

***Streptococcus pneumoniae* infection suppresses allergic airways disease by inducing regulatory T cells**

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Supported by grants from the Asthma Foundation of NSW, the Rebecca Cooper Medical Research Foundation, the University of Newcastle Project Grant, the Hunter Medical Research Institute, the Australian Research Council and the National Health and Medical research Council (project grants 401238 and 569219).

Running head: Spn and asthma

Word count for manuscript: 3,432, Max: 3,000 not including the abstract, figure legends, and references.

Abstract

Rationale: An inverse association exists between some bacterial infections and the prevalence of asthma. We investigated whether *Streptococcus pneumoniae* (Spn) infection protects against asthma using mouse models of ovalbumin (OVA)-induced allergic airway disease (AAD).

Methods: Mice were intratracheally infected, or treated with killed Spn, before, during or after OVA sensitisation and subsequent challenge. The effects of Spn on AAD were assessed.

Results: Infection, or treatment with killed Spn suppressed hallmark features of AAD including; antigen-specific T helper cell type 2 (Th2) cytokine and antibody responses, peripheral and pulmonary eosinophil accumulation, goblet cell hyperplasia, and airway hyper-responsiveness (AHR). The effect of infection on the development of specific features of AAD depended on the timing of infection relative to allergic sensitisation and challenge. Infection induced significant increases in regulatory T-cell (Treg) numbers in lymph nodes, which correlated with the degree of suppression of AAD. Tregs reduced T-cell proliferation and Th2 cytokine release. The suppressive effects of infection were reversed by anti-CD25 treatment.

Conclusion: Respiratory infection or treatment with Spn attenuates allergic immune responses and suppresses AAD. These effects may be mediated by Spn-induced Tregs. This identifies the potential for the development of therapeutic agents for asthma from Spn.

(Word count: 196, max 200)

Keywords (Max 6):

- Asthma
- Allergic airways disease
- Suppression
- Immune modulation
- Regulatory T-cell
- *Streptococcus pneumoniae*

Introduction

Asthma is a major chronic respiratory illness that has dramatically increased in prevalence over the past 30 years. Prevalence rates have recently plateaued but remain high [1, 2]. It is an inflammatory disease that is characterised by the infiltration of Th2 cells and eosinophils into the airway wall and is associated with increased mucus production and AHR. The release of the Th2 cytokines interleukin (IL)-4, IL-5 and IL-13 by activated CD4⁺ Th2 cells is instrumental in disease pathogenesis and mediates features of pathophysiology including IgE production, eosinophil accumulation in blood and lungs, mucus hypersecretion and AHR [3]. In addition, Th2 cells, eosinophils and activated inflammatory cells release mediators that damage the mucosal epithelial lining and/or promote an exaggerated repair response, resulting in airway remodelling that contributes to irreversible airflow obstruction and chronic asthma.

Increases in asthma prevalence have been associated with reduced exposure to infectious agents. Recently, extensive research has focused on the significance of both the nature and timing of respiratory infections in asthma, and if infection-induced skewing of the T-cell phenotype can influence the progression of asthma [4-8].

Although certain types of infections drive and/or exacerbate the asthma [6-10], several studies support a role for some respiratory infections, such as *Mycobacteria*, in suppressing Th2-driven allergic disease [4, 11]. Administration of mycobacterial components inhibits AAD, however, the efficacy of mycobacterial treatment in preventing allergic diseases in human trials has been varied, with decreases in the severity of atopic dermatitis but no effect on allergic asthma [11-13].

Spn is commonly carried asymptomatically in the respiratory tract and is the predominant cause of community-acquired pneumonia in children and adults [14].

However, Spn infection is not widely implicated in the pathogenesis of asthma. Spn vaccination has been recommended to prevent invasive Spn disease in asthmatics, and asthma has been suggested as an independent risk factor for invasive Spn disease.

Nevertheless, there is evidence that Spn infection or treatment may actually be beneficial in asthma [11, 14]. Spn infection suppresses parasite-induced eosinophilia [15], suggesting that Spn infection may have the potential to attenuate eosinophilic infiltration in asthma. Furthermore, Spn vaccination of asthmatic children and elderly patients reduced the number and severity of asthmatic exacerbations [14].

In this study we investigated the effect of Spn infection on OVA-induced AAD in mice and examined the potential for the attenuation of asthma with killed Spn. The role of Treg cells in Spn-mediated suppression of AAD was also investigated.

Methods

Allergic sensitisation and challenge

To induce AAD, specific pathogen free BALB/c mice were sensitised by intraperitoneal (IP) injection of OVA (day 0; 50µg; Sigma, Missouri, USA) in Rehydrogel (1mg, Reheis, Berkeley Heights, USA) and sterile saline (200µl) and subsequently challenged (progression of AAD) by intranasal delivery of OVA (day 12-15; 10µg, 50µl sterile saline, Figure 1-3) [8, 16, 17]. Mice were sacrificed and AAD was assessed one day later (day 16). Control (Saline) groups received saline sensitisation and OVA challenge. All procedures were approved by the University of Newcastle animal ethics committee.

Spn inoculation

Animals were infected or treated with Spn intra-tracheally (IT) (Figure 1-3) [16]. For treatment, mice were inoculated with ethanol killed Spn (3x, every 12h). Killed Spn was washed 3x with saline to remove residual ethanol [18]. This protocol ensured that Spn antigens were present in the lungs for the equivalent period as live Spn infection. Control groups were sham inoculated with saline. This did not alter any features of AAD and results were the same as for Saline sensitised groups, therefore, only results from Saline sensitised controls are presented.

T-cell cytokines, serum antibodies and inflammation

Ova-induced cytokine release by lung-draining mediastinal lymph node (MLN) T-cells, serum antibody titres, differential leukocyte counts in blood and BALF, eosinophils in inflamed peribronchial tissue and goblet cell hyperplasia were assessed as previously described [8, 16, 17].

Lung function

AHR was assessed by invasive plethysmography, as a change in airway function (transpulmonary resistance and dynamic compliance) following challenge with increasing doses of aerosolised methacholine [8, 16, 17].

Enumeration of Tregs

The numbers of CD4⁺CD25⁺FoxP3⁺ Tregs in MLNs were assessed by flow cytometry using the antibody conjugates; CD4-FITC, CD25-PerCP-Cy5.5 (BD Biosciences) and FoxP3-PE (eBioscience, San Diego, CA) [19]. Cells stained with isotype antibodies were used as controls.

Treg suppression assays

Treg function was assessed using suppression assays by co-culture of CD11c⁺ cells (2.5 x10⁴), CD4⁺CD25⁻ effector T-cells (5x10⁴) and CD4⁺CD25⁺ cells (0-12 x 10³) purified from MLNs using an AutoMACs Pro (Miltenyi Biotec, Auburn, CA) with OVA (5 µg/ml) or anti-CD3 (1 µg/ml, Biolegend, San Diego, CA) [20]. The purity of sorted cells was determined by FAC to be 95±0.83% for CD11c⁺ cells, 97±0.87% for CD4⁺CD25⁻ effector cells and >81-6±0.45-0.90% for CD4⁺CD25⁺ cells. Cytokine levels in supernatants were assessed [8, 16, 17].

Anti-CD25 treatment

Treg function was suppressed by treatment with anti-CD25 (100 μ g in 200 μ l saline, IP, PC61, eBioscience), 3 days before concurrent Spn infection and OVA sensitization [21].

Statistical Analysis

Results are representative of 2-3 independent experiments and are presented as mean \pm SEM where $n\geq 8$ individual mice. Suppression assays were performed in triplicate with pooled samples. AHR data were analysed by repeated measures one-way ANOVA. N.B. P values for AHR were determined for the entire dose response curve. Other data were analysed by one-way ANOVA with Tukey's post-test.

Results

Some results (Supplementary Figures 1-3) are presented in an online depository.

Induction of AAD

The development of AAD (OVA groups) resulted in significantly increased OVA-induced Th2 cytokine and IL-10 production by MLN and splenic T-cells, OVA-specific IgG1 and total IgE levels in serum and recruitment of eosinophils into the blood, peribronchial spaces (BALF) and lung tissue compared to non-allergic (Saline) controls (Figures 1-4) [8, 16, 17]. Allergic inflammation was associated with enhanced goblet cell hyperplasia in the airway epithelium and AHR (significantly increased transpulmonary resistance and decreased dynamic compliance) (Figures 1-3) [8, 16, 17]. The same OVA control data are presented in Figures 1 and 3.

Spn infection suppresses the progression of AAD

We previously showed that maximal immune responses to Spn infection occur two days after inoculation [22]. To investigate the effects of Spn infection on the progression of AAD, OVA sensitised mice were infected 2 days before challenge (Figure 1). Infection (Spn+OVA groups) significantly suppressed OVA-induced IL-5 release from MLN T-cells, OVA-specific serum IgG1 titre, the numbers of eosinophils in the blood and BALF, and AHR (reduced resistance and increased compliance) compared to uninfected, allergic (OVA) controls. Notably, the levels of eosinophils in the blood were reduced to similar levels to those of the uninfected, nonallergic (Saline) controls. By contrast, infection significantly increased OVA-induced IFN- γ release from T-cells. OVA-specific-IgG2a was not detected and infection had no effect on the numbers of neutrophils in BALF, eosinophils in peribronchial tissue or goblet cells in airway epithelium (not shown).

Treatment with killed Spn suppresses the progression of AAD

The protective properties of Spn infection suggest that Spn might have potential as a treatment for asthma. Therefore, we investigated whether a killed Spn formulation could also inhibit the progression of AAD. OVA-sensitised mice were inoculated with whole ethanol killed Spn before OVA challenge and the progression of AAD was assessed (Figure 2).

Killed Spn significantly decreased eosinophilic inflammation in the blood as well as goblet cell hyperplasia, OVA-induced IL-5 and -IFN- γ secretion by T-cells, and OVA-specific IgG1 production compared to untreated, allergic controls [16]. AHR was also suppressed: there was a trend towards a reduction in resistance, although not statistically significant, and a significant suppression of compliance.

Resolved or concurrent Spn infection suppresses the induction of AAD

Since Spn infection and killed Spn suppressed the progression of AAD in sensitised mice, we then investigated the effects of infection on sensitisation during the induction of AAD. Maximal immune responses occur 2 days after infection and recovery from inflammation is complete after 10 days [16, 22]. Therefore, mice were infected either, 10 days before (resolved infection) or during (concurrent infection) sensitisation to OVA (Figure 3). Infection concurrent with sensitisation ensured that maximal immunomodulatory effects of infection occurred during the sensitisation phase.

Resolved or concurrent infection significantly suppressed the levels of OVA-induced cytokine release from MLN T-cells, OVA-specific serum IgG1, eosinophils in blood, BALF and peribronchial tissue and goblet cells in the airways compared to uninfected, allergic controls. Resolved infection had no significant impact on AHR whereas concurrent infection reduced AHR. IL-5, IFN- γ and blood eosinophils were suppressed to the same levels as in uninfected, nonallergic (Saline) controls. Again, IgG2a was not detected and BALF neutrophil numbers did not vary between groups (not shown).

Concurrent infection suppresses the induction of additional features of AAD

Since concurrent infection during sensitisation has the most potent suppressive effects on AAD, this model was used to further investigate the mechanisms that underpin suppression. First the effects of concurrent infection on additional features of AAD were assessed (Figure 4).

Infection suppressed; local OVA-induced IL-13 release from MLN T-cells, systemic OVA-induced IL-5, IL-13 and IFN- γ release from splenocytes and total circulating IgE responses to the same level as uninfected, nonallergic controls. By contrast, infection increased both local and systemic IL-10 release. TGF- β was not detected in any sample.

Concurrent Spn infection increases the number of Treg cells in lymph nodes in AAD

IL-10 is an immunosuppressive cytokine that may be released from T-cells or Tregs [23]. The increases in IL-10 in MLN and splenocyte cultures, indicated that the suppressive effects of infection may result from the induction of Tregs. To investigate this possibility, mice were concurrently infected with Spn during sensitisation and Tregs were enumerated in MLNs.

Infection increased both the numbers and percentages of CD4⁺CD25⁺FoxP3⁺ cells in MLNs compared to uninfected, allergic controls (Figure 5 and Supplementary Figure 1). We then assessed whether infection after sensitization (which had similar suppressive effects to concurrent infection), and resolved infection (which suppressed inflammation but not AHR), also had altered numbers of Tregs in MLNs (Supplementary Figure 1). Infection after sensitisation also increased the numbers of Tregs in MLNs, which may be responsible for similar levels of suppression of AAD. Resolved infection did not significantly increase the numbers or percentages of CD4⁺CD25⁺FoxP3⁺ cells. It is likely that resolved infection induced Tregs at some time during the course of the induction and progression of AAD, which may account for the suppression of some features of AAD but not others.

Spn infection-induced Tregs suppress T-cell proliferation and Th2 cytokine release in AAD

The function and suppressive mechanisms of infection-induced Tregs was assessed using T-cell suppression assays.

Tregs from the MLNs of mice with AAD with or without concurrent infection suppressed the proliferation of CD4⁺CD25⁻ T-cells from the same lymph nodes in the presence of either OVA or anti-CD3 (Figure 6). Tregs also suppressed the release of IL-5 and IL-13 and increased IL-10 production in response to OVA stimulation. However, infection did not enhance the suppressive function of Tregs on proliferation or cytokine release. This indicates that the suppression of AAD by infection occurs through

increases in Treg numbers rather than by enhancing suppressive capacity.

Anti-CD25 treatment reverses the suppressive effects of infection on the induction of AAD

We then confirmed that Spn-induced Treg cells were responsible for the suppressive effects of infection on AAD. Anti-CD25 was administered 3 days before concurrent infection and sensitisation, which completely inhibited the development of CD4⁺CD25⁺Foxp3⁺ Tregs (not shown). The effects of administration on the suppression of AAD were assessed (Figures 7 and Supplementary Figure 2).

Anti-CD25 administration removed the infection-mediated suppression of OVA-induced IL-5, IL-13 and IFN- γ secretion by MLN and splenic T-cells, OVA-specific serum IgG1 production, the numbers of eosinophils in blood, BALF and peribronchial tissue, goblet cell hyperplasia and AHR. Administration also reversed the increased OVA-induced release of IL-10 from MLN T-cells. Blocking Tregs in infected groups allowed the induction of AAD that was similar in severity to AAD in Treg depleted, uninfected allergic groups. There were few differences between Treg depleted and non-depleted allergic controls, indicating that effector T cells were not affected by treatment. The administration of antibody isotype controls had no effect (Supplementary Figure 3). This demonstrates that it is the CD25⁺ Tregs, which are induced by infection, that mediate the suppression of AAD.

Discussion

Using a mouse model of OVA-induced Th2-driven AAD, we demonstrate that Spn infection, or whole killed Spn, suppress the hallmark features of AAD. This included inhibition of: Antigen-specific Th2 cytokine and antibody responses; peripheral and pulmonary eosinophil accumulation; goblet cell hyperplasia; and AHR. The effect of infection on some of the specific features of AAD was dependent on the timing of infection relative to allergic sensitisation and challenge. The attenuating effects of Spn result from the immunosuppressive activity of increased numbers of Tregs that are induced by Spn. These Tregs mediate suppression by reducing Th2 cell responses.

OVA-induced models of AAD have been widely employed to further the understanding of asthma pathogenesis (importance of Th2 cells and cytokines, involvement of IL-13 and IL-4R, role of eosinophils in remodelling), which has led to these molecules and events being targeted therapeutically in asthma [24]. In our model the IP delivery of OVA in alum drives the development of systemic Th2 responses and allergic sensitisation. Subsequent OVA challenge leads to the recruitment of Th2 cells into the airways that release cytokines, induce eosinophil influx and the progression of AAD. This model, therefore, enables the therapeutic manipulation of Th2-mediated allergic inflammation and the induction and progression of AAD.

We first examined whether infection would inhibit the progression of AAD in sensitised groups. Infection of sensitised mice before challenge significantly decreased the production of OVA-induced IL-5 from T-cells, eosinophil numbers in the blood and BALF, and AHR in AAD. This demonstrates that Spn infection suppresses the progression of AAD and indicates the potential for the development of Spn into a treatment for allergic asthma. However, infection significantly increased OVA-induced IFN- γ production and did not reduce the number of eosinophils in peribronchial tissue, or goblet cells in airway epithelium. Spn infection elicits protective host immune responses that involve the release of IFN- γ . An active infection, and subsequent IFN- γ production during OVA challenge may influence the expansion of T-cells and lead to enhanced OVA-induced IFN- γ production. Generally, acute respiratory infections promote increased mucus secretion, which may be responsible for maintaining goblet cell hyperplasia in AAD in our studies.

Spn infections result in serious health problems [14], therefore, to investigate the potential for utilizing Spn as a therapeutic strategy for asthma we examined the effect of treatment with killed Spn on AAD. Spn was ethanol-killed to maintain the TLR activating properties of Spn components, which are lost during heat-killing [18]. Treatment inhibited eosinophil influx into the blood and BALF, goblet cell hyperplasia and suppressed the development of AHR to similar levels to those of live infection. This suggests that it is the effects of Spn antigens, rather than an inflammatory response to infection that suppresses AAD.

The timing of infection relative to allergen sensitisation may be crucial in determining the effect on allergic outcomes [6, 7, 9]. Therefore, we then investigated the potential of resolved and concurrent Spn infections to suppress the induction of AAD. A resolved infection significantly inhibited OVA-induced IL-5 and IFN- γ production from T-cells, eosinophil accumulation in blood, BALF and peribronchial tissue and goblet cell hyperplasia, but had no significant effect on AHR although a trend towards a reduction was observed. This indicates that the suppression of inflammation and AHR may occur independently, which has also been observed clinically at times. We speculate that the attenuation of Th2 immune responses by infection may be sustained which accounts for

the reduction in Th2 cytokines, eosinophils, and goblet cells, whilst the mechanisms of direct suppression of AHR are short-lived. Alternatively, there may be a temporal dissociation between modulatory (e.g. Treg) responses that are induced following infection and the onset and persistence of AHR [25]. By contrast, concurrent infection with OVA sensitisation resulted in significant inhibition of all of the features of AAD assessed. In addition, concurrent infection suppressed OVA-induced IL-13 release from MLNs, IL-5, IL-13 and IFN- γ release from splenocytes and total circulating IgE but increased the release of IL-10.

Collectively, our results demonstrate that Spn infection suppresses key features of AAD, irrespective of the timing of infection. Importantly, from a clinical perspective, infection at all three treatment times inhibited OVA-induced IL-5 release from T-cells and blood eosinophilia. This is particularly significant as it reflects an attenuation of allergen-induced eosinophil expansion in the bone marrow. Eosinophils actively contribute to airway wall remodeling in allergic asthma in humans [26]. Suppression of blood eosinophilia by Spn infection may be significant for the long-term inhibition of eosinophil accumulation in the lungs, leading to the inhibition or resolution of pathogenesis and remodelling events [27]. Thus, the features of AAD that were not reduced in the short-term by infection may be resolved in the longer term. The suppressive effects of Spn were observed with exposure before, during and after sensitisation. This indicates the potential application of Spn to humans for both the suppression of sensitisation in naïve individuals and disease progression in sensitised patients. The beneficial effects of infection or treatment could be enhanced by the identification of the active components of Spn that are responsible for the suppressive effects [11]. These components could be delivered repeatedly and in higher concentrations to enhance the suppression of the induction and progression of asthma. The identification of these components is the subject of ongoing studies.

Suppression of AAD by Spn involves Tregs, which are significantly increased by concurrent infection. Increases in Tregs were reduced when infection had resolved prior to sensitisation and correlated with reduced suppression of AHR. Infection-induced Tregs suppressed the proliferation of both OVA-specific and anti-CD3 stimulated T-helper cells and attenuated OVA-induced Th2 cytokine release.

Importantly, the suppression of all features of AAD by concurrent infection was removed by anti-CD25 treatment. Indeed, blocking Tregs in infected mice allowed the induction of AAD that was similar in severity to AAD induced in Treg depleted groups without infection. CD25⁺ is also a marker of T-cell activation and anti-CD25 treatment may also deplete CD25⁺ effector T-cells. However, anti-CD25 treated uninfected, allergic groups still developed AAD. This shows that effector T-cells were not depleted and that the effects in infected groups were the result of reduced Treg responses. The mechanisms of how Tregs suppressed proliferation and Th2 responses remain to be determined and are under further investigation. IL-10, but not TGF- β , levels were increased in MLN and splenocyte cultures and the possibility remains that suppression could be occurring through either the release of soluble factors or cell-contact dependent processes.

Our results show that Spn may attenuate AAD by increasing Tregs in the local MLNs that suppress the proliferation of Th2 cells and eosinophils. Others have shown that the development and mobilization of CD4⁺CD25⁺ Tregs is induced by Spn infection to a substantially greater extent than by other Gram-positive bacteria and that Tregs are critical regulators of responses to Spn infection [28]. Others have also shown that Tregs

suppress the hallmark features of AAD [29]. Adoptive transfer of CD4⁺CD25⁺ Tregs attenuated the activity of allergen-specific T-cells *in vivo* leading to reduced Th2 cytokine expression in the lung, eosinophil recruitment and AHR. Thus, Tregs may inhibit over-exuberant inflammatory responses that occur during Spn lung infection and may also be important in suppressing AADs and asthma.

Immunomodulatory therapy of asthma by increasing Th1, or inhibiting Th2 responses has received considerable attention. This work has drawn on widespread acceptance of the hygiene hypothesis, which is a well recognised, if oversimplified, link between infection and allergy. Administration of mycobacteria and other Th1-inducing infectious agents for the treatment of asthma in humans have been disappointing, and successful results were dependent on the age of study subjects [4, 12, 14]. The therapeutic use of Spn antigens that may prevent Th2 and eosinophil development and mobilization by a different mechanism, i.e. the induction of Tregs, may be more effective in suppressing disease and may potentially promote the resolution of tissue remodelling of the airways and persistent AHR in chronic asthma [11].

In summary, the significance of common respiratory bacterial infections in the asthma is controversial. The present study is the first to directly examine the effect of respiratory Spn infection on AAD. Spn infection before challenge, or before or during sensitisation, with Th2-inducing antigen suppresses hallmark features of AAD. Spn infection and treatment has the potential to inhibit allergic immune responses in asthmatics, including eosinophilia that is linked to ongoing remodelling of the airway in asthma. This indicates the potential for the development of novel Spn-based therapeutic agents for asthma. A greater understanding of the fundamental mechanisms associated with the suppression of AAD, may lead to new approaches to the prevention and treatment of this disorder.

References

1. Australian Centre for Asthma Monitoring, Australian Institute for Health and Welfare. Asthma in Australia. 2008.
2. Asher M, Montefort S, Björkstén B, Lai C, Strachan D, Weiland S, Williams H, Group. IPTS. Worldwide time trends in the prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and eczema in childhood: ISAAC Phases One and Three repeat multicountry cross-sectional surveys. *Lancet* 2006: 368(9537): 733-743.
3. Temelkovski J, Hogan S, Shepherd D, Foster P, Kumar R. An improved murine model of asthma: selective airway inflammation, epithelial lesions and increased methacholine responsiveness following chronic exposure to aerosolised allergen. *Thorax* 1998: 53(10): 849-856.
4. Zuany-Amorim C, Sawicka E, Manlius C, Le Moine A, Brunet L, Kemeny D, Bowen G, Rook G, Walker C. Suppression of airway eosinophilia by killed *Mycobacterium vaccae*-induced allergen-specific regulatory T-cells. *Nat Med* 2002: 8(6): 625-629.
5. Holt P, Upham J, Sly P. Contemporaneous maturation of immunologic and respiratory functions during early childhood: implications for development of asthma prevention strategies. *J Allergy Clin Immunol* 2005: 116(1): 16-24.
6. Hansbro P, Beagley K, Horvat J, Gibson P. Role of atypical bacterial infection of the lung in predisposition/protection of asthma. *Pharmacol Ther* 2004: 101(3): 193-210.
7. Hansbro N, Horvat J, Wark P, Hansbro P. Understanding the mechanisms of viral induced asthma; new therapeutic directions. *Pharmacol Ther* 2008: 117(3): 313-353.
8. Horvat J, Starkey M, Kim R, Phipps S, Gibson P, Beagley K, Foster P, Hansbro P. Early life chlamydial lung infection predisposes to allergic airways disease through age-dependent differences in immunopathology. *J Allergy Clin Immunol* 2010: 125(3): 617-625.
9. Culley F, Pollott J, Openshaw P. Age at first viral infection determines the pattern of T cell-mediated disease during reinfection in adulthood. *J Exp Med* 2002: 196(10): 1381-1386.
10. Goldman D, Davis J, Bommarito F, Shao X, Casadevall A. Enhanced allergic inflammation and airway responsiveness in rats with chronic *Cryptococcus neoformans* infection: potential role for fungal pulmonary infection in the pathogenesis of asthma. *J Infect Dis* 2006: 193(8): 1178-1186.

11. Thorburn A, Hansbro P. Harnessing Regulatory T cells to Suppress Asthma: From Potential to Therapy *Am J Respir Cell Mol Biol* 2010: In press.
12. Shirtcliffe P, Easthope S, Cheng S, Weatherall M, Tan P, Le Gros G, Beasley R. The effect of delipidated deglycolipidated (DDMV) and heat-killed *Mycobacterium vaccae* in asthma. *Am J Respir Crit Care Med* 2001; 163(6): 1410-1414.
13. Arkwright P, David T. Intradermal administration of a killed *Mycobacterium vaccae* suspension (SRL 172) is associated with improvement in atopic dermatitis in children with moderate-to-severe disease. *J Allergy Clin Immunol* 2001; 107(3): 531-534.
14. Thorburn A, Hansbro P, Gibson P. Pneumococcal vaccines for allergic airways diseases. *Expert Opin Biol Ther* 2009; 9(5): 621-629.
15. Bass D. Behavior of eosinophil leukocytes in acute inflammation. I. Lack of dependence on adrenal function. *J Clin Invest* 1975; 55(6): 1229-1236.
16. Preston J, Essilfie A, Horvat J, Wade M, Beagley K, Gibson P, Foster P, Hansbro P. Inhibition of allergic airways disease by immunomodulatory therapy with whole killed *Streptococcus pneumoniae*. *Vaccine* 2007; 25(48): 8154-8162.
17. Horvat J, Starkey M, Kim R, Beagley K, Preston J, Gibson P, Foster P, Hansbro P. Chlamydial respiratory infection during allergen sensitization drives neutrophilic allergic airways disease. *J Immunol* 2010; 184(4): in press.
18. Malley R, Henneke P, Morse S, Cieslewicz M, Lipsitch M, Thompson C, Kurt-Jones E, Paton J, Wessels M, Golenbock D. Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection. *Proc Natl Acad Sci U S A* 2003; 100(4): 1966-1971.
19. Leech M, Benson R, De Vries A, Fitch P, Howie S. Resolution of Der p1-induced allergic airway inflammation is dependent on CD4⁺CD25⁺Foxp3⁺ regulatory cells. *J Immunol* 2007; 179(10): 7050-7058.
20. O'Sullivan B, Thomas H, Pai S, Santamaria P, Iwakura Y, Steptoe R, Kay T, Thomas R. IL-1 beta breaks tolerance through expansion of CD25⁺ effector T cells. *J Immunol* 2006; 176(12): 7278-7287.
21. Quinn K, McHugh R, Rich F, Goldsack L, de Lisle G, Buddle B, Delahunt B, Kirman J. Inactivation of CD4⁺ CD25⁺ regulatory T cells during early mycobacterial infection increases cytokine production but does not affect pathogen load. *Immunol Cell Biol* 2006; 84(5): 467-474.

22. Preston J, Beagley K, Gibson P, Hansbro P. Genetic background affects susceptibility in non-fatal pneumococcal bronchopneumonia. *Eur Respir J* 2004; 23(2): 224-231.
23. Kaiko G, Horvat J, Beagley K, Hansbro P. Immunological decision-making: How does the immune system decide to mount either a Th1 or Th2 Response? . *Immunology* 2008; 123(3): 326-338.
24. Kumar R, Herbert C, Foster P. The "classical" ovalbumin model of asthma in mice. . *Curr Drug Targets* 2008; 9: 485-494.
25. Mattes J, Yang M, Mahalingam S, Kuehr J, Webb D, Simson L, Hogan S, Koskinen A, McKenzie A, Dent L, Rothenberg M, Matthaei K, Young I, Foster P. Intrinsic defect in T cell production of interleukin (IL)-13 in the absence of both IL-5 and eotaxin precludes the development of eosinophilia and airways hyperreactivity in experimental asthma. *J Exp Med* 2002; 195(11): 1433-1444.
26. Humbles A, Lloyd C, McMillan S, Friend D, Xanthou G, McKenna E, Ghiran S, Gerard N, Yu C, Orkin S, Gerard C. A critical role for eosinophils in allergic airways remodeling. *Science* 2004; 305(5691): 1776-1779.
27. Menzies-Gow A, Flood-Page P, Sehmi R, Burman J, Hamid Q, Robinson D, Kay A, Denburg J. Anti-IL-5 (mepolizumab) therapy induces bone marrow eosinophil maturational arrest and decreases eosinophil progenitors in the bronchial mucosa of atopic asthmatics. *J Allergy Clin Immunol* 2003; 111(4): 714-719.
28. Kadioglu A, Coward W, Colston M, Hewitt C, Andrew P. CD4-T-lymphocyte interactions with pneumolysin and pneumococci suggest a crucial protective role in the host response to pneumococcal infection. *Infect Immun* 2004; 72(5): 2689-2697.
29. Kearley J, Barker J, Robinson D, Lloyd C. Resolution of airway inflammation and hyperreactivity after in vivo transfer of CD4+CD25+ regulatory T cells is interleukin 10 dependent. *J Exp Med* 2005; 202(11): 1539-1547.

Figure legends

Figure 1: Spn infection after sensitization suppresses the progression of AAD. (A) Spn infection was induced after OVA sensitization and before challenge. (B) OVA-induced cytokine release in MLN culture supernatants. (C) OVA-specific IgG1 serum antibodies. (D) Eosinophil accumulation in blood and BALF. AHR in terms of averaged peak (E) transpulmonary resistance and (F) dynamic compliance in response to increasing doses of methacholine; N.B. significant differences in resistance and compliance are for the entire dose response curves. # and * represent significant differences compared to uninfected, nonallergic (Saline) and uninfected, allergic (OVA) controls, respectively. #/* $p < 0.05$, ** $p < 0.01$, and ###/*** $p < 0.001$.

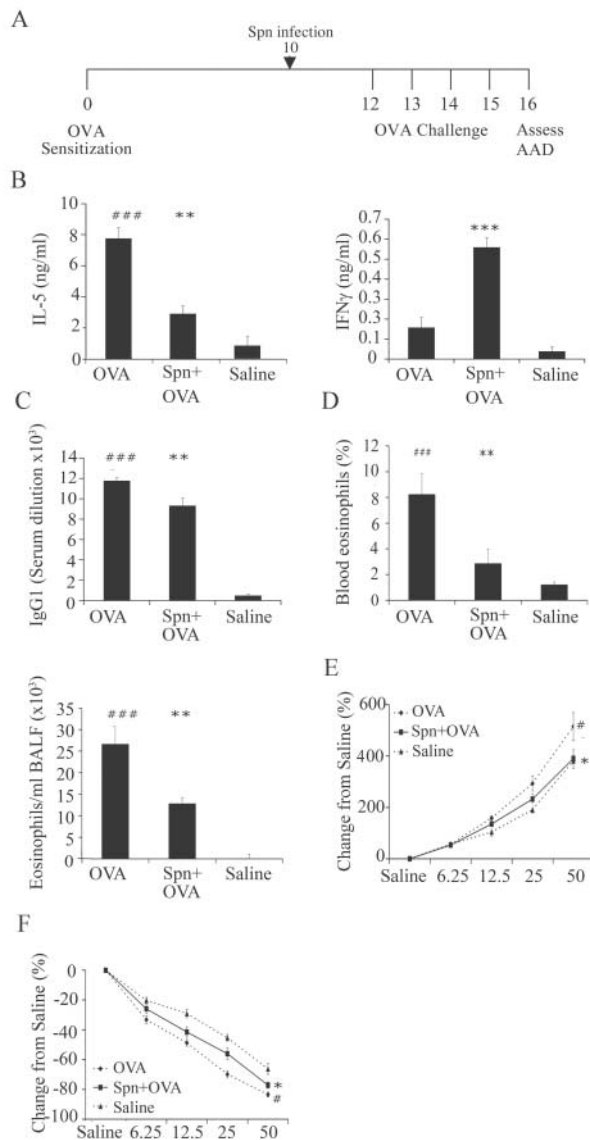


Figure 1

Figure 2: Treatment with killed Spn after sensitization suppresses AAD. (A) Killed Spn was administered after OVA sensitization and before challenge. (B) Eosinophil accumulation in blood. AHR in terms of averaged peak (C) transpulmonary resistance, and (D) dynamic compliance in response to increasing doses of methacholine; N.B. significant differences in resistance and compliance are for the entire dose response curves. # and * represent significant differences compared to uninfected, nonallergic (Saline) and uninfected, allergic (OVA) controls, respectively. #/* $p < 0.05$, ** $p < 0.01$, and ### $p < 0.001$.

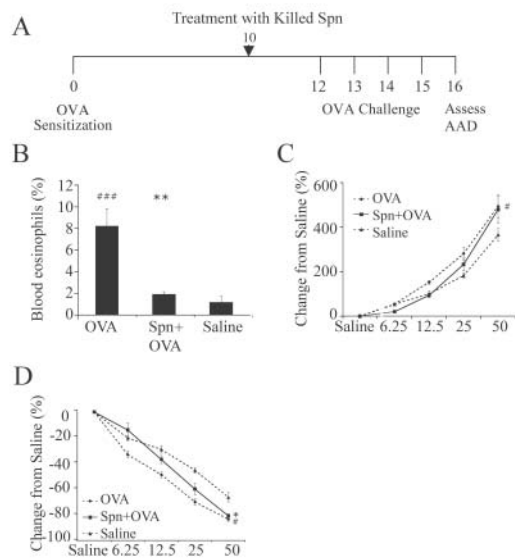


Figure 2

Figure 3: Resolved or concurrent Spn infection at sensitization suppresses the induction of AAD. (A) Spn infection was induced before or during OVA sensitization. (B) OVA-induced cytokine release in MLN culture supernatants. (C) OVA-specific IgG1 serum antibodies. (D) Eosinophil accumulation in blood, BALF and tissue. (E) Mucus secreting cell numbers surrounding the airway lumen. AHR in terms of averaged peak (F) transpulmonary resistance, and (G) dynamic compliance in response to increasing doses of methacholine; N.B. significant differences in resistance and compliance are for the entire dose response curves. # and * represent significant differences compared to uninfected, nonallergic (Saline) and uninfected, allergic (OVA) controls, respectively. #/* $p < 0.05$, ** $p < 0.01$, and ###/*** $p < 0.001$.

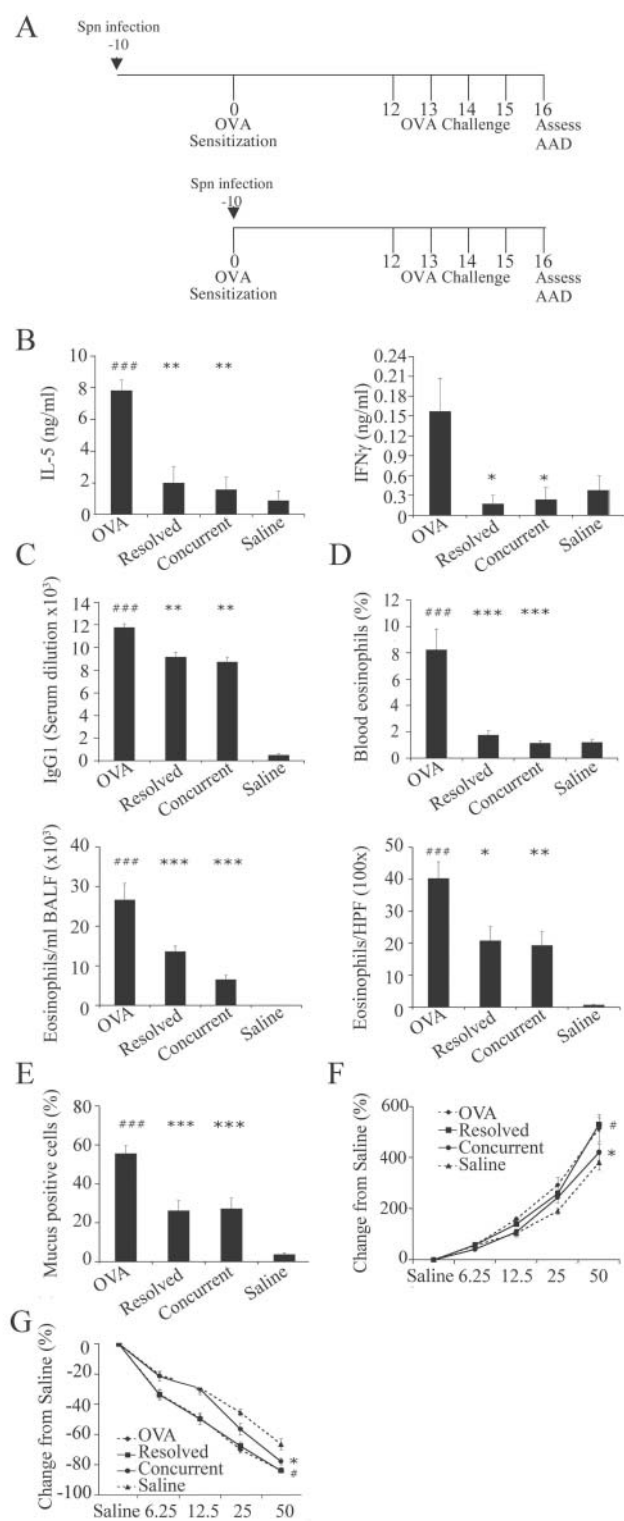


Figure 3

Figure 4: Concurrent Spn infection at sensitization suppresses the induction of additional features of AAD. (A) OVA-induced IL-13 and IL-10 release in MLN culture supernatants. (B) OVA-induced cytokine release in splenocyte culture supernatants. (C) Total IgE serum antibodies. # and * represent significant differences compared to uninfected, nonallergic (Saline) and uninfected, allergic (OVA) controls, respectively. #/* p<0.05, ###/** p<0.01, and ####/*** p<0.001.

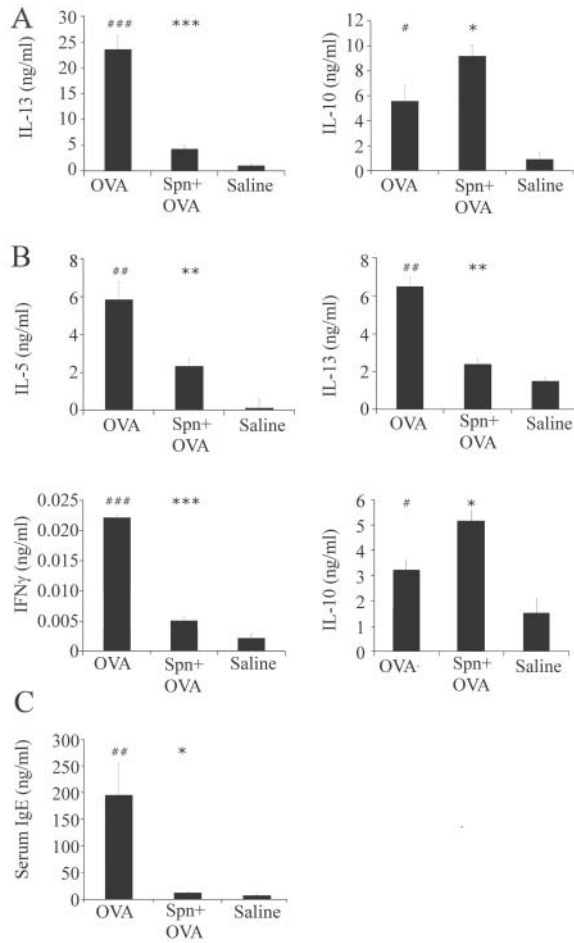


Figure 4

Figure 5: Spn infection during sensitisation induces increases in Tregs in AAD. (A) Total numbers and (B) percentages of CD4⁺CD25⁺Foxp3⁺ Treg cells in MLNs. * represent significant differences compared to uninfected, allergic (OVA) controls. * p<0.05, and ** p<0.01.

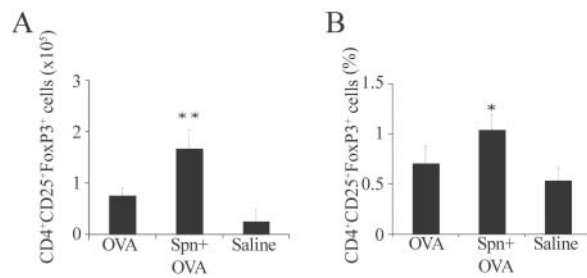


Figure 5

Figure 6: Spn infection-induced suppression of ADD occurs through increases in Treg numbers that reduce Th2 responses. Suppression of proliferation of cultures of CD4⁺CD25⁺ T-cells and CD11c⁺ dendritic cells with increasing numbers of CD4⁺CD25⁺ T-cells stimulated with either (A) OVA or (B) anti-CD3. (C) OVA-induced cytokine release from the same cultures.

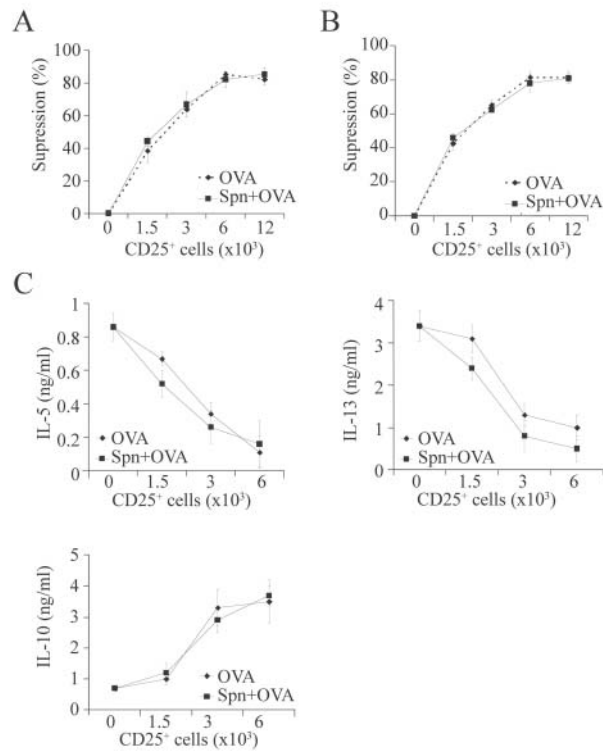


Figure 6

Figure 7: Anti-CD25 treatment reverses the suppressive effects of Spn infection on AAD. (A) OVA-induced cytokine release in MLN culture supernatants. (B) OVA-specific IgG1 serum antibodies. (C) Eosinophil accumulation in blood, BALF and tissue. (D) Mucus secreting cell numbers surrounding the airway lumen. AHR in terms of averaged peak (E) transpulmonary resistance, and (F) dynamic compliance in response to increasing doses of methacholine; N.B. significant differences in resistance and compliance are for the entire dose response curves. # and * represent significant differences compared to uninfected, nonallergic (Saline) and uninfected, allergic (OVA) controls, respectively. #/* p<0.05, ##/** p<0.01, and ###/*** p<0.001.

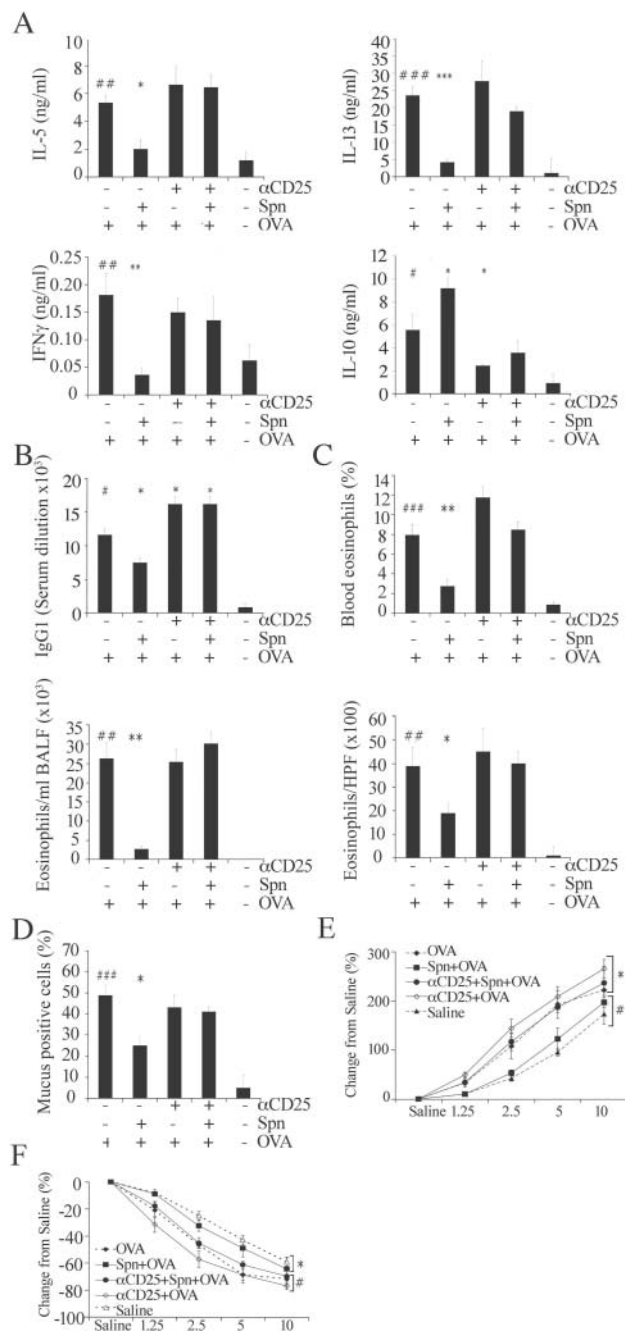


Figure 7

