Online Supplement

Methods

Experimental models

A novel model of *Chlamydia* respiratory infection-induced, severe, steroid-insensitive, neutrophilic allergic airway disease (SSIAAD) was developed to reproduce the effects of infection in established asthma. Female (6-8 week-old) BALB/c mice were intraperitoneally (IP) sensitised to Ova (50µg [Sigma-Aldrich, Castle Hill, Australia]), in the Th2-inducing adjuvant alum (1mg Rehydrogel [Reheis, Berkeley Heights, NJ] in 200µl 0.9% saline) on day 0 [1]. Mice were then intranasally (IN) challenged with Ova on d12-13 and d33-34 (10µg; 50µl sterile saline [Figure 1A]). On d14 mice were inoculated IN with the natural mouse pathogen *C. muridarum* (Cmu; 100 inclusion-forming units, ATCCVR-123, 30µl sucrose phosphate glutamate buffer [SPG]).[1, 2] Dexamethasone (DEX) was administered IN (2mg/kg; 50µl phosphate buffered saline [PBS]) on d32-34 with the Ova challenges (Figure 1A).

We also investigated the broader applicability of our data in a different, previously developed model using *Haemophilus influenzae* (*Hi*) infection. Mice were inoculated intratracheally (IT) with $5x10^5$ colony-forming units (CFU) non-typeable *Hi* (NTHi-289, 30ul PBS) 10 days prior (d-10) to Ova IP sensitisation (50µg; 200µl in alum 0.9% saline). They were then challenged with Ova (10µg; 50µl sterile saline) on d12-15 (Figure 1B). DEX (1mg/kg; 50µl PBS) was administered IN on d13-15.[3]

All controls were sham-sensitised with saline, and sham-inoculated with SPG (*Chlamydia*-induced AAD) or PBS (*Hi*-induced AAD). In both models, mice were sacrificed 24h after the final challenge and features of AAD were assessed.

Lung function

Two methods of lung function measurement were used to assess the robustness of the effects of antibiotic treatment in the two different models. Lung function for all the *Chlamydia* groups (and their controls) was analysed using the FlexiVent system. Mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg, Troy Laboratories, Smithfield, Australia) and their tracheas were cannulated. FlexiVent apparatus (FX1 System; SCIREQ, Montreal, Canada) was used to assess airways-specific resistance (tidal volume of 8 mL/kg and respiratory rate of 450 breaths/min). Three measurements per dose were taken in response to increasing doses of nebulized methacholine (Sigma-Aldrich, Sydney, Australia) and the average calculated. Lung function for all the *Haemophilus* groups and their controls was analysed using the Buxco R&C system. Mice were anesthetized with ketamine (100 mg/kg) and xylazine (20 mg/kg) and their tracheas were cannulated. R&C apparatus (BUXCO Electronics, Sharon, CT, USA) was used to assess airways resistance (at a tidal volume of 9 mL/kg and respiratory rate of 180 breaths/min). Peak airways resistance was determined in response to increasing doses of nebulized methacholine (Sigma-Aldrich).[1, 3]

Lung mRNA expression

RNA was extracted from homogenised whole lung tissue using TRIzol® according to manufacturer's instructions (Invitrogen, Mount Waverly, Australia) and reverse-transcribed using BioScript™ (Bioline Pty. Ltd., NSW, Australia) and random hexamer primers (Invitrogen, Life Technologies, Australia). Relative abundance of cytokine cDNA was determined compared to the reference gene hypoxanthine-guanine phosphoribosyltransferase (HPRT) by real-time PCR (Mastercycler® ep

realplex^2 system; Eppendorf South Pacific Pty. Ltd., NSW, Australia).[1, 4] Primers used were HPRT Fwd 5'- aggccagactttgttggatttgaa-3', Rev 5'-caacttgcgctcatcttaggcttt-3'; IL-13 Fwd 5'-tgcttgccttggtggtct-3', Rev 5'-ggggagtctggtcttgtgtg-3'; IL-5 Fwd 5'-catcacaccaaggaactcttgcag-3', Rev 5'-tggtgaaagagaccttgacacagc-3'; TNF-α Fwd 5'-tctgtctactgaacttcggggtga-3', Rev 5'-ttgtctttgagatccatgccgtt-3'; and Cmu 16s Fwd 5'-gcggcagaaatgtcgtttt-3', Rev 3'-cgctcgttgcgggactta-5'.

Mediastinal lymph node (MLN) T-cell cytokine release

MLN cells ($5x10^6$ cells) were isolated, re-stimulated with Ova (200 mg/mL; Sigma) or ethanol-killed *Hi* ($2x10^7$ CFU) and cultured for 6 days in Gibco RPMI-1640 (Invitrogen) containing 10% FCS, 20 mmol/L HEPES, 10 mg/mL penicillin/streptomycin, 2 mmol/L L-glutamine, and 50 mmol/L 2-mercaptoethanol.[5]

Statistics

Data are represented as mean ± SEM with 6-12 mice in each group. For all data represented in histograms, statistical significance was determined by one-way analysis of variance (ANOVA) with a Tukey's or Fisher's LSD post-test. AHR was analysed using two-way repeated measures ANOVA with a Tukey's post-test. (GraphPad Prism 6 Software, San Diego, California, USA).

Discussion

Use of structurally related macrolides that are non-antibiotic as a control

We considered using a number of different compounds, including rapamycin, tacrolimus and pimecrolimus, which are all structurally related to clarithromycin.

Rapamycin has been shown to reduce disease features in AAD during the induction phase, but in established disease, exacerbates clinical features. We concluded that using this molecule would complicate our study and make it difficult to interpret the outcomes. Tacrolimus has been shown to interfere with the early events of *Chlamydia* infection *in vitro*. Finally, pimecrolimus has been shown to inhibit the growth of *Malassezia* species of fungi. Thus both of these compounds have anti-microbial effects. We, therefore, have not been able to find a structurally related macrolide that does not have antibiotic properties.

IL-5 increases in clarithromycin-treated groups with *Hi*-induced SSIAAD (Figure 7)

We show that clarithromycin and DEX treatment does not inhibit IL-5 in groups with either the steroid-sensitive (Clari/Ova/Dex) or *Hi*-induced SSIAAD (Hi/Clari/Ova/Dex) compared to their untreated controls (Ova or Hi/Ova). *Hi* infection in AAD (Hi/Ova) reduces IL-5 responses almost down to baseline levels. Clarithromycin and DEX treatment of this group (Hi/Clari/Ova/Dex) does not decrease IL-5 any further. In contrast IL-5 increases in *Hi*-induced SSIAAD with clarithromycin treatment (Hi/Clari/Ova) compared to untreated controls (Hi/Ova). This is likely to result from the removal of the suppressive effects of *Hi* infection on IL-5 by clarithromycin.

References

1. Horvat JC, Starkey MR, Kim RY, *et al*. Chlamydial respiratory infection during allergen sensitisation drives neutrophilic allergic airways disease. *J Immunol* 2010;**184**(8):4159-69.
2. Horvat JC, Starkey MR, Kim RY, *et al*. Early-life chlamydial lung infection enhances allergic airways disease through age-dependent differences in immunopathology. *J Allergy Clin Immunol* 2010;**125**(3):617-25.e6.
3. Essilfie A-T, Simpson JL, Horvat JC, *et al*. Haemophilus influenzae infection drives IL-17-mediated neutrophilic allergic airways disease. *PLoS Pathog* 2011;**7**(10).
4. Beckett EL, Stevens RL, Jarnicki AG, *et al*. A new short-term mouse model of chronic obstructive pulmonary disease identifies a role for mast cell tryptase in pathogenesis. *J Allergy Clin Immunol* 2013;**131**(3):752-62.e7.
5. Thorburn AN, Foster PS, Gibson PG, *et al.* Components of Streptococcus pneumoniae suppress allergic airways disease and NKT cells by inducing regulatory T cells. *J Immunol* 2012;**188**(9):4611-20.