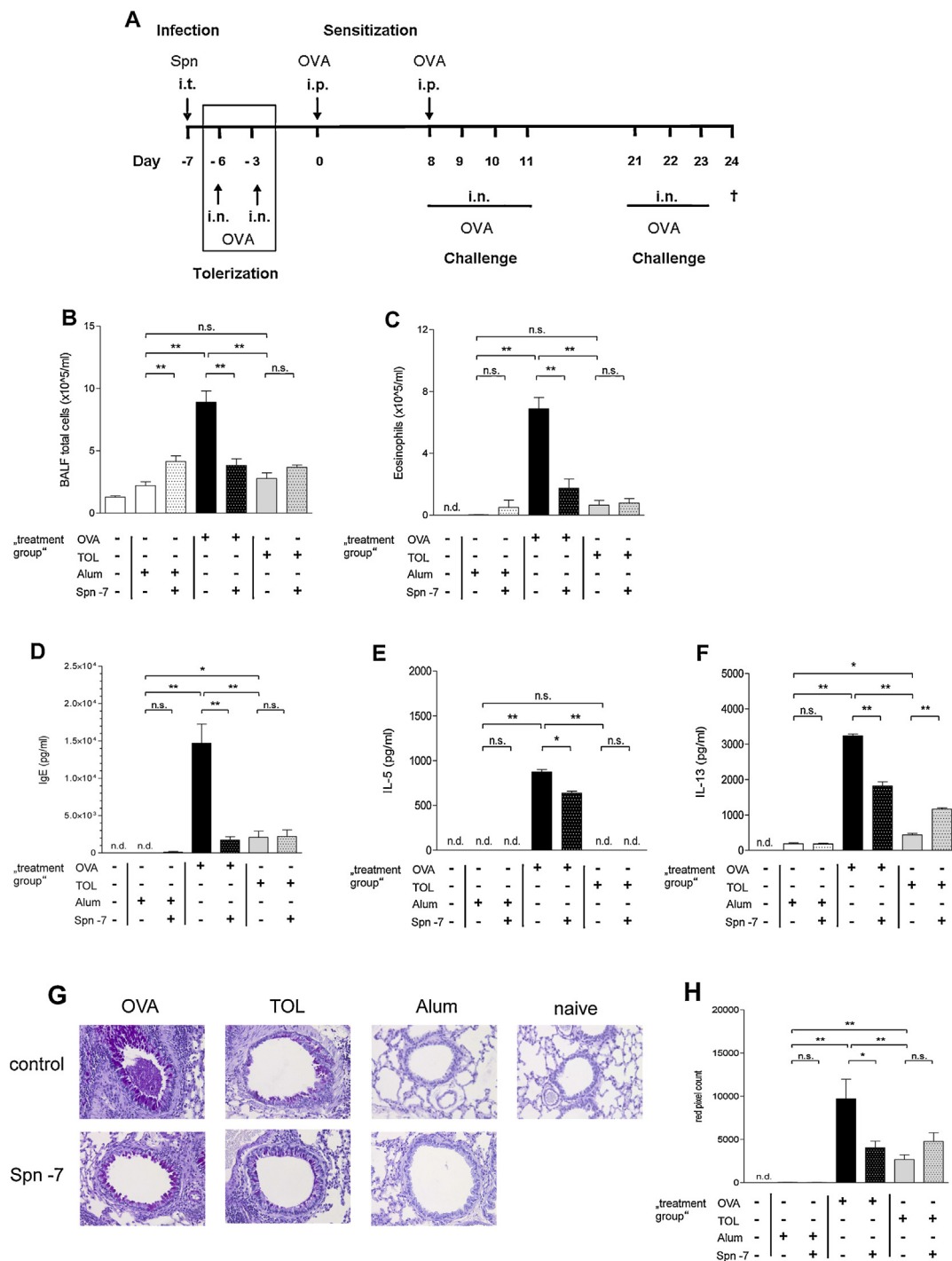


**Fig. 1.** Suppression of allergic inflammation and efficient induction of respiratory tolerance after early (Spn<sub>-7</sub>) pneumococcal pneumonia. (A) Study protocol. Intratracheal infection with *S. pneumoniae* (Spn, i.t.) at day -7. Intranasal (i.n.) tolerization with ovalbumin (OVA) in 0.9% saline at days -6 and -3 (square) in tolerized mice (TOL). Intraperitoneal (i.p.) immunization with OVA in Alum at days 0 and 8 in allergic (OVA) and tolerized mice (TOL); i.p. immunization with saline in Alum for control group (Alum). I.n. challenges at consecutive days 8–11 and 21–23 with OVA (OVA, TOL) or saline (Alum). Section of all mice at day 24. (B–C) BALF analysis. Bronchoalveolar lavage fluid (BALF) was obtained from each individual mouse to determine total cell count (B) and eosinophil cell count (C) determined on cytospins. Decreased total cell counts (B) and eosinophilic inflammation (C) in OVA/Spn<sub>-7</sub> mice vs. OVA mice. In contrast, no significant differences in total BALF cell count and eosinophils of TOL mice compared with TOL/Spn<sub>-7</sub> mice. (D) OVA-specific serum IgE levels. OVA-specific IgE (D) serum levels were measured by ELISA showing a significant decrease in IgE production for OVA/Spn<sub>-7</sub> mice compared to OVA controls, whereas levels were not altered significantly in TOL/Spn<sub>-7</sub> group compared with tolerized mice. (E–F) OVA-specific Th2 cytokine production in spleen cell culture supernatants. Production of IL-5 (E) and IL-13 (F) showed a significant decrease after early infection (OVA/Spn<sub>-7</sub>), whereas tolerized and infected mice showed an increase of IL-13 in infected animals (TOL/Spn<sub>-7</sub>) compared to the corresponding control group (TOL). One representative out of three independent experiments is shown; data are presented as mean  $\pm$  SEM;  $n = 5$ –6 animals per group; \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , n.s.  $p > 0.05$ ; Student's unpaired *t*-test. n.d. = not detectable, values below detection threshold. (G–H) Lung histology analysis. Lungs were obtained on day 24 for histological examination (G) after staining for mucus secretion (PAS), followed by computer-based quantification using an image analyzing program (H). Significantly decreased mucus production was observed in OVA/Spn<sub>-7</sub> mice compared to allergic controls (OVA). In contrast, there were no significant differences detected between TOL vs. TOL/Spn<sub>-7</sub> mice in regard to mucus hypersecretion. All data are presented as mean  $\pm$  SEM;  $n \geq 6$ –8 animals per group; three independent experiments; \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , n.s.  $p > 0.05$ ; n.d. = not detectable, values below detection threshold. Mann–Whitney–*U*-test if not described otherwise.

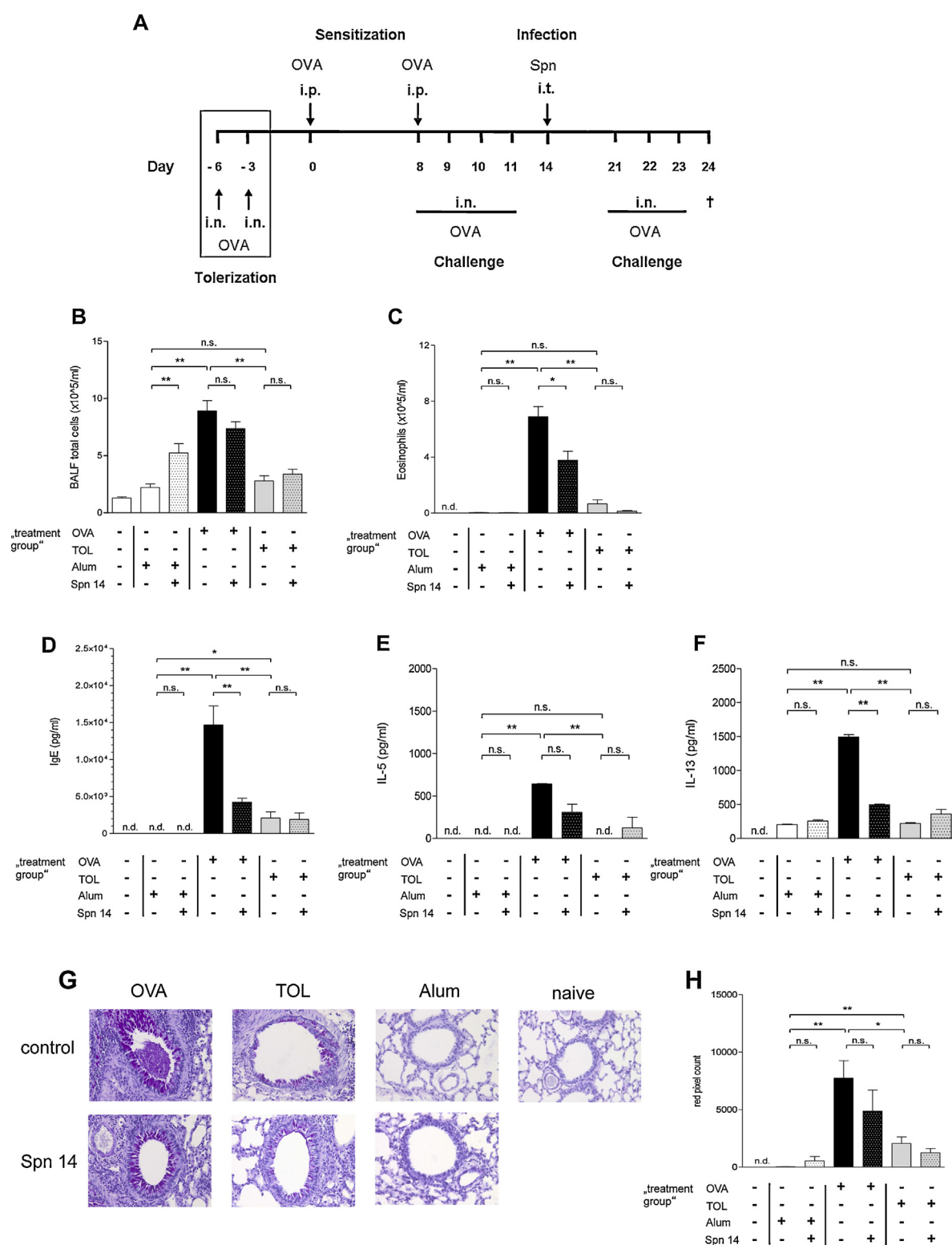


**Fig. 2.** Suppression of allergic inflammation and persistence of respiratory tolerance after late (Spn<sub>14</sub>) pneumococcal pneumonia. (A) Study protocol. Protocol according to Fig. 1A, i.t. Spn infection at day 14. (B–C) BALF analysis. Comparably enhanced total cell count (B) but decreased eosinophilic inflammation (C) were observed in OVA/Spn<sub>14</sub> mice vs. OVA mice. TOL/Spn<sub>14</sub> mice showed no significant difference in BALF total and eosinophil cell counts compared with TOL mice. (D) OVA-specific serum IgE levels. OVA-specific IgE values were significantly decreased in OVA/Spn<sub>14</sub> mice compared to OVA group. OVA-specific IgE levels did not differ between TOL/Spn<sub>14</sub> and TOL group. (E–F) OVA-specific Th2 cytokine production in spleen cell culture supernatants. Production of IL-13 (F) showed a significant decrease in OVA-sensitized and challenged mice after infection (OVA/Spn<sub>14</sub>), whereas IL-5 production was not significantly altered. Tolerized mice groups did not differ significantly (TOL/Spn<sub>14</sub> vs. TOL). One representative out of three independent experiments is shown;  $n = 5–6$  animals per group; \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , n.s.  $p > 0.05$ ; n.d. = not detectable, values below detection threshold. Student's unpaired  $t$ -test. (G–H). Lung histology analysis. Lungs were obtained on day 24 for histological examination (G), followed by computer-based quantification using an image analyzing program (H). No significant difference in mucus production between OVA/Spn<sub>14</sub> and OVA controls, neither between TOL/Spn<sub>14</sub> and TOL mice. All data are presented as mean  $\pm$  SEM;  $n \geq 6–8$  animals per group; three independent experiments; \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , n.s.  $p > 0.05$ ; n.d. = not detectable, values below detection threshold. Mann–Whitney–U-test if not described otherwise.

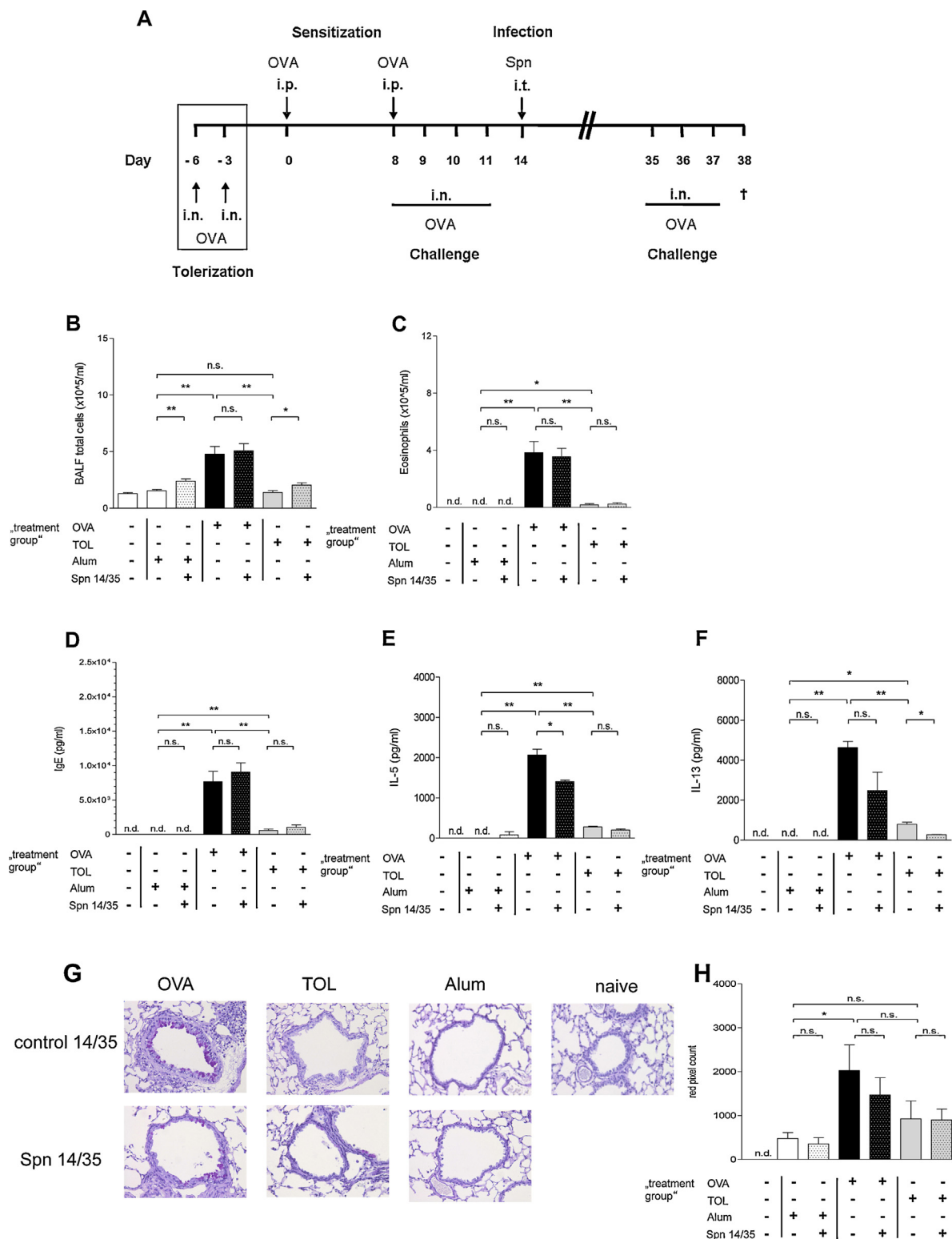


**Fig. 3.** Persistent allergic phenotype and respiratory tolerance after late *S. pneumoniae* infection and challenge (Spn<sub>14/35</sub>). (A) Study protocol. Protocol according to Fig. 2A, second challenge period at days 35–37. Section at day 38. (B–C) BALF analysis. Increased total (B) and eosinophil (C) cell counts were found in OVA/Spn<sub>14/35</sub> mice as well as allergic controls (OVA). TOL/Spn<sub>14/35</sub> mice showed a slight but significant increase in BALF total cell count though no increased eosinophilic inflammation compared with TOL group (C). 3D. OVA-specific serum IgE levels. OVA-specific IgE serum levels (D) were not significantly altered for OVA/Spn<sub>14/35</sub> and TOL/Spn<sub>14/35</sub> mice compared to the corresponding controls (OVA, TOL), respectively. (E–F) OVA-specific Th2 cytokine production in spleen cell culture supernatants. Only IL-5 production (E) showed a significant decrease in mice after late infection (OVA/Spn<sub>14/35</sub>), whereas IL-13 production was not significantly altered in OVA/Spn<sub>14/35</sub> mice (F). In contrast, IL-13 production was significantly decreased in TOL/Spn<sub>14/35</sub> mice compared to their controls (TOL). One representative out of three independent experiments is shown;  $n = 5–6$  animals per group; \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , n.s.  $p > 0.05$ ; n.d. = not detectable, values below detection threshold. Student's unpaired  $t$ -test. (G–H) Lung histology analysis. Lungs were obtained on day 38 for histological examination after PAS-staining (G), followed by computer-based quantification using an image analyzing program (H). No significant difference in mucus production in OVA/Spn<sub>14/35</sub> mice compared to OVA, neither between TOL/Spn<sub>14/35</sub> and TOL. All data are presented as mean  $\pm$  SEM;  $n \geq 6–8$  animals per group; three independent experiments; \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , n.s.  $p > 0.05$ ; n.d. = not detectable, values below detection threshold. Mann-Whitney- $U$ -test if not described otherwise.

## 2.6. Collection and analysis of bronchoalveolar lavage fluid (BALF)

The trachea was cannulated and whole lung washes were performed by repeated instillations and subsequent aspirations of increasing volumes (300–500  $\mu$ l) of ice-cold PBS supplemented with 2 mM EDTA until a total BALF volume of 5 ml per mouse was reached. Total BALF volume was centrifuged at  $300 \times g$  (4 °C, 10 min) and BALF cells were resuspended in 500  $\mu$ l PBS/EDTA. Total BALF cells were determined using an automated cell counting system based on Trypan Blue dye exclusion method (Cedex HiRes, Innovatis, Bielefeld, Germany). In addition, BALF differentials were determined on cytopspins ( $1 \times 10^5$  BALF cells per cytopspin) subjected to May–Grunwald–Giemsa staining. At least 100 cells per cytopspin were differentiated by light microscopy based on overall morphologic criteria, including cell size and shape of nuclei by a blinded examiner. Total BALF leukocyte subsets were quantified by multiplication of percent values with the respective total BALF cell counts.

## 2.7. Analysis and score-based quantification of lung histology

The lungs were inflated with 0.8 ml PBS and fixed in 4% formalin at room temperature for at least 24 h. Fixed lungs were then embedded in paraffin, deparaffinized, and cut into 2–5  $\mu$ m sections and stained with periodic acid–schiff reagent (PAS, Sigma, Taufkirchen, Germany). Evaluation and quantification of the degree of mucus production was done under blinded conditions on lung histology slides scanned with a digital camera (40 $\times$ ; 10 shots per lung) and analyzed with HistoClick Software (provided by I. Hohmann and W. Scherf, Germany), based on morphometric image analysis developed in our laboratory. Positive pixel counts were first totalled for each individual lung and then recalculated per group of mice for further statistical analysis. The pixel data were finally presented as mean  $\pm$  SEM per group. Exemplary lung sections of individual mice were scanned with a digital camera ( $\times 200$ ) for illustration of mucus production.

## 2.8. Measurement of OVA-specific immunoglobulins IgE, IgG<sub>1</sub> and IgG<sub>2a</sub>

Serum was obtained from each individual mouse 24 h after the last i.n. OVA challenge. OVA-specific IgE, IgG<sub>1</sub> and IgG<sub>2a</sub> serum levels were measured by ELISA, according to standard protocol. Using a standard curve for mouse anti-OVA IgE (AbD Serotec, Oxford, UK), we calculated OVA-specific IgE concentrations (pg/ml). Data for OVA-specific serum IgG<sub>1</sub> and IgG<sub>2a</sub> are presented as titer values derived from analysis of optical density values vs. factors of serum dilution series with a logarithmic curve fitting model.

## 2.9. Measurement of in vitro cytokine production

Spleen ( $5 \times 10^6$ /well) or mediastinal lymph node cells ( $1 \times 10^6$ /well) were collected and re-stimulated in vitro with OVA (200  $\mu$ g/ml) in culture medium (RPMI medium supplemented with 10% FCS, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin). Th2 cytokines (IL-5, IL-10, IL-13) were measured in supernatants of spleen or mediastinal lymph node cells at day 3 of OVA re-stimulation by commercially available ELISA kits (R&D Systems, Minneapolis, USA), according to the manufacturer's instructions.

## 2.10. Statistical analysis

Data are presented as mean  $\pm$  SEM. Statistical analyses were performed using GraphPad Prism V software (La Jolla, CA, USA). Cytokine levels in supernatants of spleen or mediastinal lymph node cells among the groups were calculated using Student's

unpaired *t*-test. For all other parameters a non-Gaussian distribution was assumed and significance levels among the groups were calculated using Mann–Whitney–*U*-test. *p* values of less than 0.05 were considered significant (\*); values of less than 0.01 were considered highly significant (\*\*).

## 3. Results

### 3.1. Bacterial load of *S. pneumoniae*-infected lungs and overall mortality

In pre-running experiments, we determined bacterial loads in mice infected with serotype 19 *S. pneumoniae* over time. Mice infected with  $5 \times 10^6$  CFU/mouse were found to exhibit  $(1-2) \times 10^6$  CFU/mouse by 24 h post-infection, with a sharp decline thereafter. Overall mortality rates in the described infection model ranged at approximately 10–15% within the first week post-infection.

### 3.2. Suppression of allergic inflammation after early (*Spn*<sub>7</sub>) and late (*Spn*<sub>14</sub>) pneumococcal pneumonia

OVA-immunized and -challenged mice (“OVA”) showed an allergic phenotype represented by a significantly increased influx of eosinophils as well as increased allergen-specific serum IgE levels compared to control mice (Alum), consistent with previous reports [13,14,16,17,20] (Fig. 1B–D). Furthermore, we observed significantly elevated levels of Th2 cytokines IL-5, -10 and -13 in spleen cell cultures of OVA-treated mice (Fig. 1E and F; Suppl. Fig. S1C) and increased levels of IL-13 in mediastinal lymph node cell cultures (Suppl. Fig. S2A and B). To evaluate the impact of an early lung *S. pneumoniae* infection on expression of an allergic phenotype, we compared OVA mice with mice infected with *S. pneumoniae* one week prior to immunization (OVA/*Spn*<sub>7</sub>, Fig. 1A–H). We observed a profound decrease in total BALF cell numbers by more than 50% accompanied by significantly decreased eosinophil counts in infected, allergic mice (OVA/*Spn*<sub>7</sub>, Fig. 1B and C). At the same time, BALF macrophages, lymphocytes and neutrophils did not differ significantly between groups (Suppl. Fig. S3A, D and G). Moreover, early infection with *S. pneumoniae* (OVA/*Spn*<sub>7</sub>) resulted in significantly decreased OVA-specific serum IgE levels compared to non-infected controls (OVA). In line with this, cytokine measurements in re-stimulated spleen cell cultures revealed significantly reduced IL-5 and IL-13 levels compared to allergic OVA-treated mice (Fig. 1E and F) as well as significantly decreased IL-13 levels in mediastinal lymph node cells (Suppl. Fig. S2A). Notably, IL-10 as well as serum IgG<sub>2a</sub> titers were not significantly altered by infection, whereas the IgG<sub>1</sub> titer was slightly decreased (Suppl. Fig. S1A–C).

In addition, allergic inflammation in OVA mice was characterized by eosinophilic lung inflammation with mucus hypersecretion and goblet cell hyperplasia in lung tissue sections (PAS staining) compared to control mice (Alum, Fig. 1G). Lung histology from OVA/*Spn*<sub>7</sub> treated mice revealed decreased mucus production and goblet cell hyperplasia compared to OVA controls (Fig. 1H).

To examine the effect of pneumococcal pneumonia on established allergic inflammation, mice were infected with *S. pneumoniae* at day 14 relative to i.p. sensitization (*Spn*<sub>14</sub>), followed by a second intranasal challenge period until day 24. Under these experimental conditions, we observed slightly decreased eosinophil BALF cell counts in OVA/*Spn*<sub>14</sub> mice accompanied by increased numbers of BALF macrophages and neutrophils (Fig. 2C; Suppl. Fig. S3B and H). Moreover, OVA-specific serum IgE but not IgG<sub>1</sub> as significantly decreased in OVA/*Spn*<sub>14</sub> mice compared to allergic controls (Fig. 2D; Suppl. Fig. S1D), whereas IgG<sub>2a</sub> levels were significantly

increased (Suppl. Fig. S1E). In addition, IL-10 and IL-13 levels were significantly decreased in this experimental group (OVA/Spn<sub>14</sub>), while IL-5 was only slightly reduced in spleen cell cultures (Fig. 2E and F; Suppl. Fig. S1F). Analysis of IL-13 in mediastinal lymph node cells also revealed a non-significant trend toward a reduction (Suppl. Fig. S2B). Score-based quantification of lung sections from OVA/Spn<sub>14</sub> mice revealed decreased though non-significant differences relative to mice treated solely with OVA (Fig. 2G and H). In summary, we observed a considerable reduction in a majority of overall parameters of allergic inflammation, such as BALF eosinophils, OVA-specific serum IgE, and Th2 cytokines, but no comprehensively decreased allergic phenotype.

### 3.3. Persistent allergic phenotype after late *S. pneumoniae* infection and challenge (Spn<sub>14/35</sub>)

Since late infection with *S. pneumoniae* at day 14 after i.p. sensitization decreased the allergic phenotype less effectively compared to an early infection at day –7, we questioned whether this might be due to the time point of infection relative to intranasal re-challenge. Therefore, mice were subjected to infection with *S. pneumoniae* at day 14 after immunization followed by re-challenge with OVA on days 35, 36, and 37 (Fig. 3). OVA/Spn<sub>14/35</sub> mice showed no difference in total or eosinophil BALF cell counts as well as BALF cell composition (Fig. 3B and C; Suppl. Fig. S3C, F and I) compared to non-infected OVA controls. Additionally, OVA-specific serum IgE, IgG<sub>1</sub> or IgG<sub>2a</sub> levels were not decreased between infected and non-infected OVA mice (Fig. 3D; Suppl. Fig. S1G and H). Among examined Th2 cytokines, only IL-5 was slightly reduced, with no significant decrease observed for IL-10 and IL-13 in spleen or mediastinal lymph node cells, respectively (Fig. 3E and F; Suppl. Figs. S1I and S2C). In this line, histological analysis revealed no significant reduction in mucus secretion in OVA/Spn<sub>14/35</sub> mice compared to OVA controls (Fig. 3G and H).

Collectively, we observed a significantly reduced allergic phenotype after early and late infection of mice with *S. pneumoniae* in relation to i.p. sensitization. However, any influence of a pneumococcal infection on the manifestation of an allergic airway inflammation almost completely disappeared with an increasing time interval between infection and mucosal re-challenge, overall resulting in a persistent allergic phenotype.

### 3.4. Induction of respiratory tolerance after infection with *S. pneumoniae* at day –7

We induced respiratory tolerance by i.n. application of OVA prior to immunization and i.n. challenge. Tolerized mice (TOL) are prevented from the development of an allergic-inflammatory phenotype, resulting in significantly decreased BALF total and eosinophil cell counts, OVA-specific serum IgE levels and Th2 cytokines, whereas serum IgG<sub>1</sub> and IgG<sub>2a</sub> levels both do not differ significantly between allergic and tolerized mice (Fig. 1B–F; Suppl. Fig. S1A–C).

To examine the impact of a pneumococcal lung infection on the induction of respiratory tolerance in mice, animals were infected with *S. pneumoniae* one day prior to initiation of tolerization (day –7, Fig. 1A). Analysis of BALF cell composition in TOL/Spn<sub>–7</sub> compared to TOL mice revealed no major influence of a previous infection on tolerization efficacy, as judged by total and eosinophil cell counts (Fig. 1B and C). Additionally, infection itself, represented by infected non-allergic controls (Alum/Spn<sub>–7</sub>), led to a significant increase in total BALF cell counts compared to non-infected controls (Alum, Fig. 1B). Furthermore, analysis of OVA-specific serum IgE levels, as well as PAS-stained lung histology sections did not show a significant difference between TOL and TOL/Spn<sub>–7</sub> mice (Fig. 1D, G and H). In contrast, IgG<sub>1</sub> and

IgG<sub>2a</sub> titers were significantly increased compared to TOL mice (Suppl. Fig. S1A and B). Th2 cytokines showed a variable picture: whereas IL-5 was not significantly altered in TOL/Spn<sub>–7</sub> compared to TOL mice, both IL-10 and IL-13 were noticeably increased in spleen cells of infected animals (Fig. 1E and F; Suppl. Fig. S1C).

### 3.5. Established respiratory tolerance is not affected by infection with *S. pneumoniae* at day 14

To characterize the influence of a pneumococcal lung infection on an established respiratory tolerance in mice, tolerized mice were infected at day 14 after i.p. sensitization. I.n. OVA challenges were applied thereafter on days 21–23 (TOL/Spn<sub>14</sub>) and 35–38 (TOL/Spn<sub>14/35</sub>), respectively. Though total BALF cell counts were slightly increased in TOL/Spn<sub>14/35</sub> mice relative to TOL controls, the same applied to infected (Alum/Spn<sub>14/35</sub>) vs. healthy negative controls (Fig. 3B). Furthermore, BALF differential cell counts did not differ significantly between TOL mice and TOL/Spn<sub>14/35</sub> (Fig. 3C; Suppl. Fig. S3C, F and I). TOL/Spn<sub>14</sub> mice did not show any significant difference in BALF total or eosinophil cell counts compared to their non-infected controls, whereas macrophages, lymphocytes and neutrophils were markedly increased in TOL/Spn<sub>14</sub> animals (Fig. 2B and C; Suppl. Fig. S3B, E and H). Serum Ig, IL-5 and IL-13 levels from spleen and mediastinal lymph node cell culture supernatants as well as mucus production did not differ significantly in TOL/Spn<sub>14</sub> and TOL/Spn<sub>14/35</sub> mice vs. non-infected TOL controls (Figs. 2D–H and 3D–H; Suppl. Fig. S1D, E, G and H, Suppl. Fig. S2B and C). However, IL-10 levels in cell culture supernatants were markedly increased in TOL/Spn<sub>14</sub> mice compared to TOL mice, whereas IL-10 and IL-13 were slightly decreased in TOL/Spn<sub>14/35</sub> mice (Fig. 3F; Suppl. Fig. S1F and I). In summary, despite variable values of Th2 cytokines, respiratory tolerance resisted a late infection with *S. pneumoniae*, resulting in a persistent protection against an allergic phenotype.

## 4. Discussion

In the current study, we examined the effect of lung infection with *S. pneumoniae* on both the development and persistence of an allergic phenotype as well as on the induction and maintenance of respiratory tolerance in mice subjected to defined immunization protocols. We show that infection with *S. pneumoniae* near the time of allergen exposure and after sensitization and mucosal challenge inhibited the allergic response in mice. However, increasing the time interval between infection and mucosal allergen re-challenge resulted in persistence of the allergic phenotype.

Our results are consistent with the epidemiological findings of the hygiene hypothesis, indicating that exposure to pathogens results in prevention of asthma [2]. Notably, the nature of the pathogen under consideration seems to play an important role, since early-life infections with *Chlamydia pneumoniae* was found to enhance mucus-secreting cells, IL-13 mRNA expression and airway hyper-responsiveness in OVA-based mouse models [21]. Furthermore, clinical data support the view that colonization with *S. pneumoniae*, *Moraxella catarrhalis*, and *H. influenzae* promote the risk of an asthma-like phenotype [7] which emphasizes that the interaction of multiple pathogens with a developing pulmonary immune system remains to be elucidated. The latter is of special importance, since Hilty et al. [22] recently questioned the dogma of a sterile pulmonary environment by identifying a specific pulmonary microbiome within the lungs of asthmatics. The important role of *S. pneumoniae* has been examined by several murine studies in the past. To some extent, our observations are therefore in line with studies by Preston et al. [12] who showed that the allergic inflammatory response was prevented in mice treated with either

killed or live *S. pneumoniae* before, during or after sensitization with the model allergen OVA [23], possibly involving regulatory T-cells (Treg) to suppress Th2 T-cell cytokine release [23]. This is supported by the fact that *S. pneumoniae* infection is known to promote induction of Tregs more than any other Gram-positive bacteria, resulting in a reduced Th2 response and asthma-like phenotype [24,25]. Killed pneumococcal components [12,26] or pneumococcal vaccines [12,26] have been used in a few studies as novel immunomodulatory approaches for prevention of allergic airway disease by mimicking the effect of an infection. However we only employed live *S. pneumoniae* for all infection experiments rather than killed bacterial components [23] in order to purely identify the impact of infection rather than any “pharmacologic response” [23]. In this context, it might also be important to determine a possible effect of *S. pneumoniae* infection at an even earlier time point relative to sensitization to delineate the adaptive immune response to the bacterium under such conditions, since colonization and infections at young age seem to play a key role in the development of allergic airway disease [27].

We here report that not only the nature of the pathogen but especially the timing of infection seems to be crucial for its effect on allergic asthma development [2,23]. Capitalizing on the findings of Preston et al., we for the first time examined the effect of a bacterial infection on an already established allergic phenotype by infecting mice with *S. pneumoniae* on day 14 after i.p. sensitization, i.e. three days after a first intranasal challenge period. The majority of allergy-defining parameters such as BALF eosinophilia, OVA-specific serum IgE and Th2 cytokines still demonstrated a decreased allergic phenotype, which was also true for our OVA/Spn<sub>14</sub> mice, whereas Th1-associated parameter IgG<sub>2a</sub> was significantly increased which is in line with other reports supporting the effectiveness of a Th1 skewing immunomodulatory approach to suppress Th2-mediated allergic airway inflammation [28,29]. However, not only the intrinsic ability of *S. pneumoniae* to induce Treg production [12,30], but also a toll-like receptor-mediated release of IL-23 via the recruitment of neutrophils [25] might explain the reduced allergic phenotype. Further research will therefore be necessary to determine the underlying mechanism. Our model also extends the knowledge about the time dependence of a bacterial infection, on a pre-existing allergic phenotype since we were able to show that the effect of an attenuated allergic phenotype disappeared over time: shifting the second i.n. challenge period toward day 35–38 in our allergy protocol resulted in a persistent allergic phenotype with high levels of BALF eosinophil and neutrophil counts, high OVA-specific serum IgE, IgG<sub>1</sub>, and Th2 cytokine levels in spleen cell culture supernatants.

On the other hand, at no time point of infection, we detected any boost of the allergic phenotype as might be expected from clinical experience where bacterial infections often worsen the course of disease in asthmatics [31]. Furthermore, bacteria such as *C. pneumoniae* were found to provoke a dose-dependent allergic sensitization via MyD88-dependent activation of dendritic cells [32]. Vice versa, asthma has also been described as an independent risk factor for invasive pneumococcal disease [10], and was identified as the only significant risk factor associated with pneumococcal carriage in young adult men [8].

In addition, we analyzed the influence of an infection with *S. pneumoniae* with respect to the induction and maintenance of respiratory tolerance. Mucosal tolerization results in persistent protection from the development of a Th2 driven allergic immune response. Similar results were obtained when mice were infected with *S. pneumoniae* one day prior to tolerization, indicating that a moderate bacterial infection does not counterbalance the implementation of respiratory tolerance. So far, it is not clear whether a bacterial infection interferes with the generation of respiratory tolerance to allergens, and our finding in a murine model supports the

concept that an infection with *S. pneumoniae* does not abrogate tolerance induction. Our data therefore extend reports by Preston et al. [23] about prevention of an asthma-like disease in murine models in the presence of an infection with *S. pneumoniae*. We additionally examined the interaction of bacterial infection and mucosal tolerance over time. We demonstrated for the first time that an established respiratory tolerance resists infection with *S. pneumoniae*. This might be explained by persistent suppression of Th2 cell expansion as seen by Preston et al. [23]. However, further research will be needed to determine a possible role for Tregs in this model system. Moreover, we found a persistent tolerant phenotype independent of the time point of pneumococcal infection in relation to systemic allergen sensitization or mucosal re-challenge. This is in contrast to the clear time dependence of the suppressive effect of pneumococcal lung infection on the allergic response, as found by our group and also Preston et al. [12]. Taken together, our study demonstrates for the first time, that a bacterial infection does not interfere with the generation of respiratory tolerance to allergens, since in our murine model an infection with *S. pneumoniae* does not abrogate tolerance induction.

Our study has several limitations. First, our data do not include physiological parameters such as whole-body plethysmography or invasive lung function to underline our findings. However, different studies using a comparable experimental allergy setting found altered physiological parameters that are in line with our results [12,13]. One might also argue that a more physiological allergen such as house dust mite or cockroach antigen would have been even more informative compared to our model allergen OVA. Nevertheless, the latter ensures a well-controlled and widely accepted model allergen in comparable murine experiments [12,23,33]. In order to exclude any side-effects of any LPS contamination within the employed OVA preparations on *S. pneumoniae* mediated effects on allergic sensitization, we have used LPS-depleted OVA in our study [34–36]. We conclude that in our murine allergy model, an infection with *S. pneumoniae* near the time of allergen exposure prevents the development of an allergic phenotype in adult mice. Furthermore, we demonstrate that it also attenuates an already established allergic response at an early stage, while this beneficial effect was no longer detectable when the time interval between infection and second allergen re-challenge was increased. Moreover, we demonstrate that neither induction nor maintenance of respiratory tolerance was altered by bacterial infection, thus further stressing the importance of an undisturbed mucosal tolerance for the prevention of allergic airway inflammation. However, further studies are needed to elucidate the role of bacterial infections in allergic airway disease, especially in the context of growing insights into the complex microbial environment.

## Conflict of interest

The authors declare no financial or commercial conflict of interest.

## Appendix A. Supplementary data

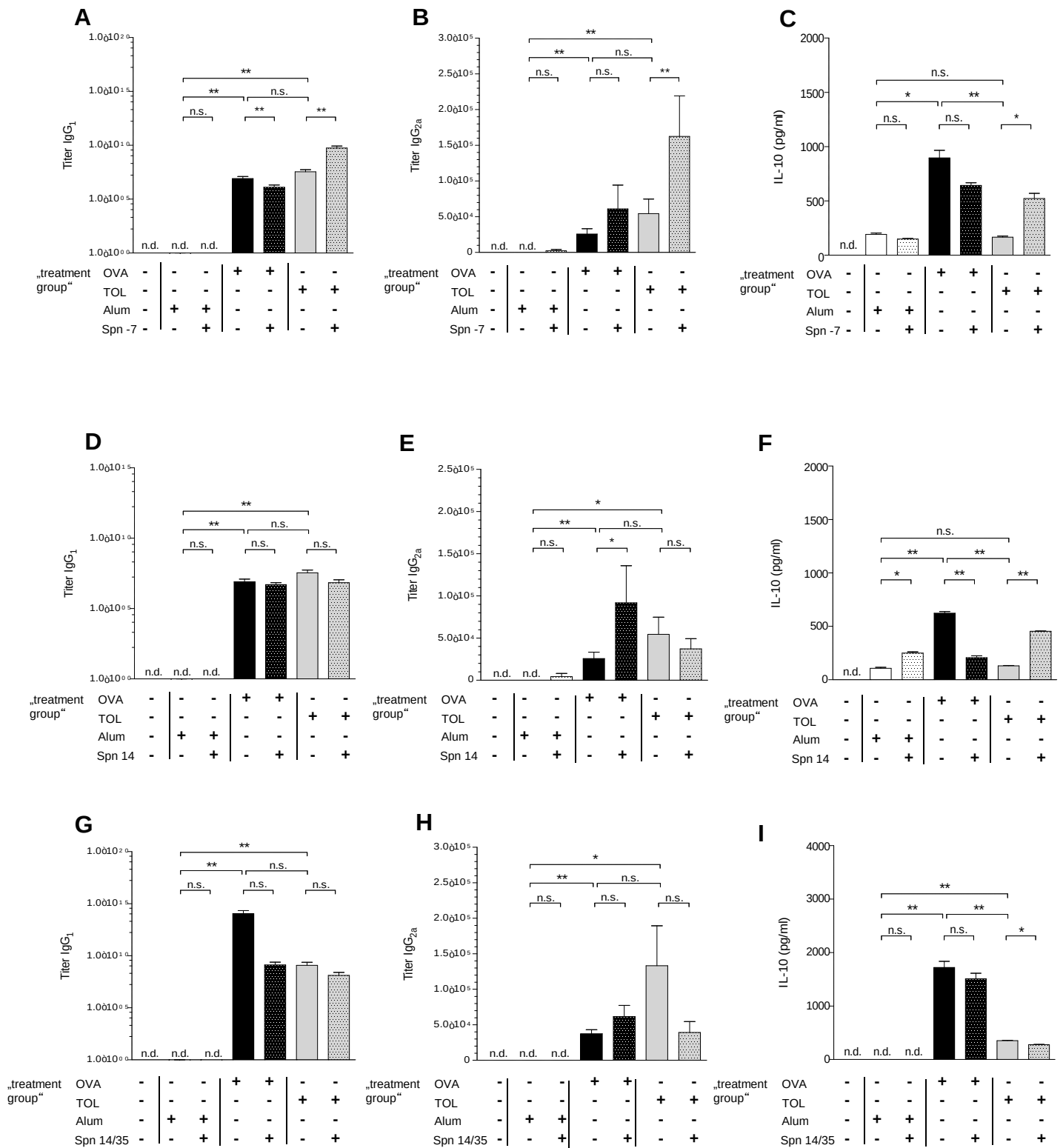
Supplementary material related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.imlet.2014.12.001>.

## References

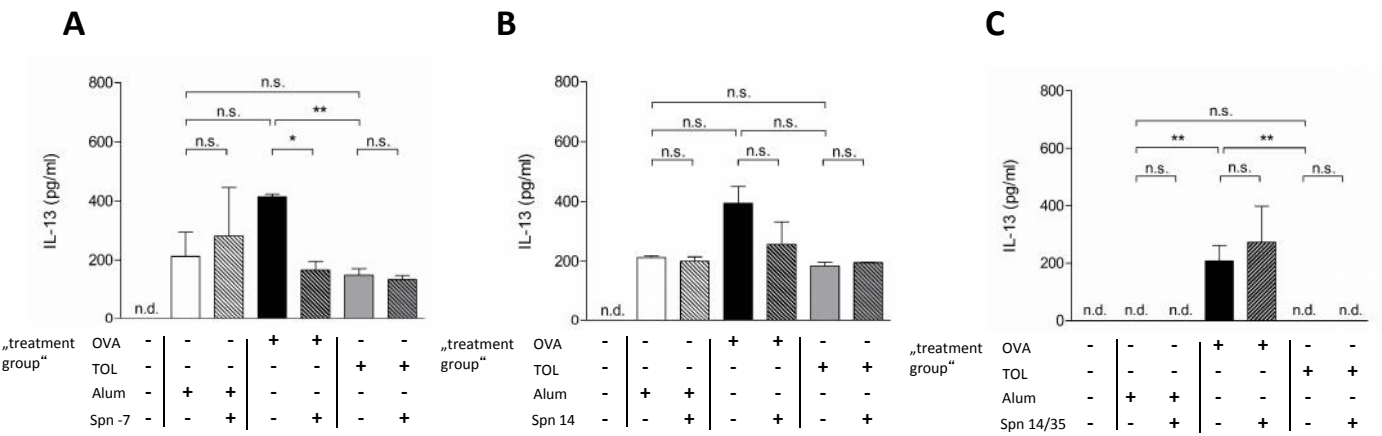
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**Suppl. Fig. 1**



Suppl. Fig. 2



Suppl. Fig. 3

